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수의학박사 학위논문

Molecular epidemiology and resistance mechanisms of extended-spectrum β-lactamase producing- and colistin-resistant *Escherichia coli* strains from Korean swine farms

한국 돼지농장 유래 ESBL 생성 및 콜리스틴 내성 대장균의 분자역학과 내성 기전 연구

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수의학과 수의병인생물학 및 예방수의학 전공

이 수 민

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이 논문을 수의학박사 학위논문으로 제출함

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Abstract

Molecular epidemiology and resistance mechanisms of extended-spectrum β-lactamase producing- and colistin-resistant *Escherichia coli* strains from Korean swine farms

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The third-generation cephalosporins and colistin have been regarded as the critically important antibiotics (CIA) for treatment of multi-drug resistant (MDR) bacterial infection diseases in human. These antimicrobial agents have been continuously prescribed to prevent and control diseases in the swine industry. This trend made swine farms one of the most important reservoirs of extended-spectrum β-lactamase (ESBL)-/AmpC β-lactamase (AmpC)-producing and mobilized colistin resistance gene (*mcr*)-carrying *Escherichia coli* (ESBL/AmpC/MCR-EC).

Pig production stages are divided into four stages, including weaning piglets, growing pigs, finishing pigs, and pregnant sows, and pigs of different stages are raised in separated barns. Since different diseases occur according to swine stages, the type and volume of antimicrobial treatment are different for

each pig stage. Therefore, the distribution and characteristics of CIA-resistant bacteria could differ at each swine stage. In that point, understanding the distribution and characteristics of ESBL/AmpC/MCR-EC by swine stages could be an important cornerstone for control and management of CIA-resistant bacteria in swine farms.

The present study aimed to investigate the risks of ESBL/AmpC/MCR-EC strains from swine farms according to swine production stages and to evaluate the potential threat of swine farm-derived strains to humans by understanding molecular epidemiological dynamics and resistance transfer mechanisms. For this study, multi-stage stratified sampling of swine feces was conducted for eleven swine farms located in South Korea between May 2017-March 2020, and whole genome sequence (WGS) of strains which uploaded in public database was utilized for comparative analysis.

The ESBL/AmpC-EC strains were distributed throughout all swine stages (total prevalence: 55.1%). Prevalence and characteristics of ESBL/AmpC-EC strains were significantly different according to stages. Weaning piglets exhibited significantly higher prevalence (86.3%) relative to finishing pigs (48.4%). The CTX-M β-lactamase was the dominant ESBL type for all swine production stages, with the dominant type of CTX-M-55. Whereas, CMY β-lactamase was identified only in growing and finishing stages with the dominant type of CMY-2. The *K*-means similarity analysis showed clonal similarity between ESBL/AmpC-EC strains from different swine production stages within farms. This result suggests there is a high potential of cross-infection between stages, which enabling spread, persistence and reintroduction of ESBL/AmpC-EC clones within swine farms. In the comparative analysis using public database, the ESBL/AmpC types and clone types were shared between strains isolated from swine farms, pork meats and humans in South

Korea. In particular, high-risk clones of swine farm-derived strains (ST101-B1, ST457-F, and ST648-F) were shared with strains from pork meats and humans. This result provides an indirect scientific evidence that swine farm-derived ESBL/AmpC-producing potentially high-risk clones could be transmitted to humans through food-chains.

Total prevalence of *mcr-1*-carrying *E. coli* (MCR1-EC) was 8.4% in swine farms, with the highest prevalence from weaning piglets (13.0%). Weaning piglet-derived strains exhibited significantly higher multi-drug resistance (MDR) rate (quinolone, aminoglycoside, and chloramphenicol, etc.) compared to other stage-derived strains. WGS-based analysis showed that *mcr*-carrying intestinal pathogenic *E. coli,* with MDR and pathogenic advantages, were highly shared between swine stages. Whereas, between strains from different pig farms and sources (humans, pigs, and pork meats), highly heterogeneous clone types were identified. It suggests the lower contribution of clonal spread to colistin resistance spreading between environments. MCR1-EC with virulence advantages (e.g., intestinal/extraintestinal pathogenic *E. coli* or robust biofilm formation) accounted for nearly half of all strains. These results imply that MCR1-EC may act as an important source of *mcr-1* horizontal transfer to other pathogenic bacteria in a harsh environment (e.g., food chain) based on its increased survivability.

The *mcr-1.1* showed high horizontal transfer frequency (6.30 logCFU/ml) and transferred with simple gene cassette without MDR and insertion sequences, "*mcr-1.1-pap2*". This result suggests that *mcr-1.1*-mediated horizontal genetic transfer may provide a high contribution for colistin resistance spreading. Whereas, the *mcr-3.1*-cassette was bracketed by multiple insertion sequences (e.g., IS26, IS4321, etc.) and mainly transferred with MDR. This result implies that the transfer of *mcr-3.1* would pose a

significant challenge on public health by spreading with MDR. From this study, it was first reported that *mcr-3.1*-cassette may be integrated into bacterial chromosome via IS26-mediated transfer. This result implies that *mcr-3.1* had dual pathways mediated by plasmid transfer (horizontal transmission) and chromosomal insertion (vertical transmission), enabling it to proliferate stably despite of its relatively lower horizontal transfer frequency (0.97 logCFU/ml). This study highlights the need for suitable strategies based on the different characteristics between *mcr* variants to control colistin resistance.

In conclusion, swine farms may act a melting pot of high-risk pathogenic *E. coli* clones and CIA-associated resistance genes. This study provided an indirect scientific evidence swine farm-derived potentially high-risk zoonotic ESBL/AmpC/MCR-EC clones may be transferred to humans through food-chains using clonal spread and horizontal genetic transfer. Prevalence and characteristics of ESBL/AmpC/MCR-EC strains were significantly different according to stages in swine farms, an important reservoir of CIA-resistant bacteria, highlighting the importance of a multi-stage systemic policy to monitor and control CIA-resistant bacteria. WGS-based genetic relatedness analysis suggested the high possibility of cross-infection within swine farms, emphasizing the need for reduction of cross-infection in farms. This study is expected to contribute to the improvement of antimicrobial resistance management strategies by presenting scientific evidence and epidemiological models for an in-depth approach to different antibiotic resistance in the livestock industry.

Keywords: ESBL, AmpC, *mcr*, swine productions stages, comparative genomic analysis, whole-genome sequencing

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List of abbreviations

3rd Cepha	3rd-generation cephalosporin class
AmpC-EC	AmpC β-lactamase-producing *Escherichia coli*
AR	antimicrobial resistance
BLAST	basic local alignment search tool
CC	clonal complex
cgMLST	core-genome multi-locus sequence typing
CLSI	Clinical Laboratory Standard Institute
CSC	complex samples crosstabs
CSLRM	complex samples logistic regression model
ED	edema disease
EPEC	enteropathogenic *Escherichia coli*
ESBL-EC	Extended-spectrum β-lactamase-producing *Escherichia coli*
ETEC	enterotoxigenic *Escherichia coli*
ExPEC	extra-intestinal pathogenic *Escherichia coli*
GEE	generalized estimating equation
HGT	horizontal gene transfer
InPEC	intestinal pathogenic *Escherichia coli*
IRL	inverted repeat left
IRR	inverted repeat right
IS	insertion sequence
LEE	locus of enterocyte effacement
LPS	lipopolysaccharide
MCR1-EC	*Escherichia coli* strains carrying *mcr-1.1*
MCR3-EC	*Escherichia coli* strains carrying *mcr-3.1*
MCR-EC	mobilized colistin resistance Escherichia coli

MDR	multi-drug resistant
MGE	mobile genetic element
MIC	minimum inhibitory concentration
MLST	multi-locus sequence typing
MR	heavy metal resistance
MST	minimum spanning tree
NCBI	National Center for Biotechnology Information
OR	odds ratio
PEtN	phosphoethanolamine
PWD	post-weaning diarrhea
SCT	Spearman's correlation test
SNP	single nucleotide polymorphism
ST	sequence type
STEC	shiga toxin producing *Escherichia coli*
Stx	shiga toxin
Tn	transposon
TSD	target site duplication
UPEC	uropathogenic *Escherichia coli*
UPGMA	unweighted pair group method with arithmetic means
UTI	urinary tract infection
VF	virulence factor
WGS	whole genome sequencing
WHO	World health organization

General Introduction

For many decades, antimicrobial resistance has been recognized as a global health problem. Currently, it has been escalated as one of the top health challenges facing the 21st century by the World Health Organization (WHO) [1]. The link between drug resistance in humans and antibiotic use in food animals remains controversial. Concerns have been raised regarding the impacts of antibiotic use in food animals on the health of people on farms and, ultimately, of consumers via the food chain [2]. Warm-blooded animals, including humans, naturally harbor gram-negative bacteria, such as *Escherichia coli*, in their guts. *E. coli* is generally a non-pathogenic commensal bacterium; however, it may act as an important intestinal and extraintestinal pathogen harboring virulence factors and presenting antimicrobial resistance and may cause diarrhea or extraintestinal infectious diseases, such as urinary tract infections, sepsis, and meningitis [3]. More importantly, due to its high genomic flexibility, *E. coli* strains tend to disseminate antimicrobial resistance through horizontal gene transfer and clonal spread.

Extended-spectrum β-lactam antimicrobials, including third generation cephalosporins, have been widely applied to treat bacterial infections both in humans and animals. Since the first description of extended-spectrum β-lactamase (ESBL) and AmpC β-lactamase (AmpC) in the mid-1980's, the global prevalence of ESBL/AmpC-producing *E. coli* (ESBL/AmpC-EC) has rapidly increased [4]. Consistently, increasing prevalence of ESBL/AmpC-EC strains on food animal farms has been reported in multiple continents, including Europe [5-7], Americas [8, 9], Africa [10-12], Australia [13, 14], and Asia [15, 16]. Although the routes of transmission to humans remain unclear, antimicrobial resistant bacteria from food-animals can

presumably pass via food chains or close contact, and can colonize the human gut. The extensively dissemination of multidrug-resistant (MDR) ESBL/AmpC-EC pathogens among food-animals may pose a serious threat to human health, since it paves the way for the limited treatment options in MDR bacterial infections.

Colistin, polymyxin E, was developed as colistimethate sodium in the 1940's and first used in the 1970's. Colistin has been prescribed as the last treatment option for MDR bacterial infections in humans, such as those caused by extended-spectrum β-lactam- or carbapenem-resistant *Enterobacteriaceae*, and is classified as a critically important antimicrobial agent (CIA) [17]. Before 2015, colistin resistance was considered to be primarily associated with mutational and regulatory changes in chromosomal genes, including *pmrAB* and *phoPQ* [18]. The mobilized colistin resistance gene *mcr-1* was first described in a plasmid carried by *E. coli* strains in 2016 [19]. Since then, it has been detected in over 50 countries across six continents, highlighting the global spread of colistin resistance via *mcr* [20]. In addition, *mcr* variant-mediated colistin resistance has been reported worldwide in diverse hosts, including humans, livestock, and companion animals, posing a severe public health threat [17].

To date, 10 *mcr* variants (*mcr-1* to *mcr-10*) have been reported [21], with *mcr-1* and *mcr-3* being the most prevalent variants. Of note, *mcr* variants are mobilized in the form of gene-cassettes containing insertion sequences (IS) or transposons (Tn) and can be transferred horizontally via mobile genetic element (MGE) vectors, particularly plasmids [22]. Recent studies have revealed that the major types of MGE vectors encoding *mcr*-carrying gene cassettes were different depending on the *mcr* types. The *mcr-1* was mobilized

by the ISApl1-mediated composite transposon Tn6330 (ISApl1-*mcr-1-pap2*-ISApl1) [23], while IS26 is responsible for the mobilization of *mcr-3* [24]. Overall, the characteristics of the MGE vectors used by mobile resistance genes may affect the expression or transferability of mobile resistance genes [22]. Therefore, understanding the differences in the genetic background and transfer characteristics of *mcr* variants may be an invaluable cornerstone to control the spread of *mcr*-mediated colistin resistance.

Among the various reported sources, the prevalence of ESBL/AmpC/MCR-EC has been reported to be highest in the swine industry. This epidemiological trend may be attributed to the long-term and extensive usage of β-lactam antibiotics and colistin in swine production [25, 26]. Both β-lactam antibiotics and colistin are routinely used for the prophylaxis of swine colibacillosis—a major porcine disease that causes tremendous economic losses in the global swine industry [27]. Although the prophylactic use of antibiotics was banned worldwide since 2016, these are commonly used for the treatment of swine colibacillosis even today.

Swine production involves four stages, including farrowing (birth to 3–4 weeks of age), weaning (4–7 weeks of age), growing (7–14 weeks of age), and finishing stage (14–24 weeks of age). In swine industry, different type and volume of antimicrobial agents are prescribed depending on the swine production stages [28-37]. The β-lactam antibiotics, aminoglycosides, and colistin are used for the treatment of bacterial diarrhea (*E. coli*/*Clostridium perfringens*) and swine colibacillosis that primarily occur in weaned pigs, and more than 80% of those antibiotics are prescribed in weaning pigs [38]. Whereas, macrolide antibiotics, including tyrosine, and lincosamide antibiotics, including lincomycin, account for over 80% of the total amount of antibiotics

used in growing/finishing pigs. Lincosamide and lincomycin are used to treat swine dysentery and ileitis, which are relatively common at the growing/finishing pig stage [36]. Different antibiotic prescriptions for each breeding stage can act as an important key-factor that makes the prevalence or characteristics of antibiotic-resistant bacteria different for each breeding stage [39]. For establishing practical strategies for controlling of ESBL/AmpC/MCR-EC strains in swine farms, a major reservoir of CIA-resistant bacteria, the studies on the differences of prevalence and characteristics of antibiotic-resistant bacteria by swine production stages are essential.

On farrow-to-finish swine farms, pigs at each stage are typically reared in three separate farrowing, weaning, and growing-finishing barns, respectively. However, as pigs age and are transferred to the next growth stage and barn, bacterial transmission may occur among animals at different swine production stages within farms, which is a significant risk factor for the high prevalence of MDR bacteria on swine farms [7, 40]. Since ESBL/AmpC-related genes (*bla*$_{CTX-M}$, *bla*$_{CMY}$, and *bla*$_{SHV}$) and *mcr* have been reported to be mainly mediated by plasmids, the important role of genetic transferability of antimicrobial resistance in the spread of ESBL/AmpC/MCR-EC has been extensively highlighted in previous studies. However, the genetic transfer of resistance genes essentially presupposes the transfer of strains and bacteria-to-bacteria interactions under favorable conditions (e.g., physical distance between strains, nutrition, and environmental conditions, etc.) [41], suggesting that bacterial transmission also provides crucial basis for the spread of ESBL/AmpC/MCR-EC. In this context, understanding the clonal distribution and dynamics of ESBL/AmpC/MCR-EC considering swine production stages may be build a foundation for developing strategies for the control of ESBL/AmpC and colistin resistance in the swine industry.

This study aimed to analyze the different risks of ESBL/AmpC/MCR-EC from swine farms by production stages and to evaluate the potential threat to humans by understanding molecular epidemiological dynamics and resistance transmission mechanisms. To this end, first, the prevalence, antimicrobial resistance, virulence characteristics, and clonal dynamics of ESBL/AmpC/MCR-EC strains were investigated in swine farms and their characteristic differences were analyzed according to swine production stages. Second, the genetic relatedness of ESBL/AmpC/MCR-EC strains isolated from swine farms and strains isolated from various sources were evaluated using public database. Finally, to understand the different threats by two major *mcr* variants, the different transfer characteristics of two major *mcr* variants, *mcr-1.1* and *mcr-3.1*, were analyzed based on the culture-based and comparative genomic analysis.

Literature Review

1. Antimicrobial use and resistance in swine industry

The misuse and abuse of antibiotics in livestock farms can increase the selective pressure of bacterial resistance, which is one of the important factors causing antibiotic resistance in food-animal husbandry [35]. Increasing evidence indicates that antimicrobial drug consumption is associated with increased antimicrobial resistance: the positive correlation between the antibiotics usage and antimicrobial resistance has been reported for various antimicrobial classes, including cephalosporins (r = 0.79, P < 0.01), aminoglycosides (r = 0.76, P < 0.01), and carbapenems (r = 0.27, P < 0.01) [42, 43].

As part of efforts to control antibiotic resistance in livestock, most countries around the world, including Korea, Denmark, France, the United States, Germany, and Japan, collect statistical data on antibiotic use and resistance and publish annual reports. The Animal and Plant Quarantine Agency and National Institute of Food and Drug Safety Evaluation investigate the antibiotic use in food and companion animals and the antimicrobial resistance of bacteria recovered from them and their products in the Republic of Korea and publish a report entitled “National antibiotic use and resistance monitoring in livestock and companion animals” annually [44]. According to this report, around 1,062 tons (1,036 tons of antibiotics and 26 tons of ionophores) of active compounds were sold in 2021. The largest volume of antimicrobials was sold for use in the swine industry (52%, 555 tons), followed by the fishery (21%, 226 tons), poultry (17%, 183 tons), and cattle (9%, 97 tons) industries.

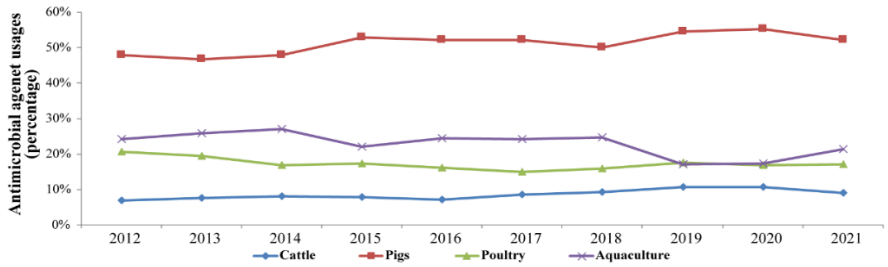


Figure 1. Antimicrobial use in food animals during 2012–2021 [44]

In pigs, comparison of the amount of antibiotics used by class showed that the most commonly used class is β-lactam antibiotics (179,033 kg), followed by phenicol (98,076 kg), macrolide (78,037 kg), and tetracycline (62,322 kg) antibiotics. Regarding the antimicrobial resistance of *E. coli* derived from healthy pig feces, resistance to four antimicrobial classes, including β-lactam (ampicillin, 76.6%), phenicol (chloramphenicol, 71.9%), tetracycline (tetracycline, 71.3%), and aminoglycoside (streptomycin, 70.1%) antibiotics, was high.

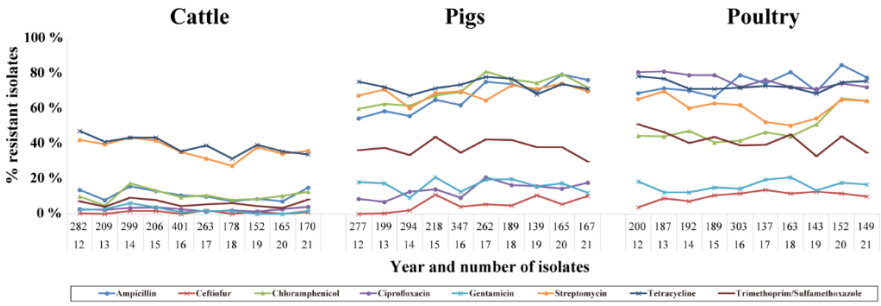


Figure 2. Antimicrobial resistance in *Escherichia coli* isolates from the feces of health food animals on farms during 2012–2021 [44]

The Danish ministry of food, agriculture, and fisheries and the Danish ministry of health publish annual report of the Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP) [38]. According this

report, the annual total antimicrobial consumption in livestock is around 100.1 tons of active compounds, and 74.6% of total consumption amount was utilized in the swine industry. The most used antimicrobial agents in swine industry were β-lactams (22.2%, 16,551 kg), tetracyclines (17.4%, 12,965 kg), and macrolides (16.2%, 12,056 kg). Accordingly, the highest resistance rate was identified for tetracyclines (33%), sulfonamides (30%), and ampicillin (27%).

The food and drug administration of United States (FDA) publish annual report on antimicrobials sold and distributed for use in food-producing animals [29]. According to this report, the annual total antimicrobial consumption in livestock is around 6,036 tons of active compounds, and 39% (2,374 tons) of total consumption amount was utilized in the swine industry. The most used antimicrobial agents in swine industry were tetracyclines (79.9%, 2,062 tons), penicillin (12%, 731 tons), macrolides (7.6%, 195 tons), and lincosamide (4.3%, 195 tons). Accordingly, the highest resistance rate was identified for tetracyclines (56.8%), and ampicillin (25.3%).

The French agency for food, environmental, and occupational health & safety (ANSES) publish annual report on the veterinary medicinal products containing antimicrobial in France [28]. According to this report, the annual total antimicrobial consumption in livestock is around 471.5 tons of active compounds, and the highest amount was prescribed in the swine industry (35.4%, 166.7 tons). The most used antimicrobial agents in swine industry were tetracyclines (38.9%), penicillin (23.4%), and polymyxins (14.0%). Accordingly, the highest resistance rate was identified for tetracyclines (67%), penicillin (58%), and streptomycin (55%).

2. Antimicrobial resistance: from swine farms to humans

Concerns have been raised regarding the impacts of antibiotic use in food animals on the health of people on farms and, ultimately, of consumers via the food chain [2]. Antibiotic resistance in pig farms may be not limited to pig farms, but spread to various ecosystems including animals, people, food, and the environment. Strong scientific evidence has been published for the possibility that resistant bacteria can be transmitted from pig farms to meat of slaughterhouse, the starting point of the food chain. These results pointed out that fecal (cross-)contamination from carcasses or intestines of food-animal is an important source of meat contamination with potential pathogens [45]. Liu et al. (2019) presented the shared distribution and abundance of antimicrobial resistance genes and dominate bacterial composition between swine feces in swine farms and pork samples from slaughterhouse [46]. Li et al. (2022) performed epidemiologic analysis with cgMLST on *mcr-1*-harboring *Salmonella* isolated from pork and confirmed clustering of strains isolated from pork from the same farm, suggesting their same origin [47]. These studies demonstrated that there is a strong indication that antimicrobial resistance genes and the associated multi-drug resistant organisms potentially spread from the pig breeding environment to meat via the pork industry chain.

Antimicrobial resistant bacteria in pig farms can also affect antibiotic resistance in agricultural crops through transmission to the nearby environment. Bacteria with various antimicrobial resistance genes are frequently identified in food animal waste as well as a wide environment near livestock farms [46]. About 85% of the antibiotics consumed by food animals are excreted, or metabolites of these antibiotics can be excreted in the animal's urine or feces and released into the environment [48]. Antibiotic usage can cause selective

pressure and lead to enrichment of these resistant strains, which can further spread to different environments [49]. Moreover, many antimicrobial resistance genes are encoded in mobile genetic elements, which are helpful for their transmission, and can spread from original bacteria to various new bacteria in a new environment [50]. Furthermore, farm feces may be processed and utilized as fertilizer for farm households, which also affects the growth of antibiotic-resistant bacteria in crops. Song et al. (2020) reported that ESBL-EC strains were isolated from the agricultural soils and vegetable (i.e., cabbages, parsleys, sweet potatoes, and lettuces) from grocery stores in South Korea, and these strains carried major human-associated extraintestinal pathogenic *E. coli* lineages [51]. These results demonstrate that MDR pathogens as well as antimicrobial resistance genes may be transmitted from swine farms to humans through various routes including food-chains, highlighting the importance of resistance monitoring and intervention in the One Health perspective.

3. Mechanisms of antimicrobial resistance

The mechanisms of antimicrobial resistance can be classified into four major categories: (1) limiting the drug uptake; (2) modifying the antibiotic target; (3) inactivating the drug; and (4) activating drug efflux [52].

3.1. Intrinsic antimicrobial resistance

Bacteria present both intrinsic and acquired resistance through genetic mutation and gene acquisition [53]. Intrinsic resistance refers to naturally resistant phenotypes to a specific antibiotic without the acquisition of chromosomal mutations or mobilizable antimicrobial resistance determinants [54]. Intrinsic resistance to antibiotics may be caused by (1) low affinity for

antibiotics, (2) inability to penetrate bacterial cells (more specifically, LPS, in gram-negative bacteria), (3) presence of an efflux pump, and (4) production of antibiotic-degrading enzymes.

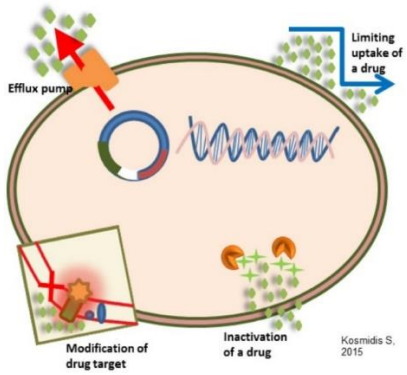


Figure 4. Common antimicrobial resistance mechanisms [54]

Intrinsic resistant phenotypes differ across bacterial species [55]. For instance, all gram-positive bacteria exhibit intrinsic resistance to aztreonams, while all gram-negative bacteria exhibit intrinsic resistance to glycopeptides and/or lipopeptides. Furthermore, *E. coli* strains exhibit intrinsic resistance to macrolides, and *Klebsiella* species exhibit resistance to ampicillins. The table below presents the intrinsic resistance phenotypes of various bacterial species.

3.2. Acquired antimicrobial resistance

3.2.1. Mutational resistance

The emergence of mutations in nucleic acids is one of the major factors underlying evolution, providing the working material for natural selection. Most bacteria allow mutations to emerge and accumulate rapidly, leading to significant phenotypic changes in what is perceived to be real time. The amino acid sequence of a protein can be altered via sequence variations in

the coding region, which can occasionally affect the functional or structural characteristics of antimicrobial resistance-associated proteins, resulting in resistance [56]. For instance, A51V, A67S, and G81C mutations in *gyrA* induce quinolone resistance [57]. In addition, S39I and R81S mutations in the *PmrA* regulator and V161G mutation in *pmrB*, the genes that control the homeostasis of *Enterobacteriaceae* cell membranes, increase the degree of polymyxin resistance [58, 59]. Furthermore, A2059G mutation in the *23S rRNA* gene induces macrolide resistance [60]. In addition, mutations are essential for continued evolution of horizontally acquired resistance genes. For instance, the TEM family of beta-lactamases has expanded to over 100 variants.

3.2.2. Horizontal gene transfer of resistance determinants

Bacteria can acquire various genes that do not exist in a single lineage through horizontal gene transfer, which is often responsible for the development of antimicrobial resistance [61]. Bacteria acquire external genetic material through three key mechanisms: (1) transformation, (2) transduction, and (3) conjugation. Transformation is the simplest way to acquire genetic material, although only a limited bacterial species (e.g., *Acinetobacter*) can naturally acquire genetic material to develop resistance [62]. Conjugation, a very efficient method of gene transfer that involves cell-to-cell contact, may frequently occur in the gastrointestinal tract of humans receiving antibiotic treatment [63]. In general, conjugation uses MGEs as vehicles to share valuable genetic information. Integron is one of the most efficient mechanisms for accumulating antimicrobial resistance genes, and it also provides necessary components to express of the acquired genes [64]. Therefore, integron is considered the major driver of bacterial genetic evolution.

4. Extended-spectrum β-lactamase (ESBL)

4.1. Resistance mechanisms of β-lactam drugs

The most widely used group of antimicrobial agents are β-lactam drugs [65]. All members of this drug group share a specific core structure, which comprises a four-sided β-lactam ring [66]. Resistance to β-lactam drugs occurs through three general mechanisms: (1) inhibition of the interaction between the target PBP and drug, typically through modifying the ability of the drug to bind to the PBP, which is mediated by alterations in the existing PBPs or acquisition of other PBPs; (2) presence of efflux pumps that can extrude β-lactam drugs; (3) hydrolysis of the drug via β-lactamase enzymes. The β-lactam ring is primarily hydrolyzed by β-lactamase, which is an ubiquitous enzymes [67]. The hydrolyzed β-lactam ring by β-lactamase cannot bind to the target PBP. Resistance to β-lactam antibiotics acquired through the production of β-lactamase is common among gram-negative bacteria, and it is the most important mechanism of resistance to penicillin and cephalosporin.

4.2. β-lactamase types

The simplest classification of β-lactamases is based on their protein sequence. Specifically, these enzymes are classified into four molecular classes, A, B, C, and D, based on amino acid motifs. Furthermore, depending on the substrate specificity of these enzymes, they are classified into three functional groups: the TEM, SHV (sulfhydryl variable), and CTX (hydrolyzing cefotaxime) families [68]. Gram-negative bacteria can produce all four enzymes from the structural classification, while most gram-positive bacteria produce group A and produce are group B enzymes. The β-lactamase genes are either present naturally on the bacterial chromosome or acquired through

plasmids. Many *Enterobacteriaceae* and gram-negative bacteria carry chromosomal β-lactamase genes. Other gram-negative bacteria carrying β-lactamase gene include *Aeromonas*, *Acinetobacter*, and *Pseudomonas* species. Plasmids carrying the β-lactamase gene are commonly found in *Enterobacteriaceae*, as well as in some gram-positive bacteria, such as *Staphylococcus aureus*, *Enterococcus faecalis*, and *Enterococcus faecium*.

4.3. ESBL β-lactamase types

ESBL is a broad-spectrum β-lactamase that hydrolyzes third-generation cephalosporins, which are broad-spectrum cephalosporins with oxyimino side chains. However, the resistance mechanisms of ESBL producers are affected by β-lactamase inhibitors. As such, β-lactamase inhibitors are structurally similar to β-lactamase, and their antibiotic action may be maximized when used together with β-lactam antibiotics. ESBLs include members of the TEM, SHV, CTX-M, CMY, and OXA enzyme families. The largest group is the CTX-Ms, which are most commonly found in *E. coli*, particularly UTI isolates. The CTX-M β-lactamase family has 236 alleles and is divided into five groups: CTX-M-1 (CTX-M-1/3/15/55), CTX-M-2 (CTX-M-2/20/31), CTX-M-8 (CTX-M-8/40/63), CTX-M-9 (CTX-M-9/13/27), and CTX-M-25 (CTX-M-25/41/91) groups. CTX-M-15 in the CTX-M-1 group and CTX-M-14 in the CTX-M-9 group are considered the most prevalent types of ESBL worldwide, including South Korea, in the past decade [69].

4.4. AmpC β-lactamase types

AmpC β-lactamases (AmpC) are class C beta-lactamases. AmpC is resistant to a wide range of β-lactam antibiotics, including third-generation cephalosporins [70]. Unlike ESBLs, AmpC mediates resistance to β-lactamase

inhibitor–β-lactam combinations. In gram-negative bacteria, AmpC β-lactamase production is mediated by chromosomal or plasmid genes. Chromosomal *ampC* genes are expressed constitutively at a low level. Some *Enterobacteriaceae* members, such as *Enterobacter*, *Citrobacter*, and *Serratia* species, carry an inducible *ampC* gene, which is regulated by *ampR*, which is overexpressed in the presence of β-lactams, such as cefoxitin and imipenem. Regulation of chromosomal *ampC* expression in *E. coli* differs considerably from that in other *Enterobacteriaceae*. *E. coli* lacks *ampR*; thus, *ampC* expression is not inducible and continuously sustained at a low level. However, various mutations in the *ampC* promoter/attenuator region of *E. coli* have been identified, which result in the constitutive overexpression of *ampC*. In addition to chromosomal *ampC*, *E. coli* may harbor plasmids carrying *ampC* (e.g., bla_{CMY}), acquired via horizontal gene transfer and derived from the chromosomal *ampC* genes of other *Enterobacteriaceae* species. The plasmid-mediated AmpC gene has been known since 1989. Plasmids may acquire a gene encoding the AmpC enzyme, which may consequently appear in bacteria lacking or poorly expressing the chromosomal *ampC* gene, such as *E. coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* [71]. The CMY β-lactamase family is the most representative of plasmid-mediated AmpC β-lactamases. There are 167 alleles in the CMY β-lactamase family, of which CMY-2 is the most reported type of plasmid-mediated AmpC β-lactamase worldwide, including South Korea.

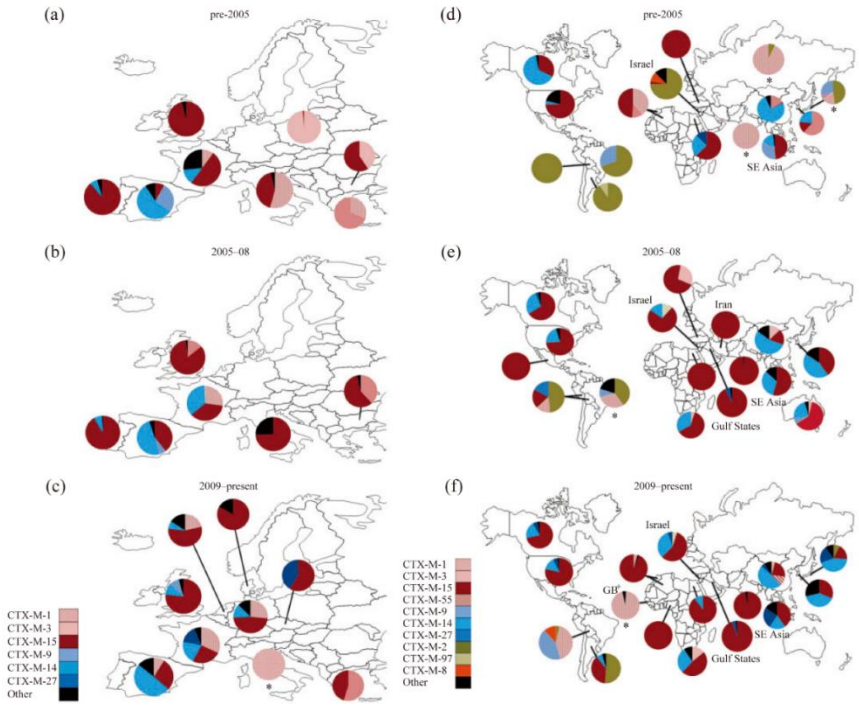


Figure 5. Epidemiological distribution of CTX-M β-lactamase types. CTX-M trends over three time periods in Europe (a, b, c) and the rest of the world (d, e, f) [69]

4.5. Global occurrence of ESBL/AmpC β-lactamase

According to the epidemiological analysis on global occurrence of ESBL/AmpC-EC in humans, the global ESBL/AmpC-EC prevalence showed an increasing trend from 2001 to 2020: the global prevalence of ESBL/AmpC-EC was 2.6% (95% CI: 1.2%-4.0%) from 2001 to 2005, and the prevalence of ESBL/AmpC-EC from 2016 to 2020 was 26.4% (95% CI: 17.0%-35.9%) [4]. In the comparison by food-animal species, the prevalence of ESBL/AmpC-EC in food-animal farms was relatively higher in swine and poultry farms than in cattle farms. The reported prevalence of ESBL/AmpC-EC was approximately 9.6–71.0% in swine farms [7, 46, 72, 73], 15.5–32.8% in poultry farms [11, 16,

74], and 3.1–12.5% in cattle farms [75-78]. The ESBL/AmpC-EC prevalence in food-animals in Korea was also similar to that of global trend. In South Korea, the reported prevalence of ESBL/AmpC-EC was 18.4 to 69.5% in swine farms [79-81], 5.0–100.0% in poultry farms [79, 82-84], and 7.6% in cattle farms[85].

A high correlation has been reported between the resistance profile of ESBL/AmpC-ECs isolated from food-animals and those isolated from humans with bloodstream infections, implying the possibility of ESBL/AmpC-EC transmission via food-chain [86]. It has been shown that genetically distinct *E. coli* isolates from humans and animals carry nearly identical IncI1 plasmids that encode third-generation cephalosporin resistance determinants, proposing the spread of ESBLs through food animals (e.g., pork, chicken) to humans through horizontal genetic transmission [87]. Some of the initial phases where pigs can be colonized by ESBL/AmpC-EC is at trading places, livestock transport vehicles, through introduction of new animals into herds, or at lairage in the slaughterhouse [7]. At slaughterhouses, a risk of cross-contamination of meat exists, especially during evisceration, where carcasses can be contaminated by ESBL/AmpC-EC from the fecal content of the same or different pigs [88]. Meat processing environments are considered to be important intermediate reservoirs and vectors of ESBL/AmpC-EC, and also food handlers pose a risk of transmission of ESBL/AmpC-EC [89]. Studies conducted from South Korea have also reported ESBL/AmpC-EC prevalence of 8.9% in pork [80], 3.0% in chicken meat [84], and 2.5% in milk [85]. The ESBL/AmpC-EC shed from livestock farms can also affect antibiotic resistance in agricultural crops through transmission to the surrounding environment. Song et. al (2021) reported that prevalence of ESBL/AmpC-EC was 4.0% in the soil of agriculture farms [90]. In addition, the national-wide research on ESBL/AmpC-EC in vegetables of retail market exhibited that the ESBL/AmpC-EC was isolated from fresh

sprouts and mixed salads sold in retail markets, with the prevalence of 10.1% [91]. These research results suggest that ESBL/AmpC-EC, which resides in livestock farms, may spread to the human through various routes including food-chains.

5. Mobilized colistin resistance gene, *mcr*

5.1. Colistin

Colistin, or polymyxin E, is a cationic polypeptide antibiotic. It binds to LPS and phospholipids of the cell membrane of gram-negative bacteria, destroys the cell membrane, and induces bacterial death [92]. Colistin use to treat bacterial infections in humans was banned in the 1970s due to its toxicity and low renal clearance. Recently, however, it has been reintroduced as last resort against infections caused by pan-drug resistant gram-negative bacteria. WHO has classified colistin among the "Highest Priority Critically Important Antimicrobials," because of the increasing usage of this antibiotic and discovery of transmissible resistance genes.

5.2. Mechanism of colistin resistance

Traditionally, bacterial resistance to colistin was considered to be acquired through chromosomal point mutations. Non-resistant bacteria can express colistin resistance naturally due to the modulation of the cell surface, including variations in the structure of LPS, or due to shedding of the capsular polysaccharides that bind colistin. However, LPS variation via the addition of 4-amino-4-deoxyl-arabinose (Lara4N) and phosphoethanolamine (PEtN) or galactosamine of lipid A was found to induce colistin resistance [93]. These

LPS variations reduce the affinity of colistin to the cell wall. Additionally, two-component regulatory systems (TCSs), such as PmrAB and PhoPQ, are involved in resistance. Moreover, the plasmid-mediated mobile colistin resistance gene *mcr* has recently been detected in animals, humans, foods, and the environment.

5.3. *mcr* variants

The *mcr* genes encode phosphoethanolamine transferase enzymes that bind the PEtN part of lipid A of the bacterial outer membrane, inducing colistin resistance. In 2015, the first plasmid-mediated colistin resistance gene, *mcr-1*, was identified in *E. coli* isolated from a swine farm in China [20]. Subsequently, Xavier et al. reported *mcr-2*, which shared 80.65% identity with *mcr*-1, from *E. coli* in Belgium [94]. Furthermore, *mcr-3* shared respectively 47.0% and 45.0% identity with *mcr-2* and *mcr-1[95]*. To date, *mcr-3* has been detected in *E. coli*, *Aeromonas*, and *Proteus* species isolated from humans and other non-human animals in Europe, South America, and Asia. The *mcr-4* gene was reported in *Salmonella* R3445 [96]. Carattoli et al. reported that 11 of the 125 isolates of their study carried the *mcr-4* gene. Subsequently, *mcr-4* was detected in *Salmonella enterica* serovar *Typhimurium* and *E. coli* isolates from humans and pigs in Italy, Spain, and Belgium. Borowiak et al. reported *mcr-5* from *Salmonella* enterica serovar *Paratyphi B* isolated from poultry in Germany [97], and AbuOun et al. reported *mcr-6* (sharing 87.9% identity with *mcr-2*) from *Moraxella pluranimalium* [98]. Further, *mcr-7* and *mcr-8* were detected in *Klebsiella pneumoniae* isolates in China, sharing respectively 70% and 39.96% identity with *mcr-3* [99, 100]. Carroll et al. identified *mcr-9* in a clinical MDR isolate (*Salmonella*) from Washington, and its amino acid sequence was similar to that of *mcr-3* (with 64.5% similarity and 99.5% coverage) [101]. Wang et al.

described *mcr-10* on the IncFIA plasmid of a clinical *Enterobacter roggenkampii* isolate. The *mcr-10* gene shared the highest nucleotide identity (79.69%) with the *mcr-9* gene [102]. Among the 10 reported MCR variants, *mcr-1* is the most predominant type worldwide, followed by *mcr-3*, *mcr-4*, and *mcr-9* [103]. In South Korea, the presence of *mcr-1*, *mcr-3*, and *mcr-9* has been reported from various sources including humans, companion animals, and livestock-related samples (i.e., food-animals, meats, and vegetables) [104-107]

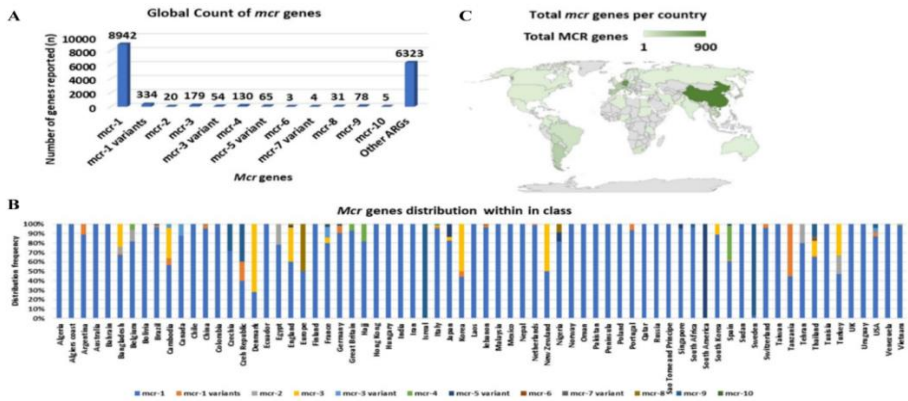


Figure 6. Global distribution and total count of *mcr* genes. (A) Total number of *mcr* genes reported globally. (B) Distribution of *mcr* genes by country. (C) Global map showing the geographical distribution of *mcr* genes [108].

5.4. Global occurrence of *mcr*

According to the epidemiological analysis on the global occurrence of MCR-EC in humans and food-animals, the prevalence of MCR-EC was significantly higher in food-animals than humans: 7.4% (95% CI: 3.9-13.6%) in humans, 14.9% (95% CI: 10.8-20.1) in pigs, and 15.8% (95% CI: 11.7-20.9%) in chickens [109]. Consistently, the researches conducted from South Korea also exhibited that the prevalence of MCR-EC was significantly higher in food-animals than humans. The prevalence of MCR-EC was 0.07-0.87% in humans

[107, 110-113], 0.03-18.89% in pigs [104, 105, 114-117], and chickens 0.31-5.88% [115, 116, 118]. Whereas, three studies on bovine derived MCR-EC strains were conducted in South Korea, but no MCR-EC strains were identified [115-117]. The higher prevalence of *mcr* in food animals compared to humans suggests that livestock farms can serve as important reservoirs of MCR-EC [108].

Raw food samples such as poultry meat, fish, lamb, fruits and vegetables have been reported as important vectors for the spread of *mcr* from food-animals to humans via food-chains [20]. Li et al. (2022) performed an epidemiologic analysis with cgMLST on *mcr-1*-containing Salmonella isolated from pork, confirming the clustering of isolates from pork from the same swine farm, suggesting a common origin [119]. It was also reported that *mcr* was confirmed in retail meat/vegetable in South Korea. Oh et al. (2020) reported that MCR-EC was confirmed in lettuce samples as a result of investigating the presence of *mcr* in fresh vegetables on the retail market [115]. Kim et al. (2020) analyzed the presence of *mcr* in retail meat and reported that *mcr* was confirmed in pork and chicken [120]. Cha et al. (2020) reported the isolation of *mcr-9*-carrying Salmonella from retail chicken meat [121]. In addition, *mcr* genes are mainly encoded in mobile genetic elements (i.e., plasmids), and can spread from original bacteria to various commensal bacteria present in food-chain environments [50]. Vines et al. (2021) analyzed the genetic relationship based on WGS for MCR-EC isolated from food-animal and farmer in the farm. As a result, the shared *mcr*-carrying plasmids were highly distributed in different *E. coli* strains from food-animals and farmers, suggesting that *mcr* was transferred between humans and animals by horizontal transfer [122]. These results suggest that the *mcr* gene can spread from food-animal farms to humans using two mechanisms: clonal expansion and horizontal transfer.

6. Comparative genomic analysis for resistance mechanisms

6.1. Single-nucleotide polymorphism (SNP)

SNPs, or single-nucleotide variants, are one of the most common type of genetic variation. SNPs are DNA sequence polymorphisms that result from the alteration of a single nucleotide at a specific locus at the genomic level. The amino acid sequence of a protein can be altered by sequence variations in the coding region, which may occasionally affect the functional or structural characteristics of antimicrobial resistance-associated proteins, resulting in resistance [56]. Using SNP analysis, the antimicrobial resistance phenotype caused by the point mutations can be predicted. In addition, SNP analysis can be applied to investigate inter-individual differences in molecular epidemiological aspects, such as the evolution and dissemination of horizontally transmitted antimicrobial resistance genes. For instance, Canton et al. [90] performed SNP-based comparative genomic analysis of CTX-M allele sequences and proposed that CTX-M has evolved from the chromosomal *bla* gene in *Kluyvera* species rather than a mutation in the plasmid-mediated gene. Furthermore, based on SNP-based *in silico* analysis, the authors suggested that the CTX-M enzyme has evolved into the five CTX-M families (CTX-M-1, -2, -9, -8, and -25) at least nine times (CTX-M-1: 3 times, CTX-M-2: 2 times, CTX-M-9: 2 times, CTX-M-8: 1 time, CTX-M-25: 1 time) [123]. Furthermore, CTX-M-74 and CTX-M-75, characterized in a survey conducted in Brazil as presenting a single one-amino acid change with respect to CTX-M-2 [124], showed some degree of nucleotide divergence and could be considered as a new cluster, reflecting the convergent evolution of these β-lactamases.

6.2. Horizontal transfer of antimicrobial resistance

Mobile AR determinants that are delivered via horizontal gene transfer using diverse MGEs, such as plasmids and transposons. In addition, AR determinants can be delivered in gene cassettes along with IS and other resistance genes. Comparative genomics from a single AR determinant gene to the entire genome of an MGE vector involves the comparison of sequenced genomes, particularly for the identification of insertions, deletions, and variations in syntenic regions, based on NCBI's basic local alignment search tool (BLAST). Visualizing the alignment between specific regions of multiple genomes is a critical step in identifying genotypic differences underlying phenotypic variations across strains or species. Currently, various tools are available for visualizing the alignment based on BLAST, such as Easyfig [125], Artemis comparison tool (ACT) [126], and Mauve [127]. These tools provide information, such as GC contents, tRNA, misc_features, and coding sequence, as well as gene features of the MGE sequence on which the AR determinants are encoded.

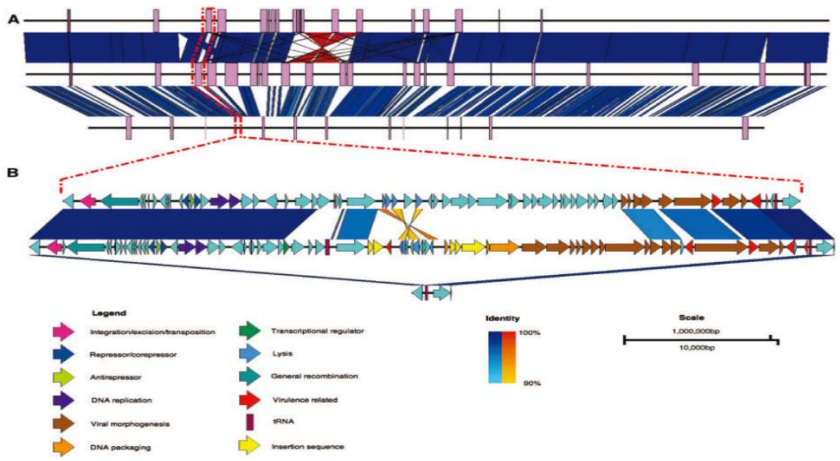


Figure 7. Comparison among the genomes of *Escherichia coli* O157:H7 strain EDL933, *E.coli* O157:H7 strain Sakai, and *E. coli* K12 strain MG1655, constructed using the Easyfig program [125]

6.3. Clonal spread of multi-drug resistant bacteria

For the molecular epidemiologic analysis of antimicrobial resistant bacteria, various fingerprinting methods have been developed. Multi-locus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and multi-locus VNTR analysis (MLVA) were the common typing method for decades. Although these methods are widely used, they have the disadvantages of being labor intensive and difficult to standardize [128]. cgMLST schemes use a fixed set of conserved genome-wide genes, which involves genome-wide gene-by-gene allele calling of hundreds to thousands of conserved genes [129]. Alleles of cgMLST reduce the confusion caused by recombination in bacterial genes and can be used for global and public nomenclature. Representative schemes of cgMLST are provided by EnteroBase [130] and Ridom SeqSphere+ [128].

High discriminatory and rapid analysis using WGS and cgMLST can be suitable for prospective studies of tracking the transmission of MDR bacteria in a hospital. Li et al. (2022) performed epidemiologic analysis with cgMLST on *mcr-1*-harboring *Salmonella* isolated from pork and confirmed clustering of strains isolated from pork from the same farm, suggesting their same origin [47]. Kurittu et al. [131] conducted epidemiologic analysis on CTX-M-27 β-lactamase-producing and ST131 ESBL-producing *E. coli* isolated from clinical samples of the “Eastern Finland Laboratory Center Joint Authority Enterprise” based on cgMLST and found them to be genetically distinct from ESBL-EC strains derived from a non-human source [131]. Furthermore, cgMLST-based epidemiological analysis can be applied to the evolutionary analysis of antimicrobial resistant bacteria. For instance, Lagos et al. [132] performed cgMLST analysis on 95 strains of MRSA isolated for 10 years from the Orebro University Hospital in Sweden and confirmed that 5.0 alleles change per year.

Chapter 1.

Prevalence, characteristics and clonal distribution of extended-spectrum β-lactamase- and AmpC β-lactamase-producing *Escherichia coli* following the swine production stages, and it's potential risks to humans

Abstract

The worldwide spread of ESBL/AmpC-EC poses serious threats to public health. Swine farms have been regarded as important reservoirs of ESBL/AmpC-EC. The present study aimed to analyze the prevalence, resistance, virulence, and clonal dynamics of ESBL/pAmpC-EC strains in swine farms according to swine production stages, and to evaluate genetic relatedness with strains from various sources using public database. Individual fecal samples (n = 292) were collected from weaning, growing, finishing, and pregnant pigs in nine swine farms of South Korea between July 2017 and March 2020. In total, 161 ESBL/AmpC-EC isolates were identified (55.1%), with the highest prevalence from weaning piglets. According to swine stages, the prevalence and characteristics of ESBL/AmpC-EC strains significantly differed. The CTX-M β-lactamase was the dominant ESBL type for all swine production stages with the dominant type of CTX-M-55. Whereas, CMY β-lactamase was identified only in growing and finishing stages, with the dominant type of CMY-2. In addition, weaning piglets-derived ESBL/AmpC-EC strains showed the statistically highest resistance rate to colistin, meanwhile growing pigs-derived strains showed the statistically highest resistance rate to ceftiofur. The *K*-means similarity analysis presented that ESBL/AmpC-EC strains exhibited clonal similarity between different swine production stages within farms, suggesting a high possibility of cross-infection. The clonal population structure analysis based on the virulence factor (VF) presented that swine ESBL/AmpC-producing ExPEC clones, especially ST101-B1, ST648-F, and ST457-F, harbored a highly virulent profile. The ESBL/AmpC types and clone types were shared between ESBL/AmpC-EC strains isolated from pigs, pork meats and humans, implying indirect scientific evidence that ESBL/AmpC-EC strains

may be transmitted to humans through the food-chains. Notably, this study showed that the prevalence and characteristics of ESBL/AmpC-EC strains in swine farms significantly differ depending on the swine production stages, highlighting the need for a multistage systematic strategy to monitor and control ESBL/AmpC-EC strains in farms.

Keywords: ESBL, AmpC, *E. coli*, ExPEC, swine production stage

1.1. Introduction

The third-generation cephalosporin-resistant *Enterobacteriaceae*, ESBL/AmpC-EC, have been reported as a serious global threat to public health. The third-generation cephalosporins show excellent activity against both Gram-positive and Gram-negative bacteria, and are particularly prescribed in treating multidrug-resistant bacterial infections [133]. The spread of ESBL/AmpC-EC is of great concern because it could aid the emergence and spread of pathogens that are difficult to treat even with an antimicrobial agent of choice regarded as a final treatment option [5, 134]. In addition, as ESBL/AmpC-producing extra-intestinal pathogenic *E. coli* (ExPEC) clones are increasingly reported worldwide, the virulence potential of ESBL/AmpC-EC strains has also become an important public health issue [135-137].

The increasing prevalence of ESBL/AmpC-EC in food-animal farms has also been reported in multiple continents including Europe [5-7], America [8, 9], Africa [10-12], Australia [13, 14], and Asia [15, 16]. In particular, pigs have been regarded as the main driver of the increasing prevalence of ESBL/AmpC-EC in food-animals [138]. The possibility of ESBL/AmpC-EC transmission from swine farms to humans has been continuously proposed and vice versa [79, 139]. Various ESBL/AmpC-EC transmission routes have been suggested, including the food-chain of pigs [46], direct contacts of farm workers with pigs [7], and manure excretion into the surrounding environment in farms such as soils, ponds, and rivers [140].

Swine production involves four stages, including farrowing (birth to 3–4 weeks of age), weaning (4–7 weeks of age), growing (7–14 weeks of age), and finishing stage (14–24 weeks of age). In farrow-to-finishing farms, pigs at each different stage in the farrow-to-finish swine farms are usually reared in

three separated farrowing, weaning, and growing-finishing barns, respectively. However, there is a generally high probability of bacterial co-transmission between the production stages within a farm [40, 141], which has been regarded as an important risk factors for the high prevalence of MDR bacteria in swine farms [7, 40].

In general, the prevalent swine disease is different according to swine production stages, thereby, antimicrobial therapy also varies depending the swine stages. The different antibiotic prescriptions may act as an important key-factor that makes the prevalence or characteristics of antibiotic-resistant bacteria different for each breeding stage in swine farms [39]. The antimicrobial prescription without considering the different characteristics and clonal dynamics of strains at each swine stage may lead to misuse and abuse of antibiotics, resulting in increased ESBL/AmpC-EC within swine farms as well as disease control failure. For establishing practical strategies for controlling of ESBL/AmpC/MCR-EC in swine farms, a major reservoir of MDR bacteria, the studies on the differences of prevalence and characteristics of antibiotic-resistant bacteria by swine production stages are essential.

The present study aimed to analyze the prevalence, characteristics and clonal dynamics of ESBL/AmpC-EC strains according to swine production stages, and evaluate their genetic relatedness with strains from various sources. To this end, first, the prevalence, β-lactamase types, resistance, virulence potential, and clonal dynamics of ESBL/AmpC-EC strains were analyzed according to swine production stages. Second, the virulence potential of swine-farm derived ESBL/AmpC-EC strains was evaluated. Finally, genetic relatedness analysis was conducted on strains isolated from swine farms and strains isolated from various sources using public database.

1.2. Materials and Methods

Sample Collection

In total, 292 swine fecal samples were collected from nine farrow-to-finish swine farms between July 2017 and March 2020 (Supplementary table 4). For this research, we selected nine swine farms satisfying three criteria: 1) located in in five provinces with the highest number of pig farms in South Korea, 2) farrow-to-finishing farm, and 3) raising pigs more than 1,000 pigs. The number of pig farms by province in South Korea was obtained from the 2017 demographic report of the Korean Statistical Information Service of Statistics Korea [142]. Total sampling size was calculated using Equation 8.13 described in the book "*Epidemiology: study design and data analysis*, " 3rd edition [143]. For calculation of sampling size, 20% precision level, 10% power, 95% confidence interval, and 50% estimated prevalence of ESBL/AmpC-EC were used as input variable for the two-side test. The estimated prevalence of ESBL/AmpC-EC was determined using previous global prevalence of ESBL/AmpC-EC in swine farms [7, 46, 72, 73].

In total, individual fecal samples were collected from 51 weaning piglets (4-7 weeks old), 96 growing pigs (7-14 weeks old), 97 finishing pigs (14-24 weeks old), and 50 pregnant sows based on the multi-stages stratified random sampling. A total of 26–34 pigs from each swine farm were included for this study, including 5–6 weaning piglets, 9–11 growing, 8–11 finishing pigs, and 3–6 pregnant sows. The sampling size for each swine stages were determined using two criteria: 1) the number of pigs for each stage, and 2) estimated prevalence of ESBL/AmpC-EC for each stage. The estimated prevalence of ESBL/AmpC-EC for each swine stages were determined with the previous researches conducted worldwide [7, 144, 145].

Isolation of ESBL/AmpC-EC and non-ESBL/AmpC-EC

To isolate ESBL/AmpC-EC, 1 g of each fecal sample was homogenized with 9 ml of *E. coli* broth (Oxoid, United Kingdom) for 1 min using a homogenizer and incubated overnight at 37°C. Approximately 100 µl of enriched *E. coli* culture suspension was spread on MacConkey agar (Oxoid). Cefotaxime (CTX) disk (30 µg/ml, Oxoid) was then placed on the plate. After overnight incubation at 37°C, 2–4 cefotaxime-resistant *E. coli* candidate isolates grown inside the CTX resistant zone (<22 mm) were selected and streaked on CHROMagar™ ESBL (CHROMagar, France) to demonstrate the morphology of cefotaxime-resistant *E. coli* colony. One strains from each sample was randomly selected if more than one strains were identified from one sample. Finally, a standard double-disk test was performed to confirm the typical ESBL and AmpC phenotype, as described in the 2016–Clinical Laboratory Standard Institute (CLSI) guideline M100S 26th Edition.

To isolate non-ESBL/AmpC-EC, 10 µl of enriched *E. coli* broth suspension was streaked on MacConkey agar. Three colonies showing typical *E. coli* morphology were randomly selected and transferred to Eosin Methylene Blue (EMB) agar (Oxoid) for purification. The suspected *E. coli* isolates on EMB agar were subjected to a standard ESBL/AmpC double-disk test, and typical non-ESBL/AmpC-EC isolates were determined as non-ESBL/AmpC-EC strain. One strains per sample was randomly selected, where more than one strains were identified from one sample. Considering the distribution of ESBL/AmpC-EC isolates by farm and production stage, a total of 81 non-ESBL/AmpC-EC isolates were selected for further analysis.

The presence of ESBL genes (*bla*CTX-M, *bla*TEM, and *bla*SHV), AmpC gene (*bla*CMY), and carbapenemase genes (*bla*KPC, *bla*NDM, and

*bla*OXA) were determined with PCR. Then, PCR amplicons were sequenced using the ABI PRISM 3730XL DNA analyzer (Applied Biosystems, United States). DNA sequences were compared with the published β-lactamase gene sequences available from the GenBank database of the NCBI using the BLAST program. Primer sequences and reaction conditions for each PCR-based genotyping of ESBL/AmpC are summarized in Supplementary Table 1.

Antimicrobial susceptibility test

Disk diffusion susceptibility test (Kirby-Bauer method) were conducted for 14 antibiotics: amoxicillin/clavulanic acid (20/10 μg), ampicillin (10 μg), cefotaxime (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), aztreonam (30 μg), imipenem (10 μg), chloramphenicol (30 μg), amikacin (30 μg), gentamicin (10 μg), tetracycline (30 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), colistin (10 μg), and trimethoprim–sulfamethoxazole (1.25/23.75 μg). *Escherichia coli* ATCC 25922 was used as the reference strain for quality control. Antimicrobial susceptibility was interpreted according to the CLSI guidelines.

Plasmid-mediated antimicrobial resistance genes, intestinal pathogenic E. *coli* typing, and dxtraintestinal pathogenic *E. coli* typing

The presence of plasmid-mediated antimicrobial resistance genes inferring resistance to chloramphenicol (*catA*, *cmlA*, and *floR*), tetracycline (*tetA*, *tetB*, and *tetD*), quinolone (*qnrA*, *qnrB*, *qnrC*, *qnrS*, and *aac(6)-Ib-cr*), aminoglycoside (*aac(3)-I*, *aac(3)-II*, and *aac(3)-IV*), colistin (*mcr-1*), and Sulfonamide/Trimethoprim (*dfrIa*, *dfrIb*, *dfrII*, *dfrVII*, and *dfrXII*) were determined using PCR. Intestinal pathogenic *E. coli* typing was conducted for the following types; shiga toxin-producing *E. coli* (*stx1* and *stx2*),

enteropathogenic *E. coli* (*eaeA* and *bfpV*), enteroaggregative *E. coli* (*aggR*), enteroinvasive *E. coli* (*ipaH*), and enterotoxigenic *E. coli* (*lt*, *sta*, *stb*, and *east-1*). The extraintestinal pathogenic *E. coli* associated virulence factors (ExPEC VFs) associated with adhesion (*fimH*, *iha*, *papC*, and *csgA*), toxin (*astA*, *hlyA*, *aat*, *tsh*, and *pic*), protectin/serum resistance (*traT* and *ompT*), and siderophore (*fyuA* and *iroNe.coli*) were also determined using PCR. The classification of ExPEC was conducted following the previously described criteria, specifically positive for ≥2 of five key markers as follows: *papA* and/or *papC*, *sfa/focDE*, *afa/draBC*, *iutA*, and *kpsMTII* [146]. The classification of UPEC was conducted following previously described criteria, specifically positive for ≥3 of four key markers as follows: *vat*, *fyuA*, *chuA*, and *yfcV* [147].

Biofilm assay

Biofilm production assays were performed following a previously described protocol with modification [148]. Approximately 120 µl of overnight culture was added into 96-well microtiter plate and incubated for 24 h at 30°C in a stationary condition. Each bacterial suspension was inoculated into three wells of the microtiter plate. Growth optical densities (ODs) were measured at λ = 595 nm with a multiplate reader (Bio-rad, United States). The wells were then washed once with 200 µl of phosphate-buffered saline (PBS) dried for 20 min, and stained with 120 µl of 1% crystal violet for 5 min. This was followed by gentle washing with 200 µl of distilled water (DW) for four times and air-drying for 1 h. The absorbed dye was solubilized in 120 µl of absolute ethanol, and ODs were read at 595 nm. The extent of biofilm formation was calculated using the formula: $\mathrm{SBF} = \frac{(AB-CW)}{G}$, where SBF is the specific biofilm formation index, AB is the OD595 of the stained bacteria, CW is the OD595 of the stained

control wells containing absolute media without bacteria, and G is the OD595 corresponding to cell growth in the media. An SBF value above 0.5 was suggested as positive biofilm formation. *E. coli* ATCC 25922 was used as the positive control, while the culture medium was used as the negative control.

Expression of biofilm-associated extracellular matrix components: curli fimbriae and cellulose

To determine the expression of biofilm-associated extracellular matrix components (cellulose and curli fimbriae), a macrocolony assay was performed following a previously described protocol with modification [137]. Approximately, 5 µl of an overnight culture from a single colony grown was dropped on YESCA agar [10 g/L casamino acids (BD Bioscience, United States), 1 g/L yeast extract (BD Bioscience), and 20 g/L agar (BD Bioscience)] with Congo red solution [0.5% Congo red (Sigma-Aldrich) and 0.25% Coomassie brilliant blue (Sigma-Aldrich) diluted in ethanol]. Plates were incubated for 5 days at 28°C, and results were interpreted using the four morphotypes: rdar (red, dry, and rough; curli and cellulose), pdar (pink, dry, and rough; cellulose only), bdar (brown, dry, and rough; curli only), and saw (smooth and white; neither curli nor cellulose). *E. coli* ATCC 25922 was used as the negative control.

Plasmid typing and conjugation assay

The replicon typing was conducted for the major plasmid incompatibility groups among *Enterobacteriaceae* (HI1, HI2, I1-Iγ, I2, X1, X2, X3, X4, L/M, FIA, FIB, FIC, FIIs, A/C, P, K B/O, and N) using a PCR-based replicon-typing method [149-151]. Conjugation assay was conducted with *E. coli* J53-AziR as the recipient and ESBL/AmpC-EC as the donors. LB agar

plates containing 4 mg/L of CTX and 100 mg/L of sodium azide were used to select the transconjugants. The presence of the ESBL/AmpC genes (*blaCTX-M* and *blaCMY*), plasmid-mediated antimicrobial resistance genes, and replicon types in the transconjugants was confirmed by PCR.

Multi-locus sequence typing and *E. coli* phylogenetic group typing

Of the 161 ESBL/AmpC-EC isolates from the present study, 138 isolates were selected based on their antibiotic resistance and ExPEC VFs to analyze multi-locus sequence typing (MLST) and *E. coli* phylogenetic group typing. The MLST of ESBL/AmpC-EC isolates was performed as described previously [152]. A detailed scheme of gene amplification, allelic type, and sequence type (ST) assignment methods is available on the pubMLST website.2 The minimum spanning tree (MST) based on allelic profiles of seven MLST housekeeping genes was constructed using the BioNumerics software, version 6.6 (APPLIED MATHS, Belgium). Seven *E. coli* phylogenetic groups (A, B1, B2, D, C, E, and F) were determined following a previously described protocol with application [153].

Similarity analysis of the clonal distribution of swine ESBL/AmpC-EC following the swine production stages within farms

We analyzed the similarity in the distribution of ST and phylogenetic groups following the swine production stages in each farm using the *k*-means similarity clustering algorithm based on Euclidean distance [154]. To find the optimal number of clusters (*k*), we applied the average silhouette method [155]. The average silhouette method presumes that the optimal number of clusters *k* is the one that maximizes the average silhouette over a range of possible values for *k*. In this study, the optimal value of *k* was nine

(Supplementary Figure 2). The *k*-means clustering analysis and the average silhouette method were conducted using the R software, version 4.3.2 (R foundation, Austria).

Based on the combination of nine swine farms ("farm A" to "farm I") and four production stages (weaning piglets to sows), a total of 36 points indicating ST composition at each farm's swine production stages were generated. Of the 36 points, three were excluded because there were no isolated ESBL/AmpC-EC strains from "growing stage" of "farm H," "pregnant stage" of "farm H," and "growing stage" of "farm I.". Finally, 33 points were clustered into nine clusters using the *k*-means algorithm based on the Euclidean distance of paired two points. The *k*-means clustering plot was generated using the R software, version 4.3.2 (R foundation, Austria).

Clonal Population Structure Analysis Based on the VFs and Phylogenetic Group Profiles

To evaluate the virulence potential of ESBL/AmpC-EC clones, a clonal population structure analysis based on their ExPEC VFs and phylogenetic group profile was conducted using program STRUCTURE [156]. 138 ESBL/AmpC strains were assigned into virulence profile populations (*k*) using a Baysian method in the program. The most likely number of populations (*k*) was defined by the value producing a maximal rate change in posterior probability, Δln (*k*) [157]. The optimal number of populations k was five in the present study (Supplementary Figure 3). Assignment coefficients (Q values such as proportions of population *k*) were generated for each strain using the Markov chain Monte Carlo searches, which consisted of 100,000 burn-in steps followed by 100,000 iteration steps.

In this clonal population analysis, 138 ESBL/AmpC-EC isolates were divided into five populations, and each population contained 24–34 ESBL/AmpC-EC isolates. Among the five populations, strains were assigned to their best-fit populations based on the highest Q value. The Q values of individual strains are presented in a 100% stacked bar chart sorted by the STs. Differences in the ExPEC VFs and phylogenetic groups in each population are presented in Table 3. The clonal population distribution of swine ESBL/AmpC-EC isolates for each ST-phylogenetic group is presented in Supplementary Table 3.

Genetic relatedness analysis of ESBL/AmpC-EC strains from various sources using public database

To identify the shared major STs between swine and human ESBL/AmpC-EC isolates, the whole genome sequence (WGS) data of human ESBL/AmpC-EC isolates registered in the NCBI database were used.

WGS data of 11,269 human ESBL/AmpC-EC isolates from human hosts whose assembly data were available (accessed on 20 May 2021) were downloaded and analyzed to determine the MLST STs (Supplementary Material 1). In total, 739 STs were identified from 11,269 human ESBL/AmpC-EC isolates. We selected the major 20 STs of human ESBL/AmpC-EC isolates covering 73.8% of the total isolates (8,320/11,269) for further analysis.

To analyze the genetic relatedness of ESBL/AmpC-EC strains isolated from this study and strains isolated from various sources in South Korea, a total of WGS of 363 human-derived ESBL/AmpC-EC strains and 15 ESBL/AmpC-EC strains isolated from South Korea and whose registered in the NCBI database.

Statistical Analyses

To adjust the farm-induced inherent factors, the comparative analyses between groups (one stage vs. other stages, ESBL/AmpC-EC vs. non-ESBL/AmpC-EC) were performed using the generalized estimating equation (GEE). In the GEE analysis, this study presumed that several characteristics of the isolates could be affected by farm factors; therefore, we set the farm as "subject variable" and the number of isolates in each farm as "within subject variables." Where zeros caused problems in calculating the odds ratio (OR) in the GEE analysis, the Fisher's exact test was conducted by adding 0.5 to each cell [158]. In the analysis of the differences in the average number of VFs and resistance to antibiotic classes between ESBL/AmpC-EC and non-ESBL/AmpC-EC, the student's *t*-test was applied. All statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS) program, version 27.0 (IBM SPSS Statistics for Windows, United States).

1.3. Results

Prevalence of ESBL/AmpC-EC in swine farms

In total, 161 (55.1%) ESBL/AmpC-EC isolates were identified from 292 individual swine fecal samples. The farm prevalence of ESBL/AmpC-EC ranged from 17.6 to 89.7% (Figure 8A). In the comparison by stages, the prevalence of weaning piglets (86.3%, 44/51, GEE, $P < 0.05$) was significantly higher than that of growing pigs (58.3%, 51/96), finishing pigs (48.4%, 45/95), and pregnant sows (43.1%, 21/50; Figure 8B).

Higher MDR of ESBL/AmpC-EC relative to non-ESBL/AmpC-EC

We investigated the antimicrobial resistance of the 161 ESBL/AmpC-EC isolates and compared them with those of the 81 non-ESBL/AmpC-EC isolates (Figure 9). Notably, ESBL/AmpC-EC isolates showed resistance to a higher number of antimicrobial classes (average: 5.7 antimicrobial classes) compared with that of non-ESBL/AmpC-EC isolates (average: 2.9 antimicrobial classes; $p < 0.05$). Furthermore, the MDR rate was significantly higher in the ESBL/AmpC-EC isolates (100%) compared with that in the non-ESBL/AmpC-EC isolates (63.0%; OR: 80.0, 95% CI: 11.68–547.88). Resistance to six antibiotics (AMC, CTX, CAZ, CRO, ATM, AK, and CT) was found only in ESBL/AmpC-EC isolates but not in non-ESBL/AmpC-EC isolates. Resistance to three antibiotic classes, which included broad-spectrum penicillin (OR: 100.8, 95% CI: 5.99–1694.62), aminoglycoside (OR: 5.6, 95% CI: 2.08–15.26), and quinolone (OR: 6.6, 95% CI: 3.31–12.99), was significantly higher in the ESBL/AmpC-EC strains compared to non-ESBL/AmpC-EC.

In the antimicrobial resistance genotyping, the aminoglycoside resistance gene, *aac(3)-II*, was significantly more prevalent in the ESBL/AmpC-EC isolates (OR: 6.2, 95% CI: 1.25–30.70) than in the non-ESBL/AmpC-EC (Supplementary Table 2).

ESBL/AmpC-EC with multiple ExPEC VFs

Among 161 ESBL/AmpC-EC strains and 81 non-ESBL/AmpC-EC strains, two strains were identified as intestinal pathogenic *E. coli*. One ESBL/AmpC-EC isolate was identified as enterotoxin producing *E. coli*, and

the other ESBL/AmpC-EC isolate was identified as enteropathogenic *E. coli*.

A significantly higher number of ExPEC VFs were identified in the ESBL/AmpC-EC isolates (average: 4.6 VFs) compared with that in the non-ESBL/AmpC-EC isolates (average: 3.6 VFs; $p < 0.05$). The OR of having 6–7 VFs were 8.8-fold greater (95% CI: 1.31–59.30, $p < 0.05$) in the ESBL/AmpC-EC isolates than in the non-ESBL/AmpC-EC isolates (Figure 10). In contrast, the ESBL/AmpC-EC isolates showed a 0.4-fold less OR to harbor three or less VFs relative to that in the non-ESBL/AmpC-EC isolates (95% CI: 0.13–0.97, $p < 0.05$). Three VFs were highly prevalent in the ESBL/AmpC-EC isolates, namely pyelonephritis-associate pilus C, *papC* (OR: 19.8, 95% CI: 2.54–153.45, $p < 0.05$), serine protease pic autotransporter, *pic* (OR: 19.6, 95% CI: 1.16–330.30, $p < 0.05$), and outer membrane protease T, *ompT* (OR: 1.9, 95% CI: 1.00–3.64, $p < 0.05$), compared with the non-ESBL/AmpC-EC isolates (Table 1).

Improved biofilm formation of ESBL/AmpC-EC

The biofilm formation rate was higher in ESBL/AmpC-EC isolates (42.2%, 68/161) than that in non-ESBL/AmpC-EC isolates (16.0%, 13/81). The OR of biofilm formation was 3.8-fold greater in the ESBL/AmpC-EC isolates than in the non-ESBL/AmpC-EC isolates (95% CI: 1.42–10.30, $p < 0.05$). No significant differences were observed in the formation of two biofilm-associated extracellular matrix components, curli fimbriae (OR: 1.80, 95% CI: 0.71–4.55, $p = 0.21$) and cellulose (OR: 0.45, 95% CI: 0.10–2.07, $p = 0.30$), from the ESBL/AmpC-EC isolates relative to the non-ESBL/AmpC-EC isolates.

Distribution of β-lactamases in ESBL/AmpC-EC according to the swine production stages

Out of the 161 ESBL/AmpC-EC isolates, 154 isolates (95.7%, 154/161) were identified as ESBL-EC carrying the CTX-M family β-lactamases (CTX-M), while seven isolates (4.3%, 7/161) were identified as AmpC-EC carrying the CMY family β-lactamases (CMY; Figure 11). None of the isolates carried both CTX-M and CMY together. The CTX-M group was found in ESBL/AmpC-EC in all production stages and occupied 88.2–100.0% of the ESBL/AmpC-EC (Figure 11B). However, the CMY group was identified in ESBL/AmpC-EC isolates only in the growing (11.8%, 6/51) and finishing stages (2.2%, 1/45).

The CTX-M-1 group β-lactamase (83.9%, 135/161) was the most prevalent CTX-M; CTX-M-55 β-lactamase (69.6%, 112/161), CTX-M-15 (11.8%, 19/161), CTX-M-64 (1.9%, 3/161), and CTX-M-146 (0.6%, 1/161) belonged to this group (Figure 11). The second most prevalent CTX-M was the CTX-M-9 group β-lactamase (11.8%, 19/161); CTX-M-14 (5.6%, 9/161), CTX-M-27 (4.3%, 7/161), and CTX-M-65 (1.9%, 3/161) belonged to this group. All seven CMY-producing *E. coli* isolates was identified to carry CMY-2 β-lactamase. All ESBL/AmpC-EC carried only one type of CTX-M β-lactamase or CMY β-lactamase, and there were no isolates carrying more than one CTX-M or CMY β-lactamase. TEM β-lactamase was found in the 31 ESBL/AmpC-EC isolates (24.8%); however, all of these TEM β-lactamases were identified as TEM-1, which was a non-ESBL type in the sequencing analysis. Other ESBL types and carbapenemases types were not identified in this study.

Antimicrobial susceptibility of ESBL/AmpC-EC according to the swine production stages

In the comparative analysis according to swine production stages, the weaning piglet-derived ESBL/AmpC-EC strains showed significantly higher resistance rate to colistin, compared to other stages (OR: 2.4, 95% CI: 1.03-5.57, $p < 0.05$, Table 2). The growing pig-derived strains exhibited significantly higher resistance rate to β-lactamase inhibitor class, compared to other stages (OR: 9.8, 95% CI: 1.14–84.70, $p < 0.05$).

Horizontal transferability of ESBL/AmpC

To evaluate the horizontal transferability of ESBL/AmpC genes, we conducted the conjugation assay on 138 ESBL/AmpC-EC strains (Supplementary Figure 1). The transferability of *blaCTX-M* was 58.8% (77/131); however, the transfer of the CMY gene *blaCMY−2* was not identified. The most prevalent replicon type in the transconjugant of CTX-M was IncFIB (90.9%, 70/77), followed by IncI1-Iγ (28%, 17/77), IncI2 (6.5%, 5/78), and IncX4 (3.9%, 3/77). Various antibiotic resistance genes were transferred with *blaCTX-M*. The highest transferability of antimicrobial resistance gene was identified in *floR* (94.9%, 56/59), followed by *aac(3)-II* (85.7%, 6/7), and *qnrS1* (75.0%, 12/16).

Clonal distribution of ESBL/AmpC-EC between swine farms

In the MLST analysis, a total of eight CCs were identified, and CC101-B1 (26.8%, 37/138), CC10-A (8.7%, 13/138), CC648-F (2.9%, 4/138),

and CC23-A (2.9%, 4/138) were the major CCs (Figure 12). Around 31 STs, including two non-typable STs, were identified, with ST101-B1 (22.8%, 31/138), ST457-F (16.2%, 22/138), ST75-B1 (12.5%, 17/138), and ST224-B1 (8.0%, 11/138) as the major STs.

Similarity of clonal distribution among swine production stages within farms

We evaluated the similarity in the clonal distribution of different production stages within the farms using the *k*-means similarity clustering algorithm (Figure 13; Supplementary Table 3). Distributions of STs and phylogenetic groups for each farm and production stage combination were presented in Supplementary Table 3. In Figure 13, 33 points represent the clonal distribution following the combination of nine farms ("farm A" to "farm I") and four stages ("weaning piglets" to "pregnant sows"). In the *k*-means clustering analysis, the points were clustered into nine clusters based on the similarity distance between them, and 2–7 points belonged to each cluster. We found that the clonal distribution of three or all stages in the same farm was clustered together, thereby showing similarity, except for "farm D." The clonal distribution of two stages in "farm D" were clustered together.

Clonal population structure analysis of swine ESBL/AmpC-EC based on ExPEC VFs and phylogenetic group profiles

Among identified clone types of swine farm-derived ESBL/AmpC-EC strains, nine MLST types were identified as ExPEC strains: ST75-B1 (15/138, 10.9%), ST101-B1 (11/138, 8.0%), ST457-F (10/138, 7.2%), ST2628-

B1 (6/138, 4.3%), ST648-F (4/138, 2.9%), ST23-A (2/138, 1.4%), ST12-B2 (1/138, 0.7%), ST3285-B1 (1/138, 0.7%), and ST744-A (1/138, 0.7%). ST648-F (4/138, 2.9%) strains were also identified as UPEC strains.

We conducted a clonal population structure analysis based on ExPEC VFs and phylogenetic group profiles of ESBL/AmpC-EC using the program STRUCTURE to evaluate the virulence potential of swine ESBL/AmpC-EC clones (Figure 15). The ESBL/AmpC-EC isolates in population 1 mainly identified as CC101-B1 and ST75-B1, and harbored the highest number (average 6.7) of VFs, which was characterized with the highest prevalence of *papC*, *hlyA*, *pic*, *iroNe.coli*, and *ompT* (Table 3). Isolates in population 2 mainly identified as CC101-B1 and CC86-B1. This population carried the second highest number of VFs (average: 4.8 VFs), characterized by the high prevalence of *tsh* and *fyuA*. Isolates in population 3 mainly identified as CC648-F and ST457-F (average: 4.2 VFs), with a high prevalence of *papC* and *ompT*. Isolates in population 4 mainly identified as CC10-A and CC23-A (average: 3.0 VFs). Finally, isolates in population 5 identified as ST224-B1 (average: 3.0 VFs). Isolates in populations 4 and 5 carried the lowest number of VFs.

Genetic relatedness of ESBL/AmpC-EC strains isolated from swine farms, pork meats, and humans

To identify the shared major STs of ESBL/AmpC-EC isolates in swine farms and human sources, we compared the STs of the swine ESBL/AmpC-EC isolates with the major 20 STs of the human ESBL/AmpC-EC isolates, registered in the NCBI Pathogen Isolates Browser database (Figure 14). Among

29 MLST STs from the swine farm-derived strains, seven STs (ST101, ST10, ST457, ST410, ST617, ST744, and ST648) were shared with the human-derived strains. These seven STs contained 68 (49.3%) of the 138 strains from the swine farms and 1,440 (17.3%) of the 8,320 strains belonging to the major 20 STs from human sources.

In the comparative analysis with South Korea-derived ESBL/AmpC-EC strains, nine STs (ST10, ST410, ST648, ST457, ST12, ST101, ST641, ST617) of swine farm-derived strains were shared with strains from humans (Supplementary table 5). In addition, seven STs (ST10, ST457, ST101, ST224, ST5229, ST48, ST1642) were shared with strains from pig farms, slaughterhouses, and pork meats.

1.4. Discussion and Conclusion

Swine farm husbandry has been regarded as an important reservoir of ESBL/AmpC-EC [159]. In the present study, the prevalence of ESBL/AmpC-EC in swine farms was 55.1%, which was comparable with the prevalence of ESBL/AmpC-EC in swine farms from previous studies conducted worldwide [5-16]. To date, three researches have been conducted for the occurrence of ESBL/AmpC-EC in pigs in South Korea [79, 80, 85]. The prevalence of ESBL/AmpC-EC in this study (55.1%) was significantly higher than in the two previous studies (18.4%, 140/760 and 21.5%, 120/558) [80, 85], and comparable to that of one study (69.5%, 41/59) in South Korea [79]. Unlike this study, swine stages were not considered in the previous studies conducted from South Korea. Tamang et al. (2008) and Song et al. (2020) conducted ESBL/AmpC-EC researches with only finishing pigs from slaughterhouses [79,

85]. Kim et al. (2021) conducted ESBL/AmpC-EC researches on swine farms without considering the swine stages [80]. In this study, the prevalence of ESBL/AmpC-EC was significantly higher in weaning piglets (86.3%), which was almost twice as high as in finishing pigs (48.4%). Studies conducted in Denmark [145], the Netherlands [144], and Germany [7] also confirmed a similar prevalence trend with a doubling of ESBL/AmpC-EC prevalence in weaning piglets compared to finishers. These results imply that the swine stages could affect the results of total prevalence of ESBL/AmpC-EC in swine farms. Taken together, this study suggests that a multi-stage systematic study design is essential for the study of CIA-resistant bacteria in food animal farms.

In the comparative analysis by swine stages, the occurrence of ESBL/AmpC-EC was significantly different depending on the stages. The occurrence of ESBL-EC was significantly higher in weaning piglets than in other stages. Whereas, the occurrence of AmpC-EC was significantly higher in growing pigs than in other stages. According to studies that investigated the patterns of antimicrobial use in a global swine industry, the types and volume of antimicrobial agents differ at different pig stages [34-36]. In particular, over 70% β-lactams, including broad-spectrum β-lactam and cephalosporins, used in the swine industry was applied between birth and 10 weeks of age [33, 39, 160, 161]. β-lactams have been commonly prescribed for the treatment and prevention of postweaning syndromes, including postweaning diarrhea, edema disease, and endotoxin shock, which are the major problems in swine industry [162]. Whereas, ceftiofur has been mainly prescribed to treat swine respiratory infection diseases, and these diseases has been reported to show higher prevalence in growing-finishing stages, compared to other stages [35, 163-165]. In general, ESBL-EC and AmpC-EC had different resistance profile against ceftiofur, a member of 3rd cephalosporin class; AmpC-EC usually carried

resistance against ceftiofur, while ESBL-EC does not [165]. The positive correlation between use of β-lactams and high occurrence of ESBL-EC and between use of ceftiofur and high occurrence of AmpC-EC have been continuously reported from various studies [35, 42, 43, 163, 165]. The different usages of antimicrobial agents according to swine stages could act as an important factor for the different distribution of ESBL/AmpC-EC according to swine stages. In addition, the different horizontal transferability of ESBL/AmpC genes could be another reason for the accumulation of AmpC-EC at growing-finishing stages, without spreading into other stages. From the conjugation assay in the present study, the AmpC genes were not horizontally transferred, whereas ESBL genes were transferred with a conjugation rate of 59.05%. Collectively, this study showed that the prevalence and characteristics of ESBL/AmpC-EC were different according to swine stages in swine farms, which are an important reservoir of ESBL/AmpC-EC.

In this study, all seven AmpC-producing isolates were identified to carry CMY-2, which was the most frequent reported CMY type found in human- and food-animal-derived AmpC producers worldwide [166]. Consistently, CMY-2 has been reported as the most prevalent CMY type of strains from South Korea [167-173]. According to global epidemiological studies, the most predominant ESBL types was CTX-M-15 and CTX-M-14 worldwide for the last decade, including South Korea [26, 174, 175]. Meanwhile, the most prevalent ESBL was CTX-M-55, followed by CTX-M-15 and CTX-M-14 in the present study. According to the recent studies, the prevalence of CTX-M-55 has been recently increasing in Europe [176, 177], North America [9], and Asia [16, 178, 179]. Consistently, CTX-M-14 and CTX-M-15 were reported to be the major types among the ESBL/AmpC-EC in pig farms in one research conducted in South Korea in 2008 [85], whereas CTX-

M-55 was the major type among ESBL/AmpC-EC from swine farms in two researches conducted in 2017-2019 [79, 80]. CTX-M-55, which differs from CTX-M-15 by one substitution, A77V, has been reported to display enhanced catalytic activity against expanded-spectrum cephalosporins [180]. The CTX-M-55 exhibits higher structure stability, most likely by forming hydrophobic interactions between A77V and various key residues in different helices, thereby stabilizing the core architecture of the helix cluster, and indirectly contributes to a more stable active site conformation, which in turn shows higher catalytic efficiency and is more tolerant to temperature change. Collectively, these results suggest the possibility of global epidemiological shift in ESBL towards types with stabilized catalytic sites.

In the comparative analysis of ESBL/AmpC-EC strains from swine farms and various sources in South Korea, the most prevalent ESBL/AmpC types in humans were CTX-M-14 (47.4%, 173/365), followed by CTX-M-15 (25.5%, 93/365), CTX-M-55 (31/365, 8.5%). Whereas, the CTX-M-55 was the major ESBL type in pigs 33.3% (4/12) and chickens 47.6% (10/21). Consistently, recent studies conducted from South Korea reported that CTX-M-55 was common type in ESBL/AmpC-EC isolated from pig carcasses of slaughterhouses and retail pork meats [80], as well as chicken carcasses slaughterhouse and retail poultry meats [84]. Collectively, CTX-M-55, which is uncommonly distributed in humans, was found to be widely disseminated in livestock farms and livestock-derived meat. This result provides the indirect scientific evidence that livestock farms can act as an important reservoir of CTX-M-55-type ESBL-EC and that swine farm-derived strains could be transmitted to humans through the food-chain such as the slaughtering and meat-processing environment.

Here, the prevalent clone types of ESBL/AmpC-EC from swine farms were ST101-B1 (CC101), ST10-A (CC10), ST457-F, and ST648-F. And these clone types were consisted of ExPEC/UPEC strains. Consistently, a study on the ESBL/AmpC-EC isolates from swine at slaughterhouses in South Korea reported CC101 as the major CC type of ESBL-EC from pigs followed by CC10 [79]. In contrast, the most common CC of ESBL/AmpC-EC was CC10 in other countries including Portugal, Netherlands, Taiwan, and China, while the second most common CC varied across studies, including CC155, CC405, or CC648 [15, 181-184]. In the clonal population structure analysis, ST101-B1 was identified as the most virulent clone type with highly virulent ExPEC profiling. In recent, ESBL/AmpC-EC clone type ST101 has attracted renewed global attention in human ESBL/AmpC-producing ExPEC infections given its enhanced virulence and pan-drug resistance [136, 185, 186], and has been reported to cause hemolytic uremic syndrome [187] and bloodstream infections [136]. The ST648 has been also reported to have a similar high MDR level and virulence compared to ST131, a global high-risk clone, and exhibit high potential biofilm formation ability and adaptability to UTI infection [188, 189]. The ST457 has been reported as a pandemic *E. coli* clone in humans and animals worldwide, and is one of the main high-risk clones that cause bacteremia/sepsis and UTI in human infections [190].

Next, we compared the MLST types of ESBL/AmpC-EC isolated in this study with strains isolated from slaughterhouses and pork of retail markers of South Korea. And it was confirmed that all MLST types identified in pork (i.e., ST101, ST457, ST10, etc.) were shared with the pig farm-derived strains in this study, with the most prevalent type of ST101. Meanwhile, the major MLST types of human derived-ESBL/AmpC-EC strains were consisted of global high-risk ExPEC clone types including ST131, ST38, ST69, ST10, and

ST410 [185], which differed from pig-derived strains (Supplementary Table 5). However, it was also confirmed that the major ExPEC clones of swine farm and pork meats-derived strains were shared with human-derived strains. These results provide indirect scientific evidence for the hypothesis that potentially high-risk zoonotic clones, including ST101, ST648, and ST457, present in pig farms could be transmitted from pig farms to humans through the food-chain. However, since MLST-based genetic relatedness analysis provide relatively lower discriminative power than WGS- and cgMLST-based phylogenetic analysis, it seems need additional WGS-based analysis to strengthen this hypothesis.

The horizontal gene transfer system plays a crucial role in the transmission of ESBL/AmpC-EC; however, the clonal transfer could also be important in their transmission [144]. Schmithausen et al. (2015) reported that ESBL-EC from individual pigs could spread into farm environments and almost the entire pigs present within the compartment could be affected by ESBL-EC from individual pigs [7]. When the clonal distribution in the swine production stages was compared *via* the *k*-means clustering analysis, we found that the clonal distribution of three or all stages from the same farm tended to be clustered together, showing similarity of clonal distribution. A similar clonal distribution between production stages implies the high possibility of cross-infection between stages within farms. Furthermore, ESBL/AmpC-EC from swine farms showed MDR, multiple virulence factors, and enhanced biofilm formation ability relative to non-ESBL/AmpC-EC. Especially, the prevalence of the three VFs, namely *papC*, *pic*, and *ompT*, which were reported to show a positive association with the high mortality in human ExPEC infection [191-194], was significantly higher in the ESBL/AmpC-EC isolates ($p < 0.05$). Biofilm formation conferred fitness advantage to the bacteria by enhancing

their survivability, increasing their virulence, and facilitating their ability to acquire virulence and antibiotic resistance genes during horizontal gene transmission due to their high microbial density [195, 196]. Collectively, these enhanced properties, namely MDR, multiple virulence factors, and enhanced biofilm formation ability, of ESBL/AmpC-EC from swine farms could make them difficult to be controlled once introduced to swine farms, allowing ESBL/AmpC-EC to survive and continue to exist within swine farms. Our result suggests that through the repeated cycle, which involved the shedding from swine through feces, survival in the farm environment, and reintroduction to swine, the ESBL/AmpC-EC could spread into other swine at different stages and could continue to exist within swine farms.

In conclusion, to the best our knowledge, this study is the first study to analyze the characteristics and dynamics of ESBL/AmpC-EC in swine farms and analyzed their differences by breeding stages. Swine farms derived ESBL/AmpC-EC shared high-risk ESBL/AmpC-producing ExPEC clone types (ST101, ST648, ST457) with human-derived strains in South Korea, proposing these clones as potentially high-risk zoonotic clones. In addition, the ESBL/AmpC types and clone types were shared between strains from pigs, porks and humans, implying indirect scientific evidence that ESBL/AmpC-EC strains may be transmitted to humans through the food-chains. In order to strengthen this hypothesis, additional comparative analysis based on WGS with higher discriminative power seems to be needed. Notably, this study showed that the prevalence and characteristics of ESBL/AmpC-EC strains in swine farms significantly differ depending on the swine production stages, highlighting the need for a multi-stage systematic policy based on the breeding stages to monitor and control potentially high-risk ESBL/AmpC-EC clones in swine farms.

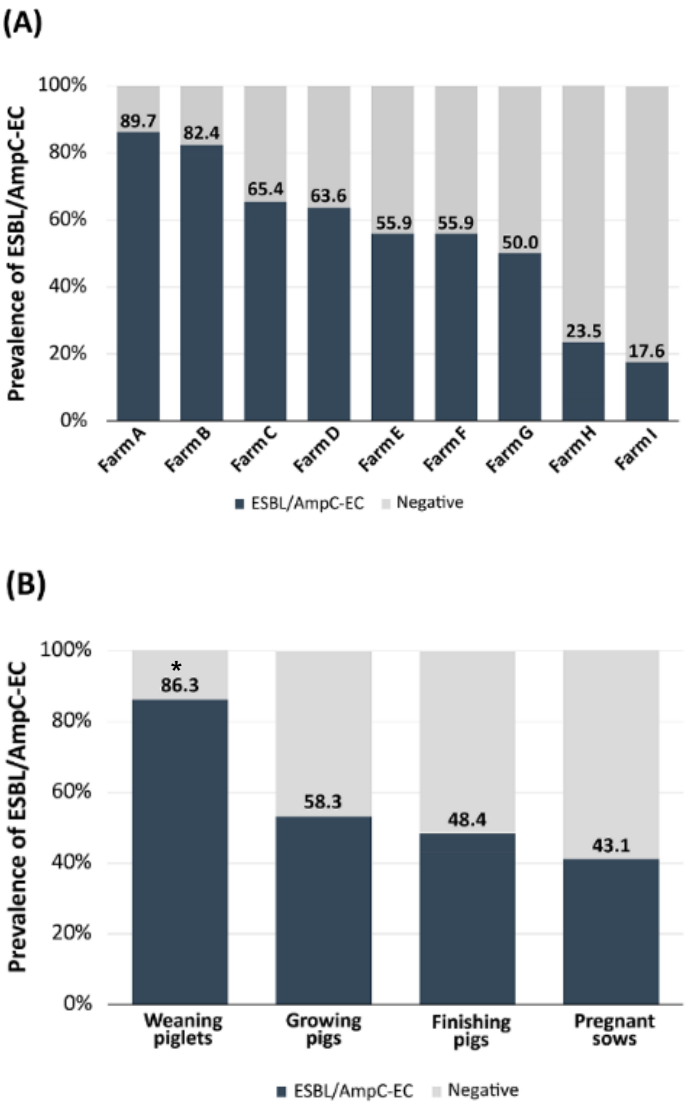


Figure 8. Prevalence of extended spectrum β-lactamase (ESBL)- or AmpC β-lactamase (AmpC)-producing *E. coli* (ESBL/AmpC-EC) according to different swine farms (A) and swine production stages (B). Prevalence of ESBL/AmpC-EC of weaning piglet was significantly higher that of growing, finishing, and pregnant sows, respectively. ($^{*}p < 0.05$, GEE)

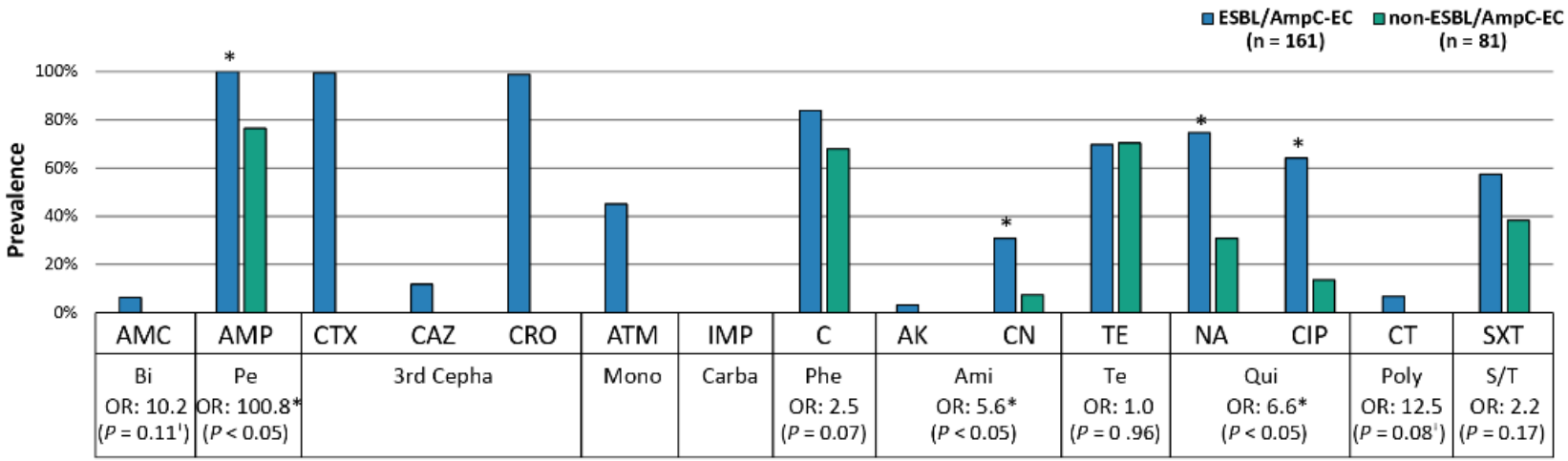


Figure 9. ESBL/AmpC-EC showed significantly higher resistance rate to antibiotics compared to non-ESBL/AmpC-EC. Statistically significant ($^{*}p < 0.05$, GEE; $^{\dagger}p < 0.05$, Chi-square test).

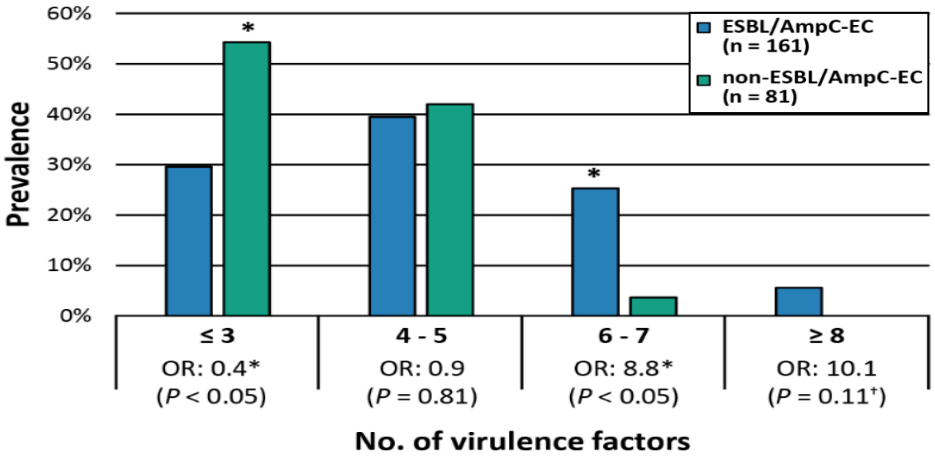


Figure 10. ESBL/AmpC-EC carried higher number of extra-intestinal pathogenic *E. coli* associated virulence factors (ExPEC VFs) compared to non-ESBL/AmpC-EC. OR, odds ratio. Statistically significant ($^{*}p < 0.05$, GEEs; $^{\dagger}p < 0.05$, Chi-square test).

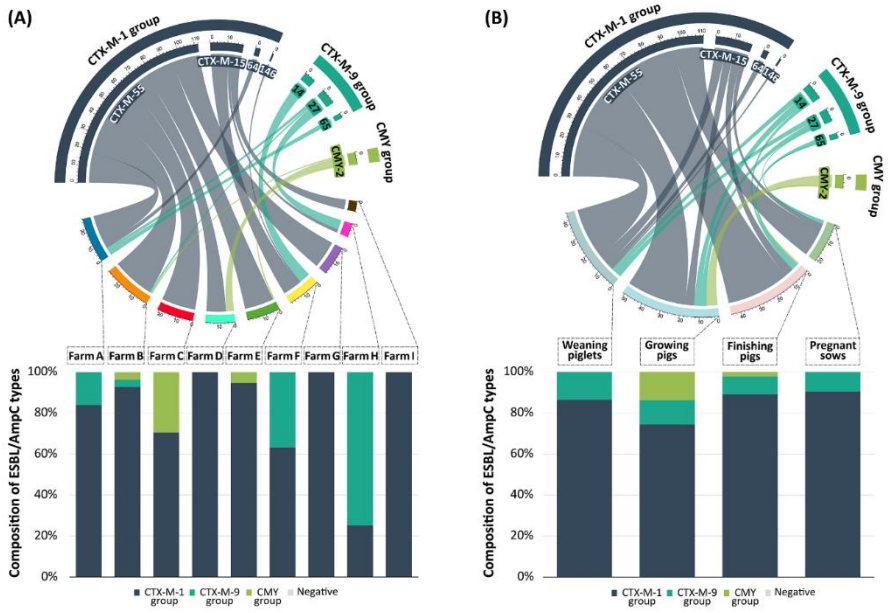


Figure 11. Prevalence and distribution of ESBL/AmpC types from swine farms. In the chord diagram, the size of segments on the top represents the number of ESBL/AmpC-EC isolates with a specific ESBL/AmpC types. Size of segments on the bottom represent the number of ESBL/AmpC-EC isolates detected in different farms (A) and production stages (B). Ribbons connecting the top and bottom segments represent the number of ESBL/AmpC-EC isolates with a specific ESBL/AmpC type found on the respective farms and production stages. The connected bar chart shows the composition of ESBL/AmpC types based on the number of ESBL/AmpC-EC isolates in different farms (A) or production stages (B). The chord diagram and bar chart were generated with R software (ver. 4.3.2).

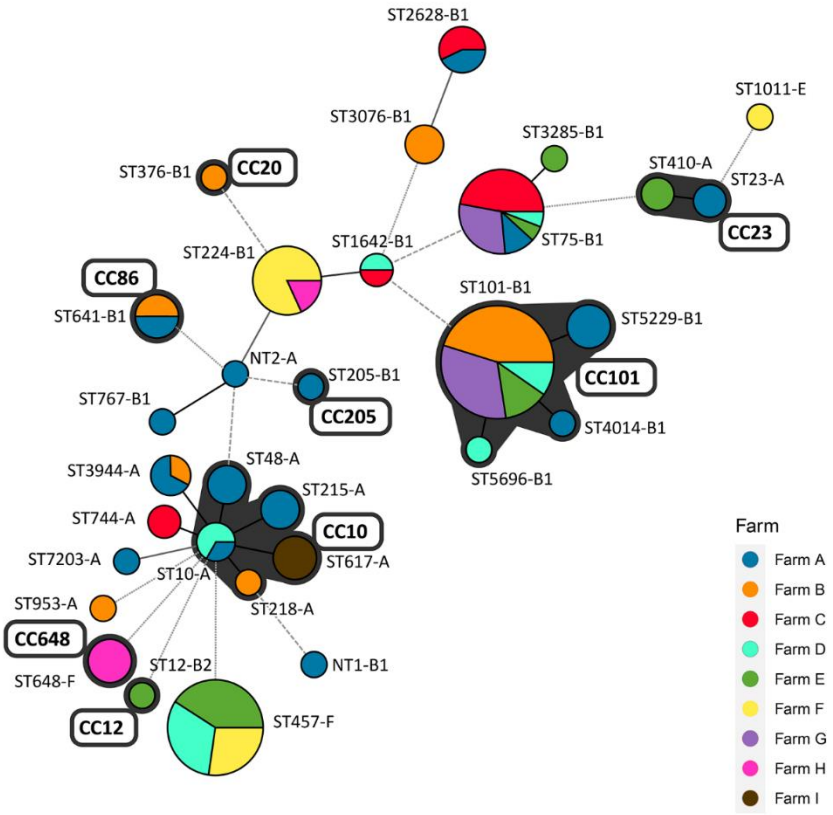


Figure 12. Minimum spanning tree (MST) based on allele profiles of multi-locus sequence type (MLST): clonal distribution of ESBL/AmpC-EC between pig farms. The number shows the sequence type of each node, and the size of the node indicates the number of strains belonging to the sequence type (ST)-phylogenetic group. The gray shadow represents the clonal complex (CC). Branch line types represent differences in the number of alleles: bold solid line (1 allele), thin solid line (2–3 alleles), dashed line (4 alleles), and dotted line (above 5 alleles). CC, clonal complex; ST, sequence type; NT, non-typable ST (including two different non-typable STs).

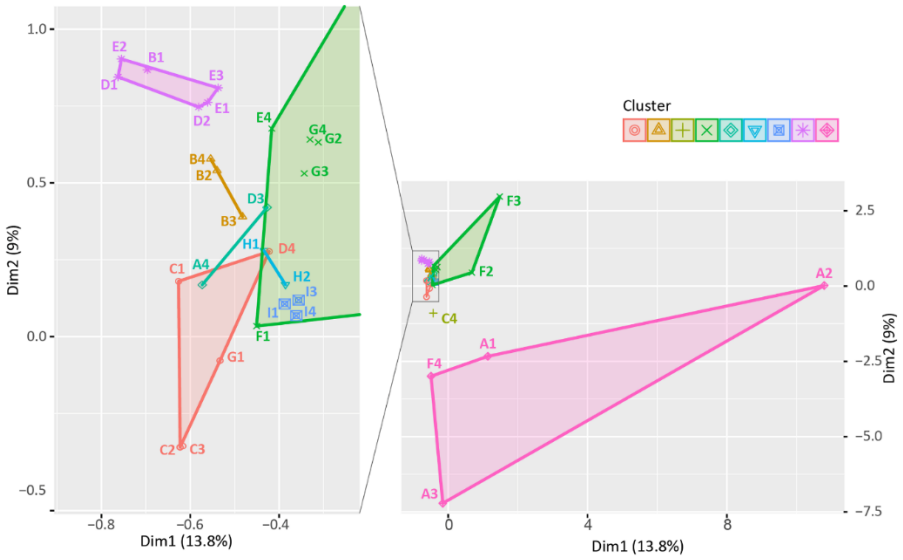


Figure 13. *k*-means similarity clustering plot: similarity in the clonal distribution among swine production stages within farms. A total of 33 points were described and clustered into 9 (symbols and colors) using the *k*-means similarity clustering algorithm based on Euclidean distance. The *k*-means cluster plot was generated using the R software (ver. 4.3.2). Each point indicates the distribution of STs among swine production stages in each farm, consisting of 36 points based on combination of 9 farms (A to I) and 4 production stages (1, weaning piglets; 2, growing pigs; 3, finishing pigs; and 4, pregnant sows; e.g., A1 presents the clonal distribution of "Weaning piglets" of "Farm A"). Three points (H2, H4, and I2) were excluded as no ESBL/AmpC-EC strains were isolated from "Growing pigs" of "Farm H", "Pregnant sows" of "Farm H," and "Growing pigs" of "Farm I."

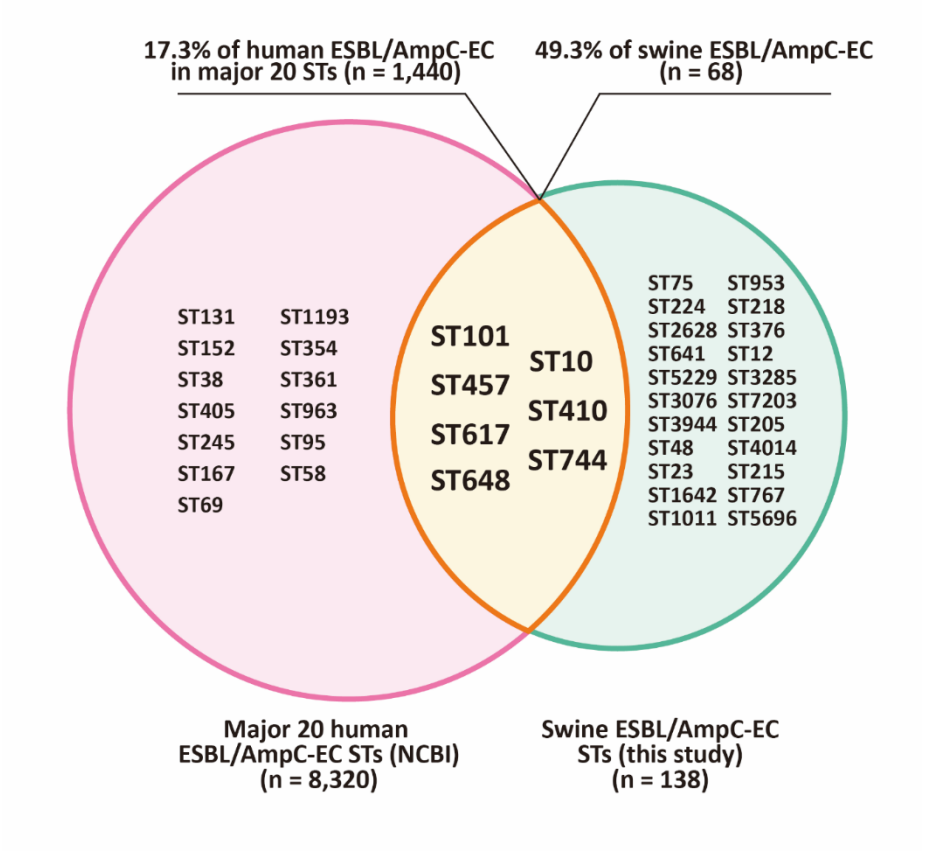


Figure 14. Shared major STs of ESBL/AmpC-EC isolated from swine farms and humans. Venn diagram shows the STs shared between swine farm derived ESBL/AmpC-ECs from this study and ESBL/AmpC-ECs from human sources which registered in the NCBI Pathogen Isolation Database. The intersection area of the two circles represents to the seven shared STs (ST101, ST10, ST457, ST410, ST617, ST744, and ST648) of ESBL/AmpC-EC from swine farms and human sources. ST, sequence type.

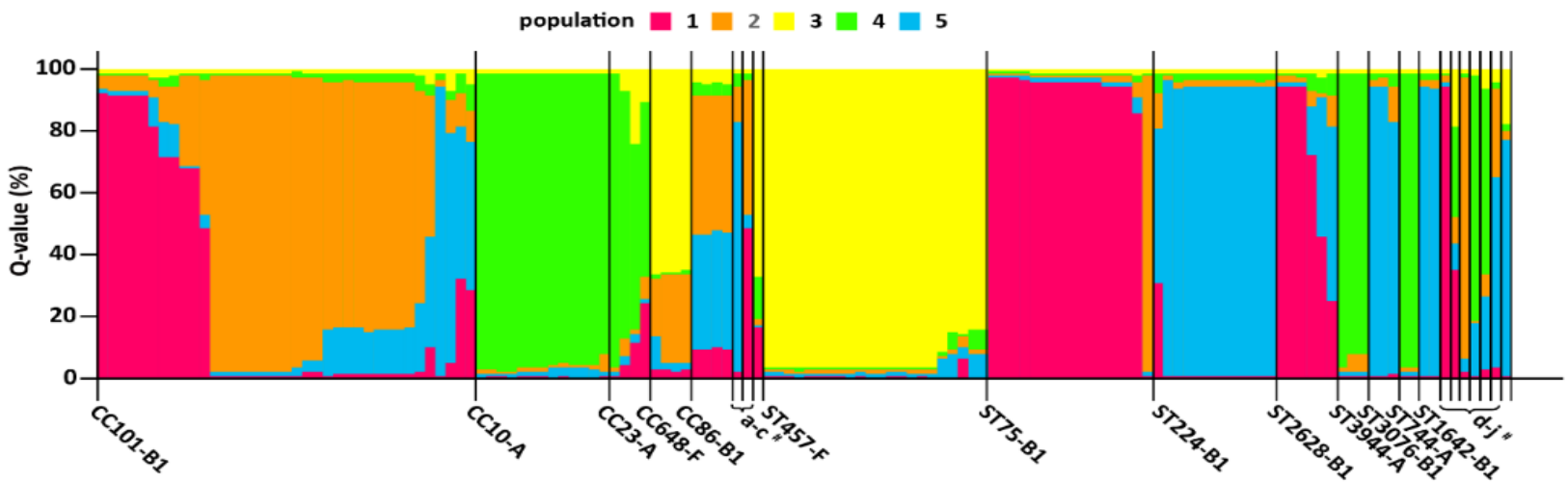


Figure 15. A clonal population analysis of swine ESBL/AmpC-EC isolates using program structure. Each isolate was assigned to five populations based on their ExPEC VFs and E. coli phylogenetic group profile. The ExPEC VFs and E. coli phylogenetic group profile for each clonal population are presented in Table 3. Each isolate is represented by a vertical segment and aligned horizontally according to CCs and STs (x-axis). The proportion of population (Q value) for each isolate is shown as 100% stacked bar plots, with proportions of colored sections representing the probability of belonging to each population within each segment (y-axis). CC, clonal complex; ST, sequence type; STNT, ST non-typable. #a, CC205-B1; b, CC376-B1; c, CC12-B2; d, ST3285-B1; e, ST953-A; f, NT-B1; g, ST7203-A; h, STNT-A; I, ST767-B1; and j, ST1011-E

Table 1. Comparison of the carriage of virulence factors between ESBL/AmpC-EC and non-ESBL/AmpC-EC isolates

Virulence factor function	Virulence factor	ESBL/AmpC-EC (n = 161)	non-ESBL/AmpC-EC (n = 81)	OR (95% CI)	*p* – value
Adhesion	*fimH*	96.3%	96.3%	1.0 (0.10-10.55)	1.00
	iha	3.1%	0.0%	5.8 (0.31-105.49)	0.24†
	papC	33.3%	2.4%	19.8 (2.54-153.45)	< 0.01*
	csgA	99.4%	95.1%	8.4 (0.72-97.63)	0.09
Toxin	*hlyA*	4.3%	6.1%	0.7 (0.17-2.75)	0.60
	astA	11.7%	19.5%	0.4 (0.12-1.04)	0.06
	aat	0.6%	3.7%	0.2 (0.02-1.60)	0.11†
	pic	10.5%	0.0%	19.6 (1.16-330.30)	0.04†
	tsh	9.3%	12.2%	0.7 (0.18-2.94)	0.65
Siderophore	*fyuA*	28.4%	11.0%	3.2 (0.57-17.78)	0.19
	iroNe.coli	27.2%	7.3%	4.7 (0.70-31.13)	0.11
Protectin / Serum resistance	*ompT*	57.4%	42.7%	1.9 (1.00-3.64)	0.049*
	traT	77.2%	63.4%	2.0 (0.69-5.74)	0.20

* Statistically significant. *$p < 0.05$, Generalized estimating equations, †*p*-value was calculated using Chi-square test. CI: Confidence interval

Table 2. Resistance rate of ESBL/AmpC-EC against antimicrobial classes following the swine productions stages

Anti-microbial classes	Anti-microbials	Weaning piglets			Growing pigs			Finishing pigs			Pregnant sows		
		Prevalence (%)	OR (95% CI)	*p* - value	Prevalence (%)	OR (95% CI)	*p* - value	Prevalence (%)	OR (95% CI)	*p* - value	Prevalence (%)	OR (95% CI)	*p* - value
Pe	AMP	100.0	-	-	100.0	-	-	100.0	-	-	100	-	-
3rd Cepha	CTX, CAZ, CRO	100.0	-	-	100.0	-	-	100.0	-	-	100	-	-
Carba	IMP	0.0	-	-	0.0		-	0.0	-	-	0.0	-	-
Mono	ATM	45.5	1.0 (0.47-2.21)	0.96	44.2	1.0 (0.42-2.18)	0.92	48.9	1.2 (0.69-2.21)	0.47	38.1	0.7 (0.27-1.95)	0.52
Bi	AMC	0.0	0.1 (0.01-2.02)	0.14†	15.4	9.8 (1.14-84.70)	0.04*	4.4	0.6 (0.09-4.65)	0.65	0.0	0.6 (0.13-3.11)	0.73†
Phe	C	88.6	1.7 (0.53-5.42)	0.38	86.5	1.3 (0.62-2.93)	0.46	77.8	0.6 (0.25-1.25)	0.16	81.0	0.8 (0.48-1.27)	0.33
Ami	AK, CN	40.9	1.9 (0.84-4.13)	0.13	28.9	0.9 (0.35-2.17)	0.76	24.4	0.7 (0.28-1.51)	0.31	28.6	0.9 (0.27-2.93)	0.84
Te	TE	70.5	1.1 (0.67-1.64)	0.84	69.2	1.0 (0.39-2.37)	0.93	66.7	0.8 (0.47-1.44)	0.49	76.2	1.5 (0.56-3.79)	0.45
Qui	NA, CIP	81.8	1.8 (0.65-4.68)	0.27	76.9	1.2 (0.54-2.63)	0.66	68.9	0.7 (0.27-1.65)	0.38	66.7	0.6 (0.17-2.32)	0.49
S/T	SXT	52.3	0.8 (0.36-1.57)	0.45	65.4	1.6 (0.61-4.37)	0.33	60.0	1.2 (0.48-2.78)	0.74	42.9	0.5 (0.15-1.69)	0.27
Poly	CT	11.4	2.4 (1.03-5.57)	0.04*	7.7	1.2 (0.39-3.83)	0.73	2.2	0.2 (0.10-0.57)	<0.01*	4.8	0.7 (0.35-1.24)	0.20

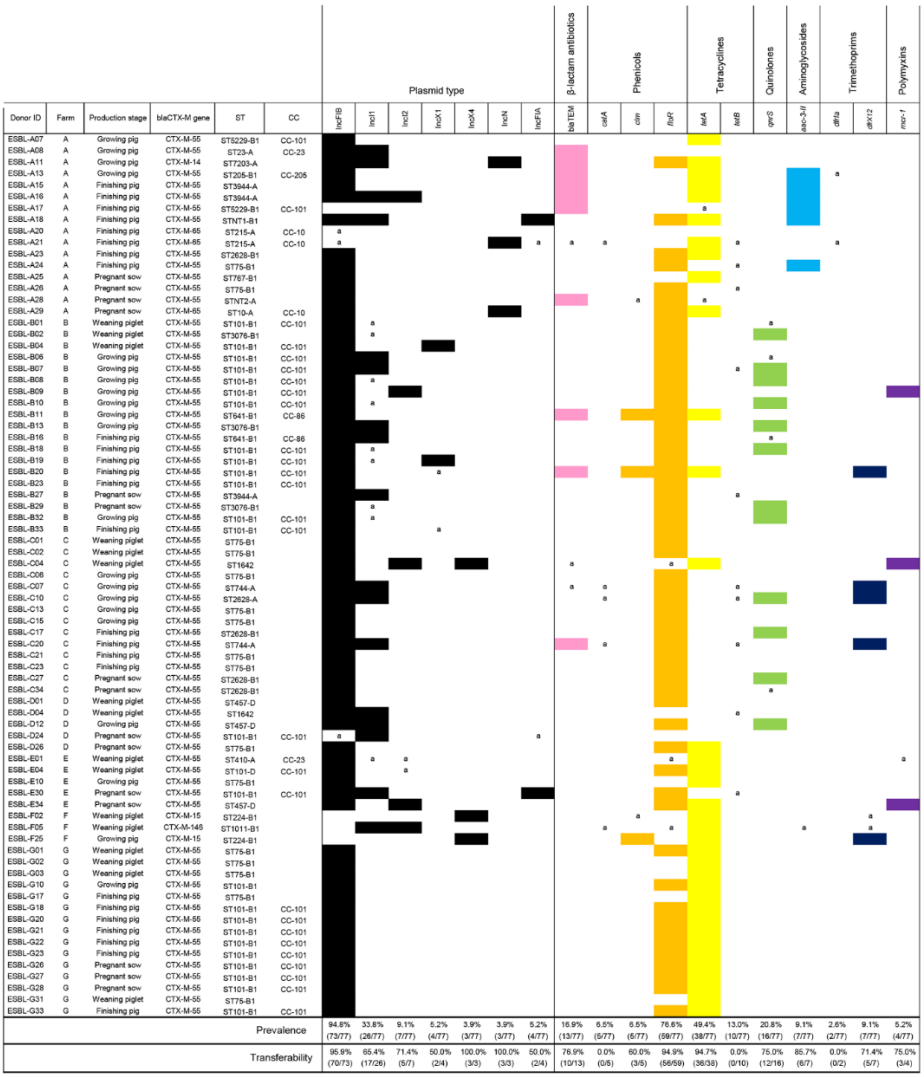
* Statistically significant. *p < 0.05, Generalized estimating equations, †p-value was calculated using Chi-square test. The odds ratio was calculated based on the resistance rate of ESBL/AmpC-EC from specific stages against those from other remaining stages.

* Abbreviation. OR, Odds ratio; Bi, β-lactamase inhibitor class; Pe, broad spectrum penicillin class; 3rd Cepha, 3rd cephalosporin class; Mono, monobactam class; Carba, carbapenem class; Phe, phenicol class; Ami, aminoglycoside class; Te, tetracycline class; Qui, quinolone class; Poly, polymyxin class; S/T, sulphoamide/trimethoprim class; AMC, amoxicillin/clavulanate; AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; ATM, Aztreonam; IMP, imipenem; C, chloramphenicol; AK, amikacin; CN, gentamycin; TE, tetracycline; NA, nalidixic acid; CIP, ciprofloxacin; CT, colistin; SXT, sulfamethoxazole/trimethoprim.

Table 3. The carriage of ExPEC VFs and phylogenetic group in each clonal population

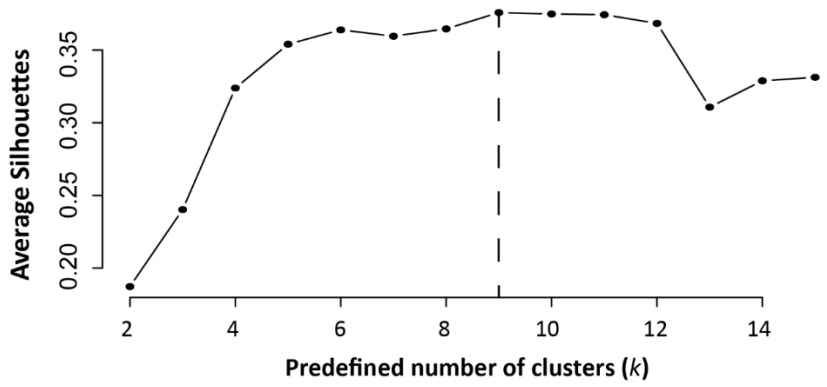
Population	Phylogenetic group (n[a])	Mean no. of VF[b]	Prevalence (%) of:												
			Adhesion				Toxin					Siderophore		Protectin / Serum resistance	
			fimH	*iha*	*papC*	*csgA*	*hlyA*	*astA*	*pic*	*tsh*	*aat*	*fyuA*	*iroN e.coli*	*ompT*	*traT*
1	B1 (33), A (1)	6.7	100.0	11.8	82.4	100.0	14.7	2.9	32.4	11.8	0.0	23.5	97.1	94.1	97.1
2	B1 (28)	4.8	100.0	0.0	0.0	100.0	0.0	0.0	0.0	39.3	0.0	78.6	0.0	60.7	100.0
3	F (26) B2 (1)	4.2	100.0	0.0	44.4	96.3	0.0	7.4	0.0	0.0	0.0	18.5	3.7	88.9	55.6
4	A (24)	3.0	83.3	0.0	8.3	100.0	0.0	4.2	0.0	0.0	0.0	16.7	0.0	12.5	75.0
5	B1 (24) E (1)	3.0	100.0	0.0	4.0	100.0	0.0	28.0	4.0	0.0	0.0	4.5	8.0	8.0	44.0

* n[a]: number of isolates, VF[b]: extra-intestinal pathogenic virulence factors

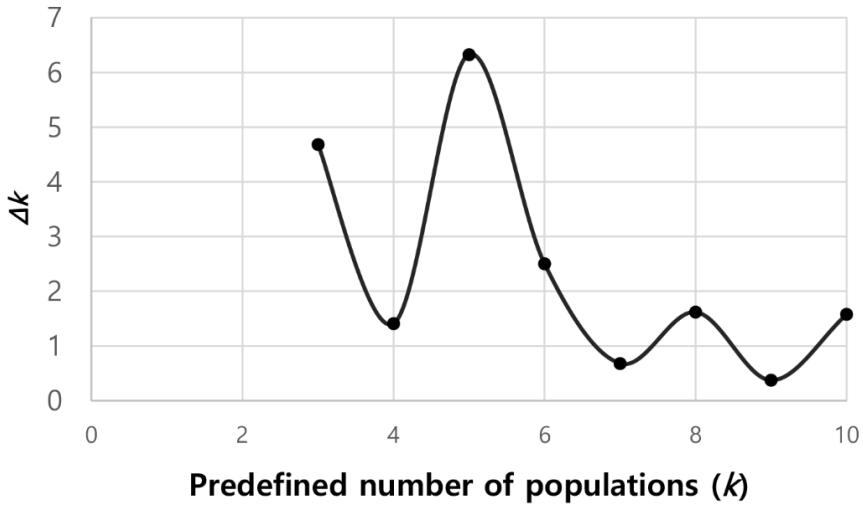


Supplementary Figure 1. Transferability of the β-lactamase gene, resistance genes, and replicon types in ESBL/AmpC-EC in the conjugation assay. Colored cells indicate the transfer of genes/plasmids represented by each column in the conjugation assay with *Escherichia coli* J53-Azi^{R} strain. The transferability of genes was confirmed by PCR.

[a] detected in donor strains but did not transfer, Blank = plasmid replicon or antimicrobial resistance genes were not detected in donor strains



Supplementary Figure 2. Determination of the best-fit cluster number by average silhouettes method for the *k*-means clustering algorithm. An average silhouette method presumes that the optimal number of clusters k is the one that maximizes the average silhouette over a range of possible values for k in the k-means clustering algorithm.



Supplementary Figure 3. Determination of the best-fit population number using the Bayesian approach for population structure analysis. Δ*k* is the second-order rate of change of *k*. A clear peak shows the most likely value of *k* (5) in the population structure analysis.

Supplementary Table 1. Oligonucleotide primers and annealing temperature used in this study.

Function	Genes		Nucleotide sequence	Size (bp)	Temp. (°C)	Reference
ESBL types	*bla*$_{CTX-M-1}$ group	F	GTTACAATGTGTGAGAAGCAG	1,041	60	[197]
		R	CCGTTTCCGCTATTACAAAC			
	bla$_{CTX-M-2}$ group	F	CGACGCTACCCCTGCTATT	832	60	[197]
		R	CAGAAACCGTGGGTTACGAT			
	bla$_{CTX-M-8}$ group	F	GGCGCTGGAGAAAAGCAG	862	60	[197]
		R	GGTTTTATCCCCGACAACC			
	bla$_{CTX-M-9}$ group	F	GTGACAAAGAGAGTGCAACGG	857	60	[197]
		R	ATGATTCTCGCCGCTGAAGCC			
	bla$_{CTX-M-25}$ group	F	GCACGATGACATTCGGG	327	60	[197]
		R	AACCCACGATGTGGGTAGC			
	bla$_{CMY}$	F	AACACACTGATTGCGTCTGAC	1,226	60	[197]
		R	CTGGGCCTCATCGTCAGTTA			
	bla$_{SHV}$	F	TCGCCTGTGTATTATCTCCC	768	54	[197]
		R	CGCAGATAAATCACCACAATG			
	bla$_{TEM}$	F	TCCGCTCATGAGACAATAACC	1,057	58	[197]
		R	ACGCTCAGTGGAACGAAAAC			
	bla$_{OXA}$	F	ACACAATACATATCAACTTCGC	813	60	[197]
		R	AGTGTGTTTAGAATGGTGATC			
Phylogenetic group	*yjaA*	F	CAAACGTGAAGTGTCAGGAG	288	55	[153]
		R	AATGCGTTCCTCAACCTGTG			
	chuA	F	ATGGTACCGGACGAACCAAC	211	55	[153]
		R	TGCCGCCAGTACCAAAGACA			
	tspE4.C2	F	CACTATTCGTAAGGTCATCC	152	55	[153]
		R	AGTTTATCGCTGCGGGTCGC			
	AceK.f	F	AACGCTATTCGCCAGCTTGC	400	55	[153]
	ArpA1.r	R	TCTCCCCATACCGTACGCTA			
	ArpAgpE.f	F	GATTCCATCTTGTCAAAATATGCC	301	55	[153]
	ArpAgpE.r	R	GAAAAGAAAAAGAATTCCCAAGAG			
	trpAgpC.1	F	AGTTTTATGCCCAGTGCGAG	219	55	[153]
	trpAgpC.2	R	TCTGCGCCGGTCACGCCC			
	trpBA.f	F	CGGCGATAAAGACATCTTCAC	489	55	[153]
	trpBA.r	R	GCAACGCGGCCTGGCGGAAG			
Virulence factors	*fimH*	F	TGCAGAACGGATAAGCCGTGG	508	63	[198]
		R	GCAGTCACCTGCCCTCCGGTA			
	iha	F	CTGGCGGAGGCTCTGAGATCA	827	55	[199]
		R	TCCTTAAGCTCCCGCGGCTGA			
	papC	F	GTGGCAGTATGAGTAATGACCGTT	200	63	[200]
		R	ATATCCTTTCTGCAGGGATGCAATA			
	csgA	F	ACTCTGACTTGACTATTACC	200	55	[198]
		R	AGATGCAGTCTGGTCAAC			
	astA	F	TGCCATCAACACAGTATATCCG	102	65	[201]
		R	ACGGCTTTGTAGTCCTTCCAT			

	hlyA	F	AACAAGGATAAGCACTGTTCTGGC	1,176	63	[199]
		R	ACCATATAAGCGGTCATTCCCGTCA			
	aat	F	CTGGCGAAAGACTGTATCAT	629	53	[202]
		R	CAATGTATAGAAATCCGCTGTT			
	tsh	F	ACTATTCTCTGCAGGAAGTC	824	55	[199]
		R	CTTCCGATGTTCTGAACGT			
	pic	F	AGCCGTTTCCGCAGAAGCC	1,111	63	[201]
		R	AAATGTCAGTGAACCGACGATTGG			
	traT	F	GGTGTGGTGCGATGAGCACAG	290	60	[198]
		R	CACGGTTCAGCCATCCCTGAG			
	ompT	F	ATCTAGCCGAAGAAGGAGGC	559	64	[199]
		R	CCCGGGTCATAGTGTTCATC			
	fyuA	F	TGATTAACCCCGCGACGGGAA	880	63	[198]
		R	CGCAGTAGGCACGATGTTGTA			
	iroNe.coli	F	AAGTCAAAGCAGGGGTTGCCCG	665	63	[199]
		R	GACGCCGACATTAAGACGCAG			
Antimicrobial resistance	*catA*	F	AGTTGCTCAATGTACCTATAACC	547	57	[203]
		R	TTGTAATTCATTAAGCATTCTGCC			
	cmlA	F	CCGCCACGGTGTTGTTGTTATC	698	57	[203]
		R	CACCTTGCCTGCCCATCATTAG			
	floR	F	TATCTCCCTGTCGTTCCAG	399	52	[203]
		R	AGAACTCGCCGATCAATG			
	tetA	F	GCTACATCCTGCTTGCCTTC	210	58	[203]
		R	CATAGATCGCCGTGAAGAG			
	tetB	F	TTGGTTAGGGGCAAGTTTTG	659	56	[203]
		R	GTAATGGGCCAATAACACCG			
	tetD	F	AAACCATTACGGCATTCTGC	787	60	[203]
		R	GACCGGATACACCATCCATC			
	qnrA	F	ATTTCTCA CGCCAGGATTTG	516	53	[204]
		R	GATCGGCAAAGGTTAGGTCA			
	qnrB	F	GATCGTGAAAGCCAGAAAGG	469	53	[204]
		R	ACGATGCCTGGTAGTTGTCC			
	qnrC	F	GGGTTGTACATTTATTGAATC	447	50	[204]
		R	TCCACTTTACGAGGTTCT			
	qnrS	F	ACGACATTCGTCAACTGCAA	417	53	[204]
		R	TAAATTGGCACCCTGTAGGC			
	aac(6)-Ib-cr	F	TTGCGATGCTCTATGAGTGGCTA	482	50	[204]
		R	CTCGAATGCCTGGCGTGTTT			
	aac(3)-I	F	ACCTACTCCCAACATCAGCC	169	60	[203]
		R	ATATAGATCTCACTACGCGC			
	aac(3)-II	F	ACTGTGATGGGATACGCGTC	237	60	[203]
		R	CTCCGTCAGCGTTTCAGCTA			
	aac(3)-IV	F	CTTCAGGATGGCAAGTTGGT	286	60	[203]
		R	TCATCTCGTTCTCCGCTCAT			
	mcr-1	F	CGGTCAGTCCGTTTGTTC	309	61	[205]
		R	CTTGGTCGGTCTGTA GGG			
	dfrIa	F	GTGAAACTATCACTAATGG	474	55	[203]
		R	TTAACCCTTTTGCCAGATTT			

	dfrIb	F	GAGCAGCTICTITTIAAAGC	393	60	[203]
		R	TTAGCCCTTTIICCAATTTT			
	dfrII	F	GATCACGTGCGCAAGAAATC	141	50	[203]
		R	AAGCGCAGCCACAGGATAAAT			
	dfrVII	F	TTGAAAATTTCATTGATT	474	55	[203]
		R	TTAGCCTTTTTTCCAAATCT			
	dfrXII	F	GGTGSGCAGAAGATTTTTCGC	319	60	[203]
		R	TGGGAAGAAGGCGTCACCCTC			
Replicon types	IncHI1	F	GGAGCGATGGATTACTTCAGTAC	471	60	[151]
		R	TGCCGTTTCACCTCGTGAGTA			
	IncHI2	F	TTTCTCCTGAGTCACCTGTTAACAC	644	60	[151]
		R	GGCTCACTACCGTTGTCATCCT			
	IncI1-Iγ	F	CGAAAGCCGGACGGCAGAA	139	60	[151]
		R	TCGTCGTTCCGCCAAGTTCGT			
	IncI2	F	CTGTCGGCATGTCTGTCTC	553	55	[149]
		R	CTGGCTACCAGTTGCTCTAA			
	IncX1	F	GCTTAGACTTTGTTTTATCGTT	461	62	[150]
		R	TAATGATCCTCAGCATGTGAT			
	IncX2	F	GCGAAGAAATCAAAGAAGCTA	678	63	[150]
		R	TGTTGAATGCCGTTCTTGTCCAG			
	IncX3	F	GTTTTCTCCACGCCCTTGTTCA	351	63	[150]
		R	CTTTGTGCTTGGCTATCATAA			
	IncX4	F	AGCAAACAGGGAAAGGAGAAGAC	569	62	[150]
		R	TACCCCAAATCGTAACCTG			
	IncL/M	F	GGATGAAAACTATCAGCATCTGAA	785	60	[151]
		R	CTGCAGGGGCGATTCTTTAGG			
	IncFIA	F	CCATGCTGGTTCTAGAGAAGGTG	462	60	[151]
		R	GTATATCCTTACTGGCTTCCGCAG			
	IncFIB	F	GGAGTTCTGACACACGATTTTCTG	702	63	[151]
		R	CTCCCGTCGCTTCAGGGCATT			
	IncFIC	F	GTGAACTGGCAGATGAGGAAGG	262	60	[151]
		R	TTCTCCTCGTCGCCAAACTAGAT			
	IncFIIs	F	CTGTCGTAAGCTGATGGC	270	60	[151]
		R	CTCTGCCACAAACTTCAGC			
	IncA/C	F	GAGAACCAAAGACAAAGACCTGGA	465	60	[151]
		R	ACGACAAACCTGAATTGCCTCCTT			
	IncP	F	CTATGGCCCTGCAAACGCGCCAGA	534	60	[151]
		R	TCACGCGCCAGGGCGCAGCC			
	IncK	F	GCGGTCCGGAAAGCCAGAAAAC	160	60	[151]
		R	TCTTTCACGAGCCCGCCAAA			
	IncB/O	F	GCGGTCCGGAAAGCCAGAAAAC	159	60	[151]
		R	TCTGCGTTCCGCCAAGTTCGA			
	IncN	F	GTCTAACGAGCTTACCGAAG	559	55	[151]
		R	GTTTCAACTCTGCCAAGTTC			

Supplementary Table 2. Comparison of the prevalence of plasmid-mediated antimicrobial resistance genes between ESBL/AmpC-EC and non-ESBL/AmpC-EC isolates

Antibiotic classes	Antibiotic resistance gene	Prevalence (%) of.		OR (95% CI)	*p* - value
		ESBL/AmpC-EC (n = 161)	non-ESBL/AmpC-EC (n=81)		
Phenicol	*catA*	4.4	0.0	7.9 (0.45-140.30)	0.16†
	cml	15.5	25.9	0.5 (0.10-2.89)	0.46
	floR	68.8	61.7	1.4 (0.54-3.46)	0.51
	catA, clmA, or floR	80.1	70.4	1.8 (0.48-6.53)	0.39
Tetracycline	*tetA*	52.8	56.8	0.9 (0.24-3.01)	0.80
	tetB	22.4	14.8	1.7 (0.60-4.60)	0.33
	tetD	3.7	0.0	6.8 (0.38-122.47)	0.19†
	tetA, tetB, or tetD	69.6	71.6	0.9 (0.28-3.14)	0.91
Quinolone	*qnrA*	0.0	0.0	-	-
	qnrB	0.6	2.5	0.3 (0.02-2.84)	0.26
	qnrC	0.0	0.0	-	-
	qnrS	14.3	19.8	0.7 (0.10-4.52)	0.69
	aac(6)-cr-Ib	0.0	0.0	-	-
	qnrA, qnrB, qnrC, qnrS, or aac(6)-cr-Ib	14.3	22.2	0.6 (0.09-3.99)	0.58
Aminoglycoside	*aac(3)-I*	0.0	0.0	-	-
	aac(3)-II	19.3	3.7	6.2 (1.25-30.70)	0.03*
	aac(3)-IV	8.1	1.2	7.0 (0.61-81.36)	0.12
	aac-(3)-I, II, or IV	23.6	4.9	5.9 (1.36-25.98)	0.02*
Sulfonamide/ Trimethoprim	*dfrIa*	1.2	7.3	0.2 (0.05-0.52)	< 0.01*
	dfrIb	0.0	0.0	-	-
	dfrII	0.0	0.0	-	-
	dfrVII	0.0	0.0	-	-
	dfrXII	19.9	28.1	0.6 (0.17-2.26)	0.47
	dfrIa, Ib, II, VII, or XII	21.1	32.9	0.5 (0.16-1.80)	0.31
Polymyxin	*mcr-1*	6.2	0.0	12.5 (0.72-214.09)	0.08†

* Statistically significant.

*$p < 0.05$, Generalized estimating equations, †*p*-value was calculated by Chi-square test. CI: Confidence interval

Supplementary Table 3. Distributions of ST and phylogroup for each farm and production stage combination

		Clone type No. of isolate (%)					
Farm A (n = 24)	Stage 1[a] (n = 5)	ST48-A 3 (60.0)	ST5229-B1 2 (40.0)				
	Stage 2[b] (n = 7)	ST641-B1 2 (28.6)	ST5229-B1 1 (14.3)	ST23-A 2 (28.6)	ST7203-A 1 (14.3)	ST205-B1 1 (14.3)	
	Stage 3[c] (n = 9)	ST3944-A 2 (22.2)	ST75-B1 1 (11.1)	ST5229-B1 1 (11.1)	ST4014-B1 1 (11.1)	ST215-A 2 (22.2)	ST2628-B1 2 (22.2)
	Stage 4[d] (n = 3)	ST75-B1 1 (33.3)	ST767-B1 1 (33.3)	ST10-A 1 (33.3)			
Farm B (n = 23)	Stage 1 (n = 5)	ST101-B1 3 (60.0)	ST3076-B1 1 (20.0)	ST376-B1 1 (20.0)			
	Stage 2 (n = 8)	ST101-B1 6 (75.0)	ST3076-B1 1 (12.5)	ST641-B1 1 (12.5)			
	Stage 3 (n = 8)	ST101-B1 5 (62.5)	ST641-B1 1 (12.5)	ST953-A 1 (12.5)	ST218-A 1 (12.5)		
	Stage 4 (n = 2)	ST3076-B1 1 (50.0)	ST3944-A 1 (50.0)				
Farm C (n = 15)	Stage 1 (n = 3)	ST75-B1 2 (66.7)	ST1642-B1 1 (33.3)				
	Stage 2 (n = 5)	ST75-B1 3 (60.0)	ST2628-B1 1 (20.0)	ST744-A 1 (20.0)			
	Stage 3 (n = 5)	ST75-B1 3 (60.0)	ST2628-B1 1 (20.0)	ST744-A 1 (20.0)			
	Stage 4 (n = 2)	ST2628-B1 2 (100.0)					
Farm D (n = 15)	Stage 1 (n = 3)	ST457-F 1 (33.3)	ST5696-B1 1 (33.3)	ST1642-B1 1 (33.3)			
	Stage 2 (n = 7)	ST101-B1 1 (14.3)	ST457-F 6 (85.7)				
	Stage 3 (n = 4)	ST101-B1 2 (50.0)	ST10-A 2 (50.0)				
	Stage 4 (n = 1)	ST75-B1 1 (100.0)					
Farm E (n = 18)	Stage 1 (n = 5)	ST101-B1 1 (20.0)	ST457-F 2 (40.0)	ST410-A 2 (40.0)			
	Stage 2 (n = 7)	ST457-F 5 (71.4)	ST75-B1 1 (14.3)	ST12-B2 1 (14.3)			
	Stage 3 (n = 3)	ST101-B1 1 (33.3)	ST457-F 1 (33.3)	ST3285-B1 1 (33.3)			
	Stage 4 (n = 3)	ST101-B1 2 (66.7)	ST457-F 1 (33.3)				

			Clone type No. of isolate (%)		
Farm F	(n = 16)	Stage 1 (n = 6)	ST224-B1 2 (33.3)	ST457-F 3 (50.0)	ST1011-E 1 (16.7)
		Stage 2 (n = 5)	ST224-B1 3 (60.0)	ST457-F 2 (40.0)	
		Stage 3 (n = 3)	ST224-B1 3 (100.0)		
		Stage 4 (n = 2)	ST224-B1 1 (50.0)	ST457-F 1 (50.0)	
Farm G	(n = 15)	Stage 1 (n = 4)	ST75-B1 4 (100.0)		
		Stage 2 (n = 1)	ST101-B1 1 (100.0)		
		Stage 3 (n = 7)	ST101-B1 6 (85.7)	ST75-B1 1 (14.3)	
		Stage 4 (n = 3)	ST101-B1 3 (100.0)		
Farm H	(n = 6)	Stage 1 (n = 4)	ST224-B1 2 (50.0)	ST648-F 2 (50.0)	
		Stage 2 (n = 2)	ST648-F 2 (100.0)		
		Stage 3 (n = 0)			
		Stage 4 (n = 0)			
Farm I	(n = 4)	Stage 1 (n = 1)	ST617-A 1 (100.0)		
		Stage 2 (n = 2)	ST617-A 2 (100.0)		
		Stage 3 (n = 0)			
		Stage 4 (n = 1)	ST617-A 1 (100.0)		

Stage 1[a], Weaning piglets; Stage 1[b], Growing pigs; Stage 1[c], Finishing pigs, Stage 1[d], Pregnant sows.

Supplementary Table 4. Farm and sampling information for nine swine farms included in this study and prevalence of ESBL/AmpC-EC across farms.

Farm	Farm ID	Total	Farm A	Farm B	Farm C	Farm D	Farm E	Farm F	Farm G	Farm H	Farm J
	Province	-	Gyeonggi-do	Gyeonggi-do	Gyeonggi-do	Gyeonggi-do	Gyeongsang-nam-do	Jeolla-nam-do	Jeolla-buk-do	Jeolla-nam-do	Chungcheong-nam-do
	Sample collection time	-	Oct 2018	Mar 2018	Jan 2019	Mar 2020	May 2018	July 2017	May 2018	Aug 2017	April 2018
	Prevalence of ESBL/AmpC-EC		89.7%	82.4%	65.4%	63.6%	55.9%	55.9%	50.0%	23.5%	17.6%
Number of pigs	Weaning pigs		5,000	2,000	2,100	200	3,200	4,000	2,000	1,200	4,800
	Growing pigs		4,000	1,500	1,260	300	2,400	3,000	1,500	900	3,600
	Finishing pigs		15,000	1,950	1,640	500	3,120	3,900	1,950	1,170	4,680
	Pregnant sows		2,100	500	350	200	800	1,000	500	300	1,200
	Total		26,100	5,950	5,350	1,200	9,520	11,900	5,950	3,570	14,280

Abbreviation. No., Number; ESBL/AmpC-EC, extended-spectrum β-lactamase- or AmpC type β-lactamase producing *Escherichia coli*

Supplementary Table 5. Clone types of South Korea-derived ESBL/AmpC-EC strains, which registered in NCBI database

	Human	Pig		Chicken		Cattle
Sequence type	Human (n = 387)	Pig (n = 12)	Pork (n = 1)	Chicken (n = 4)	Chicken meat (n = 18)	Cattle (n = 1)
131	93	0	0	0	0	0
517	19	0	0	0	0	0
38	18	0	0	0	0	0
1491	16	0	0	0	0	0
10	13	2	0	0	0	0
69	13	0	0	0	0	0
1193	11	0	0	0	0	0
34	10	0	0	0	0	0
6272	9	0	0	0	0	0
4	8	0	0	0	0	0
410	7	0	0	1	0	0
405	7	0	0	0	0	0
1312	7	0	0	0	0	0
414	7	0	0	0	0	0
648	7	0	0	0	0	0
2178	6	0	0	0	0	0
616	5	0	0	0	0	0
443	4	0	0	0	0	0
40	4	0	0	0	0	0
1380	4	0	0	0	0	0
31	4	0	0	0	0	0
457	3	1	0	0	1	0
20	3	0	0	0	0	0
2040	3	0	0	0	0	0
88	3	0	0	0	0	0
68	3	0	0	0	0	0
12	3	0	0	0	0	0
393	3	0	0	0	0	0
167	2	0	0	0	0	0
155	2	0	0	0	1	0
95	2	0	0	0	2	0
156	2	0	0	0	1	0
641	2	0	0	0	0	0
642	2	0	0	0	0	0
3570	2	0	0	0	0	0
6955	2	0	0	0	0	0
803	2	0	0	0	0	0
382	2	0	0	0	0	0
337	2	0	0	0	0	0
752	2	0	0	1	0	0
28	2	0	0	0	0	0
328	2	0	0	0	0	0
1125	2	0	0	0	0	0
6303	2	0	0	0	0	0
4119	2	0	0	0	0	0
2003	2	0	0	0	0	0
152	1	0	0	0	0	0
101	1	1	0	0	0	0
617	1	0	0	0	0	0
720	1	0	0	0	0	0
453	1	0	0	0	1	0
354	1	0	0	0	0	0
70	1	0	0	0	0	0
746	1	0	0	0	0	0
442	1	0	0	0	0	0
173	1	0	0	0	0	0
1490	1	0	0	0	0	0
590	1	0	0	0	0	0
795	1	0	0	0	0	0
189	1	0	0	0	0	0
1136	1	0	0	0	0	0
381	1	0	0	0	0	0
32	1	0	0	0	0	0
793	1	0	0	0	0	0
327	1	1	0	0	0	0
1201	1	0	0	0	0	0
4213	1	0	0	0	0	0
218	1	0	0	0	0	0
800	1	0	0	0	0	0
2346	1	0	0	0	0	0
799	1	0	0	0	0	0
940	1	0	0	0	0	0
1295	1	0	0	0	0	0
450	1	0	0	0	0	0
4060	1	0	0	0	0	0
7937	1	0	0	0	0	0
335	1	0	0	0	0	0
1849	1	0	0	0	0	0
2356	1	0	0	0	0	0
686	1	0	0	0	0	0
3303	1	0	0	0	0	0
2088	1	0	0	0	0	0
3933	1	0	0	0	0	0
2332	1	0	0	0	0	0
226	1	0	0	0	0	0
1722	1	0	0	0	0	0
1638	1	0	0	0	0	0
404	1	0	0	0	0	0
6999	1	0	0	0	0	0
93	0	0	0	0	4	0
1721	0	0	0	0	0	0
224	0	1	0	0	0	1
23	0	0	0	0	1	0
602	0	0	0	0	2	0
117	0	0	0	0	0	0
1286	0	0	0	0	0	0
58	0	0	0	0	0	0
1485	0	0	0	0	1	0
744	0	0	0	0	1	0
5229	0	0	1	0	0	0
2170	0	0	0	0	2	0
2607	0	0	0	0	1	0
10064	0	0	0	0	0	0
48	0	1	0	0	0	0
1196	0	0	0	0	0	0
1737	0	0	0	0	0	0
162	0	0	0	1	0	0
100	0	2	0	0	0	0
542	0	1	0	0	0	0
1642	0	1	0	0	0	0
2216	0	1	0	0	0	0
295	0	0	0	0	0	0
1146	0	0	0	0	0	0
372	0	0	0	0	0	0
485	0	0	0	0	0	0
961	0	0	0	0	0	0
-	15	0	0	1	0	0
Total	387	12	1	4	18	1

Chapter 2.

Prevalence, Characteristics, and Clonal Distribution of *Escherichia coli* Carrying Mobilized Colistin Resistance Gene *mcr-1.1* in Swine Farms and Their Differences According to Swine Production Stages

Abstract

Global spread of *Escherichia coli* strains carrying the mobilized colistin resistance gene *mcr-1.1* (MCR1-EC) poses serious threats to public health. Colistin has been generally prescribed for swine colibacillosis, having made swine farms as major reservoirs of MCR1-EC. The present study, the prevalence, characteristics and clonal dynamics of MCR1-EC strains were analyzed according to swine production stages, and genetic relatedness were evaluated using public database. Individual fecal samples (n = 360) were collected from asymptomatic weaning-piglets, growers, finishers, and sows from 11 farrow-to-finishing farms in South Korea between 2017 and 2019. The weighted prevalence of MCR1-EC was 8.4%, with the highest prevalence at weaning stage (13.0%). In the WGS-based analysis, MCR1-EC strains having MDR and pathogenic advantages (intestinal-/extraintestinal pathogenic *E. coli*-associated virulence factors or robust biofilm formation) were highly shared between pig stages within farms. Furthermore, core genomes of MCR1-EC isolated from individuals within closed environments (same farms or human hospitals) were highly shared (genetic distance < 0.01), suggesting a high probability of clonal expansion of MCR1-EC within closed environments such as livestock husbandry. Whereas, MCR1-EC strains isolated from various sources such as pigs, pork, and humans in South Korea exhibited a highly heterogeneous MLST types without a dominant type or a shared type, thereby providing indirect scientific evidence that MCR1-EC strains have a low possibility of inter-environmental transmission via clonal spreading. Instead, the *mcr-1.1* was transferred from more than 90% of MCR1-EC strains via conjugation, implying horizontal gene transfer plays a more important role than clonal diffusion in colistin propagation between environments. Notably, this

study showed that weaning piglets were an important reservoir of CIA-resistant bacteria such as ESBL/AmpC/MCR-EC, proposing that the need for special attention in the weaning stages to manage antimicrobial resistance of pig farms.

Keywords: *mcr*, *E. coli*, intestinal pathogenic *E. coli*, swine production stage

2.1. Introduction

Colistin is regarded as a last resort for the treatment of multi-drug resistant (MDR) bacterial infections in humans and has been classified as a critically important antimicrobial agent by the World Health Organization [17]. Before 2016, colistin resistance was mainly considered to be associated with mutational and regulatory changes in chromosomal genes, including *pmrAB* and *phoPQ* [18]. The mobilized colistin resistance gene *mcr-1* was first described in a plasmid carried by *Escherichia coli* strains in 2016 [18], and has since been found in more than 50 countries across six continents [19], highlighting the global spread of colistin resistance *via mcr-1*.

Swine colibacillosis is a major disease in pigs that causes huge economic losses for the global swine industry [27]. Colistin has been generally used for the treatment of swine colibacillosis, leading to an increased prevalence of *E. coli* strains carrying *mcr-1* (MCR1-EC) in swine farms [206-209]. Given that colistin has been considered a recommended treatment option for swine colibacillosis and that intestinal pathogenic *E. coli* (InPEC) comprises major causative pathogens of swine colibacillosis [206-209], the presence of intestinal pathogenic MCR1-EC in pig husbandry represents a severe challenge for the swine industry. Colistin administration during the treatment of swine colibacillosis caused by intestinal pathogenic MCR1-EC can lead to disease treatment failure, as well as complications, resulting in serious economic losses for pig farms [210]. To establish suitable strategies to control intestinal pathogenic MCR1-EC in swine farms, an in-depth characterization of intestinal pathogenic MCR1-EC should be performed, and whole-genome sequence (WGS)-based analysis might provide valuable insights.

In pig production systems, pigs at different stages of growth, referred to as weaning piglets, growers, finishers, and pregnant pigs, are usually raised in separate barns [211]. However, as pigs age and transition to the next growth stage and next stage barn, bacterial transmission can occur between animals at different swine production stages within farms, which has been reported to be a significant risk factor for the high prevalence of MDR bacteria in swine farms [7, 40]. Since *mcr-1* is mainly mediated by plasmids, the important role of genetic transferability of *mcr-1* in the spread of MCR1-EC has been continuously highlighted in various studies [19, 210, 212, 213]. However, genetic transfer essentially presupposes the transfer of strains and bacteria-to-bacteria interactions under favorable conditions (e.g., physical distance between strains, nutrition, and environmental conditions, etc.; [41], which suggests that bacterial transmission also provides a crucial basis for the spread of MCR1-EC. Understanding the genetic characteristics and distribution of MCR1-EC considering swine production stages, which is an important reservoir of MCR1-EC, could be a cornerstone to establish strategies for the control of colistin resistance in the swine industry. However, despite its importance, the characteristics and distribution of MCR1-EC based on different swine production stages within farms have rarely been studied.

The present study aimed to investigate the risks of MCR1-EC strains according to swine production stages, an important reservoir of CIA-resistant bacteria, and to evaluate the potential threat of swine farm-derived strains to humans by understanding molecular epidemiological dynamics and resistance mechanisms. For this, first, the prevalence, antimicrobial resistance, and genetic and phenotypic virulence characteristics of MCR1-EC isolated from swine farms were investigated, and differences according to swine production

stages were analyzed. Second, we performed WGS for all intestinal pathogenic MCR1-EC isolated in this study and conducted an in-depth genetic characterization. Finally, to understand spread characteristics of MCR1-EC, genetic relatedness analysis based on the clone types and WGS were conducted for MCR1-EC strains from swine farms and strains from various sources using public database.

2.2. Materials and Methods

Sample collection

In total, 360 swine fecal samples were collected from eleven farrow-to-swine farms in South Korea between May 2017 and August 2019 (Figures 1, 2; Supplementary Table 6). For this study, eleven swine farms were selected for three criteria: 1) located in in five provinces with the highest number of pig farms in South Korea, 2) farrow-to-finishing farm, and 3) raising pigs more than 1,000 pigs. The number of pig farms by province in South Korea was obtained from the 2017 demographic report of the Korean Statistical Information Service of Statistics Korea [142].

The sampling for each swine farms was conducted through multi-stage stratified random sampling. The sampling size for each swine stages were determined using two criteria: 1) the number of pigs for each stage, and 2) estimated prevalence of MCR1-EC for each stage. The estimated prevalence of MCR1-EC for each swine stages were determined with the previous researches conducted worldwide [19, 117, 206-209, 214]. In total, individual fecal samples were collected from 64 weaning piglets (4–7 weeks old), 117 growing pigs (7–

14 weeks old), 117 finishing pigs (14–24 weeks old), and 62 pregnant sows. The sampling was conducted with similar numbers from each swine farm; a total of 26–34 pigs from each swine farm, including 5–6 weaning piglets, 9–11 growing, 8–11 finishing pigs, and 3–6 pregnant sows.

Isolation of MCR1-EC

The isolation of MCR1-EC was conducted following previously described protocols for the isolation of antimicrobial resistant *E. coli*, with slight modifications [215-218]. Approximately, 1 g of each sample was resuspended in 9 ml of *Escherichia coli* broth (BD Biosciences, New Jersey, United States) and incubated overnight at 37°C. Thereafter, 100 µl of culture suspension was spread on MacConkey agar (BD Biosciences), and a colistin disk (10 µg/ml, Oxoid, Cheshire, United Kingdom) was placed on the plate. After overnight incubation at 37°C, 1–4 colistin-resistant *E. coli* candidate isolates grown inside the colistin-resistant zone (≤10 mm) were selected and streaked on Eosin Methylene Blue agar (BD Biosciences) containing 2 mg/L colistin (Sigma Aldrich, Massachusetts, United States) for further confirmation. The diameter (≤10 mm) of the candidate colistin-resistant zone was set with reference to the disk diffusion quality control range of *E. coli* reference strain ATCC 25922 described in the Clinical Laboratory Standard Institute (CLSI) guidelines M100S 31th Edition (2021). Then, the presence of *mcr-1* and *mcr-1*-encoded replicon types was determined *via* PCR and sequencing as previously described [212]. The sequenced PCR amplicons were compared with the reference sequences from the NCBI GenBank database using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the replicon types, as well as *mcr-1* variants from *mcr-1.1* to *mcr-1.32*. The PCR was performed using a SimpliAmp Thermal Cycler (Thermo

Fisher Scientific, Massachusetts, United States), and sequencing was performed using an ABI PRISM 3730XL DNA analyzer (Thermo Fisher Scientific). *Escherichia coli* isolates carrying *mcr-1* were confirmed as MCR1-EC, and one MCR1-EC strain per sample was randomly selected if more than one isolates were identified from a sample. Primer sequences and reaction conditions are summarized in Supplementary Table 7.

Antimicrobial susceptibility assay

Minimum inhibitory concentration (MIC) tests were conducted to evaluate colistin resistance using the Trekstar Sensititre KNIHCOL custom panel (colistin test range: 0.25–128 μg/ml, Trek Diagnostic Systems, Ohio, United States) according to the manufacturer's instructions. Each isolate was tested in duplicate for the MIC of colistin. Kirby-Bauer disk diffusion susceptibility tests (KBTs) were conducted for 11 antimicrobial classes comprising 14 antimicrobial agents using antimicrobial disks from Oxoid (Cheshire, United Kingdom) as follows: ampicillin (10 μg/ml), cefotaxime (30 μg/ml), ceftazidime (30 μg/ml), ceftriaxone (30 μg/ml), amoxicillin/clavulanate (20/10 μg/ml), aztreonam (30 μg/ml), imipenem (10 μg/ml), chloramphenicol (30 μg/ml), amikacin (30 μg/ml), gentamycin (10 μg/ml), tetracycline (30 μg/ml), nalidixic acid (30 μg/ml), ciprofloxacin (5 μg/ml), and sulfamethoxazole/trimethoprim (1.25/23.75 μg/ml). The MIC tests and KBs results were interpreted according to the CLSI guidelines M100S 31th Edition (2021), and the *E. coli* reference strain ATCC 25922 was used for quality control. When the isolate was resistant to at least one antimicrobial agent belonging to the antimicrobial class, we determined that this isolate was resistant to this antimicrobial class. Then, we calculated the average number of antimicrobial classes to which MCR1-EC strains were resistant. Extended-

spectrum β-lactamase (ESBL) phenotypes were determined *via* a standard double-disk test according to CLSI guidelines using four antimicrobial disks from BD Bioscience (New Jersey, United States) as follows: cefotaxime (30 μg/ml), ceftazidime (30 μg/ml), cefotaxime/clavulanate (30/10 μg/ml), and ceftazidime/clavulanate (30/10 μg/ml).

Antimicrobial resistance genes and replicon typing

The presence of genes conferring resistance to β-lactams, chloramphenicol, aminoglycoside, quinolones, and sulfonamide/trimethoprim was determined by PCR. The ESBL genotypes were determined by PCR and sequencing as previously described [197]. PCR-based replicon typing was conducted as previously described [149-151]. Primer sequences and reaction conditions are summarized in Supplementary Table 7.

Classification of pathogenic *Escherichia coli*

To analyze the genotypic virulence characteristics of MCR1-EC, we investigated the presence of virulence factors associated with InPEC, extra-intestinal pathogenic *E. coli* (ExPEC), and uro-pathogenic *E. coli* (UPEC). The classification of InPEC was conducted by PCR for the following five InPEC types: shiga toxin-producing *E. coli* (STEC) carrying *stx1* or *stx2*, enteropathogenic *E. coli* (EPEC) carrying *eaeA* or *bfpB*, enteroaggregative *E. coli* (EAEC) carrying *aggR*, enteroinvasive *E. coli* (EIEC) carrying *ipaH*, and enterotoxigenic *E. coli* (ETEC) carrying *lt*, *sta*, or *stb*. The carriage of 21 ExPEC-associated virulence factors associated with adhesion (*csgA*, *fimH*, *sfa/focDE*, *afa/draBC*, *papC*, *papAH*, *yfcV*, and *iha*), toxins (*hlyF*, *astA*, *pic*, *vat*, and *aat*), protectin/serum resistance (*traT*, *ompT*, *iss*, and *kpsMTII*), and siderophores (*fyuA*, *iroNE.coli*, *iutA*, and *chuA*) were

investigated using PCR. The classification of ExPEC was conducted following the previously described criteria, specifically positive for ≥2 of five key markers as follows: *papA* and/or *papC*, *sfa/focDE*, *afa/draBC*, *iutA*, and *kpsMTII* [146]. The classification of UPEC was conducted following previously described criteria, specifically positive for ≥3 of four key markers as follows: *vat*, *fyuA*, *chuA*, and *yfcV* [147]. Finally, since all pigs included in this study were healthy, without showing any disease symptoms, *E. coli* isolates that not classified as InPEC, ExPEC, or UPEC were then classified as commensal *E. coli* strains. Primer sequences and reaction conditions are summarized in Supplementary Table 8.

Phenotypic assay: 1) conjugation assay

Conjugation assays were conducted to evaluate the horizontal genetic transferability of *mcr-1* with the *E. coli* J53-AziR strain as the recipient and 53 MCR1-EC strains as the donors. The conjugation assay was conducted following a previously described protocol with modifications [219]. Briefly, overnight cultures of donor and recipient strains in Luria-Bertani broth were mixed at a ratio of 1:1, followed by incubation at 37°C for 18 h with constant shaking. Then, 100 µl of the mixture of donor and recipient cells were spread on LB agars supplemented with 2 mg/L colistin (Sigma Aldrich, Massachusetts, United States) and 100 mg/L sodium azide (Sigma Aldrich), followed by overnight incubation at 37°C. The presence of *mcr-1* in conjugants was confirmed *via* PCR.

Phenotypic assay: 2) biofilm assay

To analyze the phenotypic virulence characteristics of MCR1-EC, biofilm production assays were performed following a previously described

protocol with modifications [220]. Briefly, overnight M9 minimal medium [200 ml/L of M9 media (5X, Sigma-Aldrich), 0.4 g/L of glucose (Sigma-Aldrich), 2 ml/L of MgSO4 solution (1 M, Sigma-Aldrich), and 100 µl/L of CaCl2 solution (1 M, Sigma-Aldrich)] culture was diluted in fresh M9 minimal medium to a McFarland scale of 0.5. Approximately, 100 µl of this dilution was added into a 96-well microtiter plate and incubated for 24 h at 28°C under stationary conditions. Each bacterial suspension was inoculated into three wells of a microtiter plate. Growth optical densities (ODs) were measured at $\lambda = 595$ nm with a multiplate reader (Bio-Rad, California, United States). The wells were then washed once with 200 µl of phosphate-buffered saline, dried for 20 min, and stained with 100 µl of 1% crystal violet for 1 h. This was followed by gentle washing with 200 µl of distilled water four times and air-drying for 1 h. The absorbed dye was solubilized in 100 µl of absolute ethanol, and ODs were read at 595 nm. The extent of biofilm formation was calculated using the following formula: SBF = (AB−CW)/G, where SBF is the specific biofilm formation index, AB is the OD595 of the stained bacteria, CW is the OD595 of the stained control wells containing absolute media without bacteria, and G is the OD595 corresponding to cell growth in the media. *Escherichia coli* ATCC 25922 was used as the positive control, whereas the culture medium was used as the negative control. The degree of biofilm production was classified into three categories, weak (SBF < 0.5), moderate (0.5 ≤ SBF < 1.0), and strong (SBF ≥ 1.0).

Clonal distribution analysis of MCR1-EC based on multi-locus sequence typing and *E. coli* phylogroup typing

Multi-Locus Sequence Typing (MLST) was performed as previously described [152]. A detailed scheme describing gene amplification, allelic type,

and sequence type (ST) assignment methods is available on the pubMLST website. The minimum spanning tree (MST) based on allelic profiles of seven MLST housekeeping genes was constructed using BioNumerics software (v6.6, Applied Maths, Sint-Martens-Latem, Belgium). The PCR-based PG typing was conducted as previously described [153], and primer sequences and reaction conditions are summarized in Supplementary Table 8.

Further, we analyzed the clonal distribution of 1,652 MCR1-EC strains, of which WGS was publicly available in the NCBI database (accessed on 07 Jan 2020, https://www.ncbi.nlm.nih.gov/pathogens/isolates/), including strains isolated from humans ($n = 940$), chickens ($n = 446$), and pigs ($n = 226$). In addition, we also analyzed the clonal distribution of 17 South Korean-derived MCR1-EC strains, of which WGS was available in the NCBI database, including strains isolated from humans ($n = 13$), chickens ($n = 2$), a pig ($n = 1$), and a dog ($n = 1$). The *in silico* MLST and *E. coli* phylogenetic typing were performed using the MLST 2.0 (v2.0.4) program at the CGE website and the Clermont typing program (v21.03) provided by the website http://clermontyping.iame-research.center/ [221]. The assembly accession numbers of strains used in this study are summarized in Supplementary File 1.

WGS-based in-depth characterization of intestinal pathogenic MCR1-EC

We conducted WGS for all intestinal pathogenic MCR1-EC strains isolated in this study. Total genomic DNA was extracted using the Nucleospin Microbial DNA kit (Macherey-Nagel, North Rhine-Westphalia, Germany) following the manufacturer's instructions. Genomic DNA was sequenced *via* NextSeq® 500 technology (Illumina, California, United States).

The nucleotide sequences have been submitted to the NCBI sequence read archive with the assigned Bioproject no. PRJNA757225. The sequence reads were assembled into contigs using the CLC Genomics Workbench program (Qiagen, Hilden, Germany) with default setting. The assembled contigs were analyzed using the bioinformatics tools of the Center for Genomic Epidemiology2 for the presence of resistance genes (ResFinder V4.1.), virulence factors (VirulenceFinder v2.0.), and plasmid replicon types (PlasmidFinder 2.1).

Genetic relatedness analysis based on WGS

For genetic relatedness analysis based on WGS, we conducted core genome multi-locus sequence typing (cgMLST) to focus on the genetic relatedness between the core genomes of strains, not the genetic difference that occurs through the acquisition or loss of accessory genomes such as plasmids. The cgMLST was performed using the Ridom SeqSphere+ program (v8.2.0; [128]). In this analysis, first, we conducted cgMLST among all 12 intestinal pathogenic MCR1-EC strains isolated from this study and 17 MCR1-EC strains isolated in South Korea published in the NCBI database to assess the genetic relatedness among strains isolated in South Korea. Second, for genetic relatedness analysis of global MCR1-EC strains, we performed cgMLST on MCR1-EC isolated from humans, pigs, and chickens worldwide and harboring a major clone type. Based on clonal distribution analysis, 154 strains carrying the major clone type ST10-A were identified among 1,652 MCR1-EC strains published in the NCBI database. Moreover, 80 strains were selected among 154 MCR1-EC isolates of clone type ST10-A using a simple random sampling procedure with Statistical Package for the Social Sciences (SPSS) program (v27.0, IBM SPSS Statistics for Windows, New York, United

States). Then, the genetic relationships among 82 MCR1-EC strains harboring ST10-A (two intestinal pathogenic MCR1-EC strains isolated in this study and 80 MCR1-EC strains published in the NCBI database) were analyzed based on cgMLST. Then, we clustered strains with a genetic relatedness distance of less than 0.01 in cgMLST, and a total of eight clusters were identified.

Statistical analysis

All statistical analyses included in this study were conducted using the SPSS program (IBM SPSS Statistics for Windows). Since the sampling number was similar for each farm with different number of total pigs, which providing different sampling probabilities for each swine farm, we performed the weighted prevalence analysis of MCR1-EC [complex samples crosstabs (CSC) and complex samples logistic regression model (CSLRM)] based on the unbiased Horvitz-Thompson estimator [222], setting farm. Weighted prevalence of MCR1-EC by stage and 95% confidence interval (95% CI) were calculated using CSC. In addition, differences in the prevalence of MCR1-EC according to swine stage were evaluated using the CSLRM setting stage as a covariate parameter.

To adjust the farm-induced factor, we conducted the generalized estimating equation (GEE) for comparative analyses of antimicrobial resistance and virulence factors of MCR1-EC isolates by swine stages. The GEE analysis was used for the calculation of odds ratios (ORs) and 95% CIs setting weaning stages as a reference. To adjust the farm-induced factors, farm was set as the "subject variable" and number of MCR1-EC strains per each farm was set as "within subject variables." If the zero value of the cross-tab caused a problem in the GEE-based OR calculation, Fisher's exact test was performed by adding

0.5 to each cell instead of GEE [158]. To evaluate the correlation between antimicrobial resistance genes and the expected phenotypic resistance, Spearman's correlation test (SCT) was performed.

2.3. Results

Prevalence of MCR1-EC isolates according to four swine production stages

MCR1-EC strains were isolated from 55 of 360 pigs (15.3%), from four of 11 swine farms (Figure 17; Supplementary Table 6). The weighted prevalence of MCR1-EC was 8.4% (95% CI: 2.1%–28.0%), and weaning piglets had the highest weighted prevalence of MCR1-EC (13.0, 95% CI: 2.3%–48.9%). The second highest weighted prevalence MCR1-EC was identified in growing pigs (10.8, 95% CI: 2.8%–33.5%), followed by sows (6.0, 95% CI: 1.2%–24.6%), and finishing pigs (4.7, 95% CI: 1.1%–17.9%). There were no significant differences in the prevalence of MCR1-EC between four swine stages (CSLSM, $p > 0.05$).

We included 53 MCR1-EC stains for further analysis, since two MCR1-EC isolates were not recovered. Among reported 32 *mcr-1* variants (*mcr-1.1–mcr-1.32*), all 53 MCR1-EC strains were found to carry *mcr-1.1.* In the conjugation assay of MCR1-EC strains, *mcr-1.1* was transferred from 90.6% (48/53) of donor strains to the recipient strain J53-Azi^{R}.

Among 53 MCR1-EC isolates, 16 strains (30.2%, 16/53) were identified as pathogenic *E. coli*, including InPEC (22.6%, 12/53) or ExPEC (7.5%, 4/53; Figure 18). Among 12 InPEC strains, 10 MCR1-EC (18.9%, 10/53) was identified as STEC and two strains (3.8%, 2/53) were identified as EPEC.

Ten STEC were isolated from two weaning piglets, six growing pigs, and two finishing pigs. Two EPEC were isolated from one weaning piglet and one growing pig. Four ExPEC were isolated from one weaning piglet, one growing pig, one finishing pig, and one sow.

Antimicrobial resistance of MCR1-EC isolates from swine farms

All 53 MCR1-EC isolates were resistant to colistin, with MICs of 4 μg/ml (17.0%, 9/53) or 8 μg/ml (83.0%, 44/53). Through KBTs for 11 antimicrobial classes, 96.2% (51/53) of MCR1-EC strains exhibited MDR, showing resistance to three or more antimicrobial classes (average: 4.8 classes; Figure 18). Among the 14 antimicrobial agents tested, the resistance rate of tetracycline was highest (86.8%, 46/53), followed by that of ampicillin (81.1%, 43/53) and chloramphenicol (66.0%, 35/53; Figure 19A). Nine MCR1-EC strains (17.0%, 9/53) were resistant to cefotaxime and had a typical phenotype of ESBL. Imipenem- or amikacin-resistant MCR1-EC isolates were not found. In comparison by pathogenic *E. coli* types, ExPEC strains showed resistance to average 7.0 antimicrobial classes, and InPEC strains showed resistance to average 4.0 antimicrobial classes. The resistant rate of ExPEC strains against third generation cephalosporins was 75.0% (3/4), whereas, all InPEC strains were susceptible to third cephalosporins. The antimicrobial susceptibility results of InPEC, ExPEC, and commensal *E. coli* were described in Supplementary Table 9.

In the comparative analysis based on the four swine stages, the prevalence of isolates showing resistance to seven or more antimicrobial classes was highest in the weaning stage (42.9%, 6/14) compared to that in other stages,

which was statistically significant compared to that in finishing pigs (OR: 3.8, 95% CI: 1.73–8.11, $p < 0.05$, GEE) and sows (OR: 5.25, 95% CI: 2.04–13.50, $p < 0.05$, GEE; Figure 19B). Meanwhile, the prevalence of isolates showing resistance to three or fewer antimicrobial classes was highest in pregnant sows (62.5%, 5/8), and it was significantly higher than that in weaning piglets (OR: 21.7, 95% CI: 8.77–53.50, $p < 0.05$, GEE). Compared to that in weaning pigs, the resistance rate of aminoglycoside was significantly lower in growing pigs (OR: 0.3, 95% CI: 0.12–0.51, $p < 0.05$, GEE), and the resistance rate of quinolone was significantly lower in finishing pigs (OR: 0.1, 95% CI: 0.07–0.18, $p < 0.05$, GEE; Table 4). Compared to those in weaning pigs, the resistance rate of chloramphenicol (OR: 0.1, 95% CI: 0.04–0.28, $p < 0.05$, GEE) and tetracycline (OR: 0.03, 95% CI: 0.001–0.78, $p < 0.05$, Fisher's exact test) were significantly lower in pregnant sows.

MCR1-EC carried a variety of antimicrobial resistance genes, including *tetA* (79.2%, 42/53, against tetracyclines), *floR* (69.8%, 37/53, against phenicols), *blaTEM-family* (58.8%, 31/53, against narrow-spectrum β-lactams), *sul2* (50.9%, 27/53, against sulfonamides), *qnrS1* (41.5%, 22/53, against quinolones), and $bla_{CTX\text{-}M\text{-}55}$ (17.1%, 9/53, against third Cephalosporins; Supplementary Table 10). Resistance genes were strongly associated with expected phenotypic resistance to all antimicrobial classes included in this study ($p < 0.05$, SCT), with the exception of quinolones. Among the 14 replicon types investigated in this study in 53 MCR1-EC isolates, the predominant replicon types were IncI2 (94.3%, 50/53), IncFIB (84.9%, 45/53), IncFII (67.9%, 36/53), and IncFIC (43.4%, 23/53; Supplementary Table 11).

Genotypic and phenotypic virulence of MCR1-EC isolates from swine farms

Among the four investigated virulence factor classes, all 53 MCR1-EC strains carried one or more adhesion-associated virulence factors, including *fimH* (90.6%, 48/53) and *csgA* (84.9%, 45/53) (Table 5). Toxin virulence factors were identified in 54.7% of MCR1-EC (29/53), with *hlyF* (26.4%, 14/53) and *astA* (7.5%, 4/53) present. Protectin virulence factors were identified in 90.6% of MCR1-EC (48/53), with *traT* (88.7%, 47/53), *ompT* (26.4%, 14/53), and *iss* (13.2%, 7/53) present. Siderophore virulence factors were identified in 35.8% of MCR1-EC (19/53), with *iutA* (26.4%, 14/53) and *iroNE.coli* (9.4%, 5/53) present. In addition, four (7.5%, 4/53) MCR1-EC were identified as having two UPEC-associated virulence factors, although this did not satisfy the criteria of UPEC (≥3 UPEC virulence factors). In the comparison based on the four swine stages, no significant differences were identified in the prevalence of the four virulence factor classes between stages ($p > 0.05$, GEE; Supplementary Table 12). In the biofilm assay, eight MCR1-EC strains (15.1%, 8/53) showed medium-to-strong biofilm formation (Figure 18), including four strains with strong biofilm formation and four strains with moderate biofilm formation. In contrast, 84.9% (45/53) of MCR1-EC showed weak biofilm formation.

WGS-based in-depth characterization of intestinal pathogenic MCR1-EC Strains

All 10 STEC isolates harbored *stx2e*, and two EPEC strains harbored the locus of enterocyte effacement (LEE), including *eae*, *tir*, *esp*,

and *nle* (Figure 20). Intestinal pathogenic MCR1-EC carried a variety of InPEC-associated virulence factors, including *terC* (100.0%, 12/12), *gad* (33.3%, 4/12), and *katP* (16.7%, 2/12). In addition, ExPEC-associated virulence factors, including *traT* (91.7%, 11/12), *ompT* (16.7%, 2/12), *iss*, (16.7%, 2/12), *sepA* (167%, 2/12), and *cia* (8.3%, 1/12), were also identified.

In the analysis of antimicrobial resistance genes, all 12 intestinal pathogenic MCR1-EC strains carried resistance genes to five or more antimicrobial classes, including *tetA/B* (100%, 12/12, against tetracyclines), *mdf(A)* (100%, 12/12, against macrolides), *dfrA1* (83.3%, 10/12, trimethoprims), *ant(3")-Ia* (75.0%, 9/12, aminoglycosides), *sul2* (66.7%, 8/12, sulfonamides), *floR* (25.0%, 3/12, phenicols), and *blaTEM-family* (25.0%, 3/12, narrow-spectrum β-lactams). All carried IncI2, accompanying by a variety of replicon types, including IncFIB (83.3%, 10/12), IncFII (83.3%, 10/12), IncR (66.7%, 8/12), ColE10 (66.7%, 8/12), and IncI1-Iγ (33.3%, 4/12).

In the comparative genomic analysis based on swine production stages, intestinal pathogenic MCR1-EC showed highly shared virulence factor characteristics between strains with the same clone type. In addition, the patterns of replicon types and antimicrobial resistance genes were also identical with slight differences between strains with the same clone types.

Clonal distribution of MCR1-EC strains from swine farms and strains from various sources

Among 53 MCR1-EC strains, 38 strains were identified as *E. coli* phylogenetic group A (71.7%, 38/53), 11 strains (20.8%) were identified as

group B1, and four strains (7.5%) were identified as group D (Figure 21). In total, 17 clone types were identified among 53 MCR1-EC strains isolated in this study, and the major clone types were ST10-A (28.3%, 15/53), ST1112-A (15.1%, 8/53), ST744-A (7.5%, 4/53), ST101-B1 (5.7%, 3/53), and ST457-D (5.7%, 3/53). The other clone types included only one or two MCR1-EC strains. In a comparison by swine farm, all clone types were not shared between pig farms with the exception of ST101-B1, which was isolated from three pig farms. In a comparison by swine production stage, the clone types were shared between pigs of different stages within farms (Figure 18).

In the clonal distribution analysis of MCR1-EC published in the NCBI database, 17 MCR1-EC strains derived from South Korea harbored 15 clone types, including ST10-A (11.8%, 2/17) and ST11124-A (11.8%, 2/17; Supplementary File 1). In the clonal distribution analysis of human-, pig-, and chicken-derived 1,652 MCR1-EC, 248 clone types were identified among 940 human-derived MCR1-EC, and major clone types were ST10-A (9.6%, 90/940), ST152-A (3.5%, 33/940), ST206-A (3.0%, 28/940), and ST101-B1 (2.9%, 27/940). Among 266 pig-derived MCR1-EC strains, 101 clone types were identified, and major types were ST10-A (11.3%, 30/268), ST206-A (4.1%, 11/266), and ST101-B1 (3.8%, 10/266). Among 446 chicken-derived MCR1-EC isolates, 118 clone types were identified and major types were ST10-A (7.6%, 34/446), ST156-B1 (6.7%, 30/446), and ST93-A (4.7%, 21/446).

cgMLST-based genetic relatedness analysis of MCR1-EC strains from swine farms and strains from various sources

In the cgMLST-based genetic relatedness analysis of intestinal pathogenic MCR1-EC isolated from this study and South Korea-derived MCR1-EC published on the NCBI database, the genetic relatedness distances between strains ranged from 0 to 0.961 (average 0.720, 95% CI: 0.694–0.746; Figure 22). We clustered strains with a genetic relatedness distance of less than 0.01 in cgMLST, and a total of four clusters (clusters I–IV) were identified. Cluster I included two ST20-A MCR1-EC strains (MCR1-A03 and MCR1-A10) isolated from one weaning piglet and one growing pig in Farm A. Cluster II included eight ST1112-A MCR1-EC strains (MCR1-B07, B08, B14, B16, B17, B18, B20, and B22) isolated from six growing and two finishing pigs in Farm B. Cluster III included two ST10-A MCR1-EC strains (MCR1-A04 and MCR1-A36) isolated from two weaning piglets in Farm A. Cluster IV included two ST11124-A MCR1-EC strains (GCA_013390695.1 and GCA_013391045.1) published in the NCBI database. All strains of four clusters were identified as being isolated from individuals from the same farm or hospital. According to the metadata in the original report, two South Korean-derived ST11124-A MCR1-EC strains in cluster IV were reported to be isolated from two patients in the same hospital but at different collection times for each strain [107]. Except for MCR1-EC strains belonging to four clusters, the genetic relatedness distance was confirmed to have an average value of 0.771 (95% CI: 0.752–0.790), and the average value was 0.397 (95% CI: 0.317–0.477) even among six MCR1-EC isolates carrying the same clone type, ST10-A.

In the genetic relatedness analysis of 82 ST10-A MCR1-EC strains from humans, chickens, and pigs worldwide, the genetic relatedness distance

between strains ranged from 0 to 0.525 (average 0.309, 95% CI: 0.305–0.312; Figure 23). We clustered strains with a genetic relatedness distance of less than 0.01 in cgMLST, and a total of five clusters (cluster III, V, VI, VII, and VIII) were identified. Cluster III included two MCR1-EC strains (MCR1-A04 and MCR1-A36) isolated from Farm A in this study. Cluster V included two chicken-derived strains (GCA_013072745.1 and GCA_013072725.1) from China. Cluster VI included two human-derived strains (GCA_003290855.1 and GCA_003290875.1) from China. Cluster VII included two human-derived strains (GCA_003291515.1 and GCA_003290695.1) from China. Cluster VIII included two chicken-derived strains (GCA_014900955.1 and GCA_014900935.1) from China. According to the metadata in the original report, MCR1-EC, belonging to the four clusters V, VI, VII, and VIII, was isolated from individuals in the same hospital or farm, with strains in the same cluster [213, 223]. Except for MCR1-EC isolates belonging to four clusters, the genetic relatedness distance between the other MCR1-EC isolates was confirmed to have an average value of 0.309 (95% CI: 0.305–0.313).

2.4. Discussion and Conclusion

The global emergence and spread of MCR1-EC represent a serious threat for public health [17]. Although the use of colistin for the prevention of swine colibacillosis has been banned from multiple countries worldwide since 2016, colistin has been generally used for the treatment of swine diseases, leading to an increased prevalence of MCR1-EC in swine farms worldwide [117, 206-209, 214]. In this study, the weighted prevalence of MCR1-EC was 8.4% (95% CI: 2.1%–28.0%) and it was comparable with that in previous

reports conducted from Belgium (13.2%; [206]), Japan (20.4%; [207]), Taiwan (29.2%; [208]), and China (76.2%; [209]). To the best our knowledge, this is the first study to investigate the prevalence of MCR-EC in food-animal farms in South Korea. Unlike this study, previous studies conducted in South Korea only investigated the presence of *mcr* variants in previously isolated *E. coli* strains isolated from various sources including humans, livestock, and food-animal products [104, 105, 107, 111-113, 116-118, 120], but did not evaluate the prevalence of MCR-EC. Considering that colistin is a member of CIA and that livestock could play an important reservoir of MCR1-EC, further studies of MCR1-EC in food animal farms, especially pig farms, seem to be needed.

Comparison of the four swine production stages showed that weaning piglets exhibited the highest prevalence of MCR1-EC compared with pigs at other stages. This result was consistent with that of previous studies conducted worldwide, in which MCR1-EC was isolated mainly from weaning piglets [19, 117, 206-209, 214]. Considering that colistin has been reported to be mainly prescribed in weaning stages for the treatment of swine colibacillosis, which exhibits higher incidence during the weaning stage [36, 38, 39], high colistin at weaning stage could be one of the important causes for the high prevalence of MCR1-EC at this stage. In the comparative analysis based on the four swine stages, it was also found that the weaning piglets exhibited significantly higher resistance rates to various antimicrobial agents than other stages, especially sow. Furthermore, the previous chapter showed that the prevalence of ESBL/AmpC-EC was significantly higher in weaning piglets compared to other stages including sows. This result suggests that the antimicrobial resistance found in weaning piglets may not be inherited from sow. According to recent studies, colonization of ESBL/AmpC/MCR1-EC could last longer than 6 months even

without antibiotic selection pressure, ESBL/AmpC/MCR1-EC colonies in the intestinal tract of swine at the weaning stage could persist until the date of their slaughter (about 150–230 days old; [175, 224, 225]). In that point, we suggest that ESBL/AmpC/MCR1-EC prevalence at the weaning stage could be a reflection of that of the entire farm, and that weaning stage should serve as the critical point in controlling the prevalence of ESBL/AmpC/MCR1-EC in swine farms.

Notably, 96.2% of MCR1-EC exhibited MDR, with resistance against average 4.8 antimicrobial classes. Among tested antimicrobial agents, resistance rates to ampicillin, tetracycline, chloramphenicol, were remarkably high, which was consistent with the antimicrobial resistant patterns of *E. coli* strains from healthy pigs in the National Antimicrobial Resistance Monitoring Report conducted by the KFDA [226]. Based on these results, the MDR pattern of MCR1-EC isolates identified from this study may be the result of the accumulation of colistin resistance to the antibiotic-resistant *E. coli* strains prevalent in swine farms in South Korea. Among 53 MCR1-EC strains, 17.0% of strains were identified to produce CTX-M-55-type ESBL. Wu et al. (2018a) suggested that colistin and β-lactam antibiotics have been commonly prescribed together in food-animal husbandry, and resistance to colistin and third generation cephalosporins emerged and increased together under the heavy selection pressure of antibiotics over the last few decades. In our previous study, interestingly, the prevalence of ESBL/AmpC-EC in the four farms, where MCR1-EC was found in the present study, was significantly higher (76.4%; OR: 3.2, 95% CI: 1.40-7.18, $p < 0.05$, GEE) than in farms where MCR1-EC was not identified (50.5%). Hence, these findings were consistent with the conclusion of Wu et al. (2018a). The potential of MDR bacterial transmission from food-

animal husbandry to humans and vice versa has been continuously proposed by various studies [18, 166, 227]. Considering that both third generation cephalosporins and colistin are classified as critically important antimicrobial agents for livestock and humans [17], the high prevalence of MDR bacteria carrying both *mcr-1.1* and CTX-M-55-type ESBL implies the potential for the emergence of MDR pathogens, which can hardly be treated, even by last resort antimicrobials. Collectively, we suggest that pig farms, which are important reservoirs of MDR bacteria, require special attention at the weaning stage to control CIA-resistant bacteria including ESBL/AmpC/MCR1-EC.

Swine colibacillosis is one of the major swine diseases impacting the global swine industry and is associated with huge economic losses; edema disease (ED) and post-weaning diarrhea (PWD) belong to the classification of swine colibacillosis [27]. Given that colistin has been considered a recommended treatment for swine colibacillosis and InPECs are major causative bacteria of swine colibacillosis [27], the presence of intestinal pathogenic MCR1-EC in pig husbandry could represent a major challenge for the swine industry. In this study, 22.6% of MCR1-EC strains were identified as InPECs including STEC carrying *stx2e* and EPEC carrying LEE-encoded virulence factors. The *stx2e* gene is key virulence factor causing damage to arterioles and edema at various sites, eventually leading to death associated with ED [228]. The LEE-encoded VFs are responsible for the characteristic histopathological lesion of PWD, termed attaching/effacing lesions [229]. Among various identified virulence factors from intestinal pathogenic MCR1-EC strains, the presence of *katP* might especially increase the risk presented by the strains, since it has been reported to promote the virulence of InPECs by supporting their colonization of the host intestine [230]. In addition to virulence

factors, all intestinal pathogenic MCR1-EC strains were identified as MDR bacteria harboring five or more antimicrobial class resistance genes. Comparative genomic analysis according to the stage of pig development revealed that the genetic characteristics of the intestinal pathogenic MCR1-EC strains were highly shared among pigs at different stages, suggesting that there is a high potential for the transmission of intestinal pathogenic MCR1-EC within farms. Although *E. coli* is a major organism carrying *mcr* genes, other *Enterobacterales* species have also been reported to carry the *mcr* genes and inhabit the intestinal tract of pigs [231, 232]. In addition, *mcr* genes have been reported to be highly transferred from *E. coli* to other pathogens, causing swine diseases, such as *Salmonella*, *Klebsiella*, and *Pseudomonas* [219]. Thus, in cases of swine diseases caused by pathogens harboring these multiple virulence factors and MDR, the prescription of colistin may simply impose selection pressure, leading to disease treatment failure and the spread of colistin resistance in swine farms. To the control these highly virulent and MDR pathogens, it may be necessary to establish a strategy based on in-depth characterization, such as WGS analysis, rather than blindly using antibiotics for the treatment of swine diseases.

In the investigation of genotypic and phenotypic virulent characteristics, MCR1-EC isolates carried multiple ExPEC-associated virulence factors, including *traT*, *hlyF*, and *kpsMTII*, and four MCR1-EC isolates were identified as ExPEC. A high rate of ExPEC-associated virulence factors has been reported to correspond with high potential for survival in the harsh environments and pathogenicity of the bacteria against the host immune system [233]. The expression of TraT protein, an outer membrane lipoprotein, has been linked to improved serum resistance [234]. The hemolysin production

regulator *hlyF* create pores in the membrane of host cells, which increasing the permeability of host cells and ending cell lysis [235]. The *kpsMTII* has been reported to encoding capsular polysaccharides acting protect the bacteria from environment by covering bacteria and helping to form biofilm [236]. In addition to genotypic virulence, eight MCR1-EC strains showed moderate-to-strong biofilm formation capacity. Biofilm formation has been reported to confer a fitness advantage to bacteria by enhancing their survivability, increasing their virulence, and facilitating their ability to acquire virulence and antibiotic resistance genes during horizontal gene transmission owing to their high microbial density [195, 196]. Based on fitness advantages, such as strong biofilm formation or harboring multiple ExPEC virulence factors, MCR1-EC could survive better in an environment of swine farm husbandry and continuously exist through a repeated cycle, which involves the shedding from swine through feces, survival in the farm environment, and reintroduction to swine. In addition, although MCR1-EC might not be directly transmitted from pig farms to humans through the food-chain, these fitness advantages could provide MCR1-EC strains possibility to survive better in the food-chain and serve as an important source of *mcr-1.1* for various other pathogens in food-chains through genetic transmission mechanisms, such as conjugation.

In the analysis of clonal distribution of MCR1-EC, ST10-A was the most prevalent clone type of MCR1-EC strains in this study, as well as in the human, pig, and chicken-derived MCR1-EC strains described on the NCBI database. However, ST10-A represented only 28.3% of the MCR1-EC samples isolated in this study and 9.8% of 1,562 MCR1-EC samples described in the NCBI database. Other clone types, such as ST101-B1, ST744-A, and ST206-A, also accounted for a significant proportion of total strains. Consistently, the

epidemiological analyses of MCR1-EC global clonal distribution revealed that ST10-A was the most prevalent clone type of MCR1-EC in humans and food-animals, whereas the other clone types also accounted for a significant proportion among total strains [206-210, 237]. Furthermore, one recent study in Thailand showed that the dominant clone type of MCR1-EC in swine farms was ST101, followed by ST10 [238].

Interestingly, the results of clonal distribution analysis of MCR1-EC isolated from pig farms in this study revealed that the clone types were highly shared among MCR1-EC strains isolated from the same farm, but were heterogeneous between farms. Comparison by swine farms showed that all clone types, including the most predominant clone type ST10-A, were not shared between pig farms with the exception of ST101-B1, which was identified in three pig farms. In the analysis of clonal distribution of MCR1-EC isolated from South Korea, the clone types were highly heterogenous with 16 MLST types (i.e., ST10-A, ST131B2, ST155-B1, ST156B1, etc.) among 17 MCR1-EC strains. In consistent, highly heterogenous MLST types of MCR1-EC strains were also identified from researches conducted from South Korea [112, 115, 117, 120]. Collectively, our study suggests that clonal types of MCR1-EC may vary widely between studies, and that it may be shared within closed environments such as a pig farm, but not between environments such as different pig farms or food-chains. Hence, this suggestion may imply that that clonal expansion alone may not have a direct role in MCR1-EC propagation between environments.

Instead, the *mcr-1.1* was transferred from more than 90% of MCR1-EC strains via conjugation, implying horizontal gene transfer plays a more important role than clonal diffusion in colistin propagation between

environments. Vines et al. (2021) analyzed the genetic relationship based on WGS for MCR-EC isolated from food-animal and farmer in the farm. As a result, the shared *mcr*-carrying plasmids were highly distributed in different *E. coli* strains from food-animals and farmers, suggesting that *mcr* was transferred between humans and animals by horizontal transfer [122].

The cgMLST-based genetic relatedness analysis of intestinal pathogenic MCR1-EC strains isolated in this study, as well as those published in the NCBI database, revealed that MCR1-EC strains isolated from individuals within closed environment (such as hospitals or farms) were highly clustered, showing a genetic distance lower than 0.01. Noteworthy, clustered strains were isolated within the same hospitals or farms, but in separate spaces or at different time points. According to the original metadata of the two strains in cluster IV, they were isolated from patients in the same hospital but with a time interval of 2 months [107]. In addition, two strains in cluster I and eight strains in cluster II, isolated in the present study, were isolated from different swine stages, which mean that they were isolated from pigs living in separate barns, including weaning, growing, and finishing barns. These results suggest that the clonal expansion may have a relatively high contribution to the propagation of MCR1-EC between individuals in closed environments. Since *mcr-1* is mainly transmitted by plasmids, the important role of genetic transferability of *mcr-1* in the spread of MCR1-EC has been continuously highlighted in various studies. However, genetic transfer essentially presupposes the transfer of strains and bacteria-to-bacteria interactions under favorable conditions, such as physical distance between strains, nutrition, and environmental conditions, among others [41], which suggests that bacterial transmission also provides a crucial basis for the spread of MCR1-EC. It was previously reported that

bacterial transmission between swine production stages within farms may probably occur through farm worker/veterinarian handling, equipment contamination, and transference of manure excretions between different stage barns [7, 40]. Our results suggest that bacterial cross-infection between different stages, pigs may act as an important risk factor for the prevalence of MCR1-EC. Swine farms have been continuously reported as an important reservoir of MCR1-EC [206-209]. Our findings highlight that efforts to reduce bacterial cross-infection between stages are imperative to control MCR1-EC prevalence in swine farms, one of major reservoir of MCR1-EC.

Among reported *mcr* variants, the present study focused on the most predominant variant type, *mcr-1*. Recent studies have shown that the mobile genetic elements associated with the *mcr* genes may differ between variant types, which may lead to different genotypic and phenotypic traits in bacteria [19, 24, 95, 239-241]. In this study, we conducted the comparative analysis of prevalence, characteristics, and clonal distribution of MCR1-EC according to swine production stages by excluding other *mcr* variants, which could be potential confounding factors. For further study, it would be interesting to analyze the characteristic differences of the other major *mcr* variants, such as *mcr-3* or *mcr-9*, according to food-animal production stages in livestock husbandry.

In conclusion, to the best our knowledges, this is the first study that analyzed the prevalence, characteristics, and clonal dynamics of MCR1-EC strains in swine farms from South Korea. Our study showed that that MCR1-EC isolates having MDR and pathogenic advantages (InPEC/ExPEC-associated virulence factors or robust biofilm formation) were highly shared between pig stages within farms, as suggested by WGS-based analysis. In this

study, MCR1-EC strains isolated from various sources such as pigs, pork, and humans in South Korea exhibited a very heterogeneous MLST types without a dominant type or a shared type, thereby providing indirect scientific evidence that MCR1-EC strains have a low possibility of inter-environmental transmission via clonal spreading. Whereas, high genetic closeness was identified between MCR1-EC strains isolated from closed environments such as livestock farms and human hospitals, suggesting a high possibility of cross-contamination within these environments. In particular, this study showed that weaning piglets were an important reservoir of CIA-resistant bacteria such as ESBL/AmpC/MCR-EC. Considering the previous research results that these CIA-resistant bacteria can exist in the pig intestine for more than 6 months without antibiotic administration, it suggests that CIA-resistant bacteria in weaned pigs can persist until the slaughterhouse. Our study highlights the need to manage pig farms, an important reservoir of CIA-resistant bacteria, with special attention in the weaning stage.

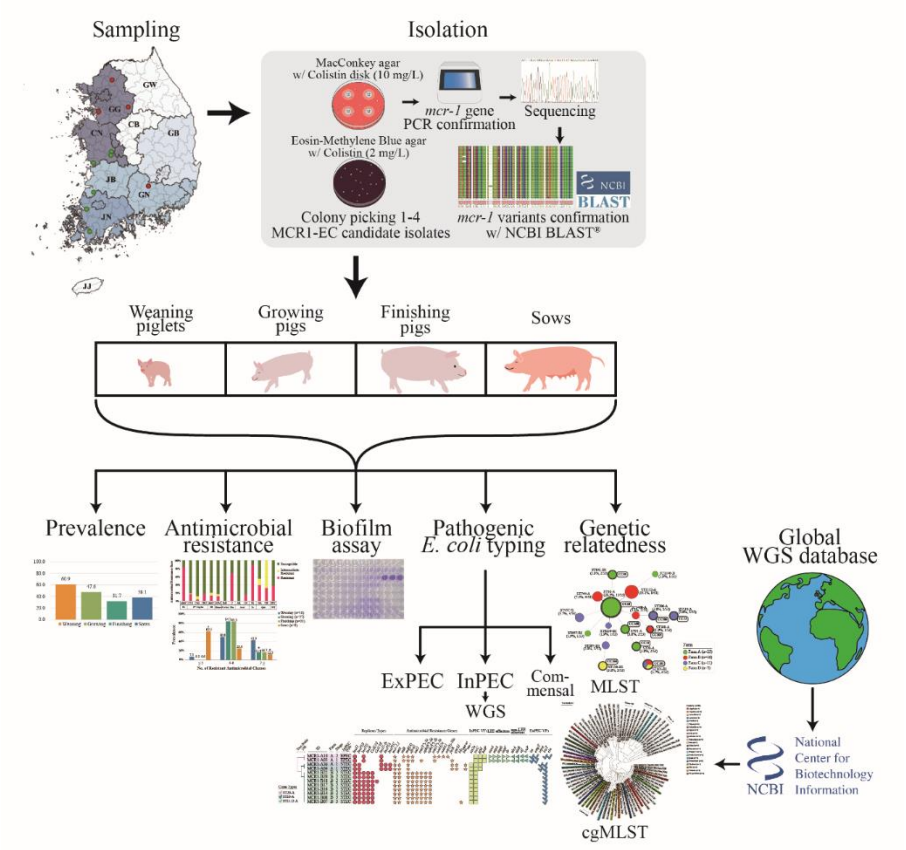


Figure 16. Flow chart of the present study design (Chapter 2)

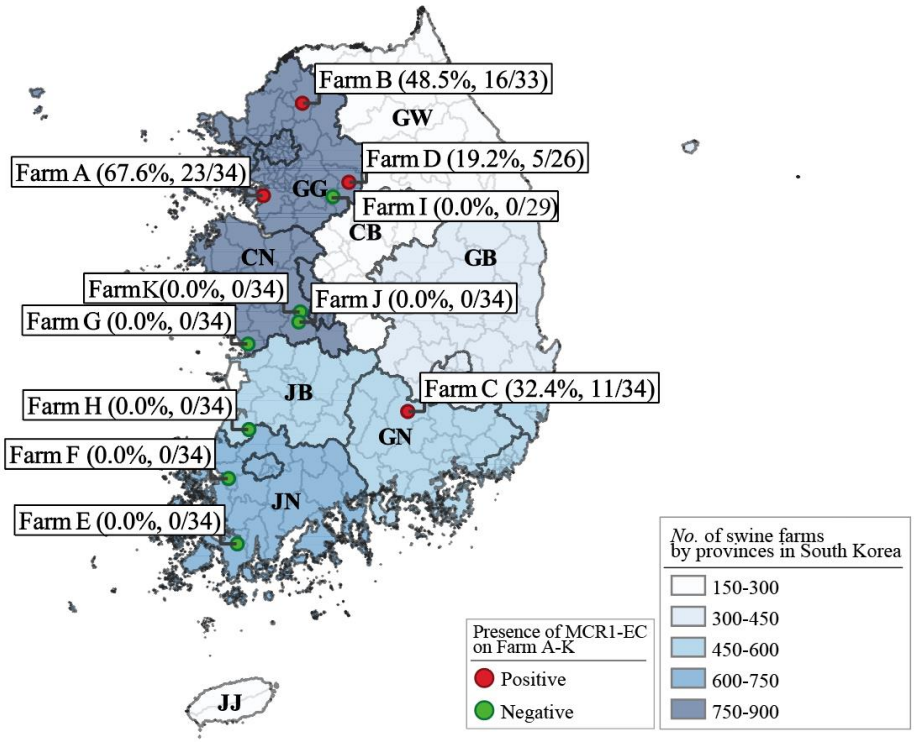


Figure 17. *Escherichia coli* strains carrying *mcr-1* (MCR1-EC) prevalence in Farms A–K and number of swine farms by South Korean province. The numbers in parentheses show the unweighted prevalence of MCR1-EC and the number of MCR1-EC-positive samples versus that of total samples for each farm. The number of pig farms by province in South Korea was obtained from the 2017 demographic report of the Korean Statistical Information Service of Statistics Korea. Visualization was conducted using the QGIS geographic information system program (v3.16.15). MCR1-EC, *Escherichia coli* carrying the mobilized colistin resistance gene *mcr-1.1*; GG, Gyeonggi-do; CN, Chungcheong-nam-do; JB, Jeolla-buk-do; JN, Jeolla-nam-do; GN, Gyeongsang-nam-do; GB, Gyeongsang-buk-do; CB, Chungcheong-buk-do; and GW, Gangwon-do; JJ, Jeju-do.

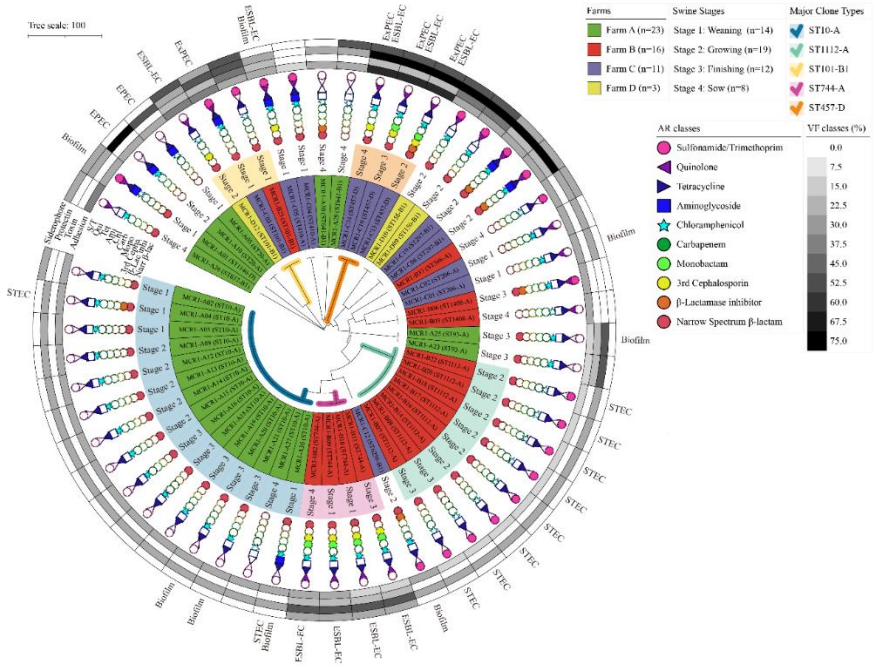


Figure 18. Antimicrobial resistance, genotypic/phenotypic virulence factors, and clone types of 53 MCR1-EC isolates from swine farms. Visualization was conducted using the online visualization tool iTOL (v6, https://itol.embl.de/). MCR1-EC, *Escherichia coli* carrying the mobilized colistin resistance gene *mcr-1.1*; EPEC, enteropathogenic *E. coli*; STEC, shiga toxin-producing *E. coli*; and ESBL-EC, extended-spectrum β-lactamase producing *E. coli*, Biofilm, *E. coli* with moderate/strong biofilm formation ability.

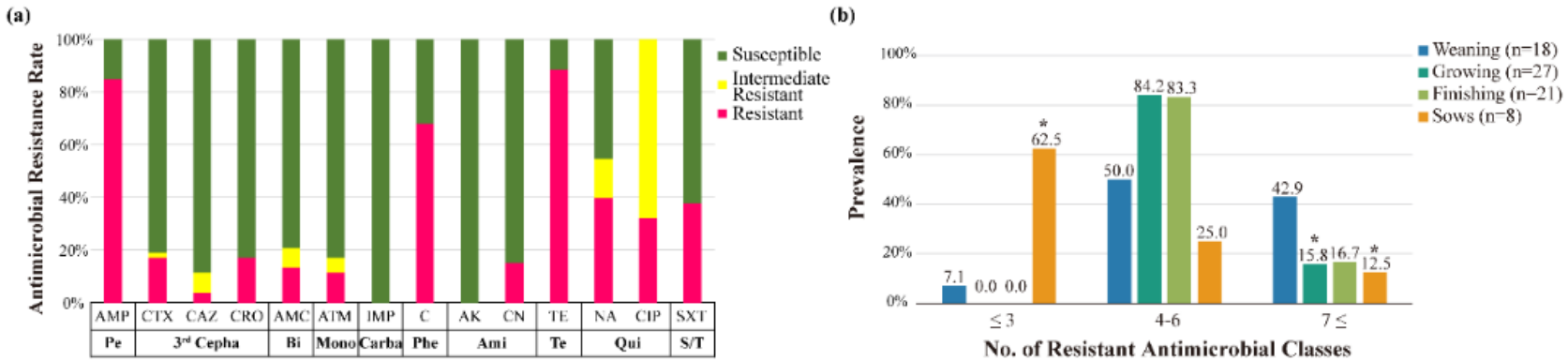


Figure 19. Antimicrobial susceptibility of MCR1-EC strains and prevalence of resistant MCR1-EC. Antimicrobial susceptibility of MCR1-EC from swine farms (A) and prevalence of MCR1-EC isolates resistant to different numbers of antimicrobial classes by swine production stage (B). MCR1-EC, *Escherichia coli* carrying the mobilized colistin resistance gene *mcr-1.1*; Pe, broad spectrum penicillin class; third Cepha, third-generation cephalosporin class; Bi, β-lactamase inhibitor class; Mono, monobactam class; Carba, carbapenem class; Phe, phenicol class; Ami, aminoglycoside class; Te, tetracycline class; Qui, quinolone class; S/T, sulfonamide/trimethoprim class; AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; AMC, amoxicillin/clavulanate; ATM, aztreonam; IMP, imipenem; C, chloramphenicol; AK, amikacin; CN, gentamycin; TE, tetracycline; NA, nalidixic acid; CIP, ciprofloxacin; and SXT, sulfamethoxazole/trimethoprim. $^{*}p < 0.05$, significantly different prevalence relative to that of weaning piglets, calculated *via* GEEs.

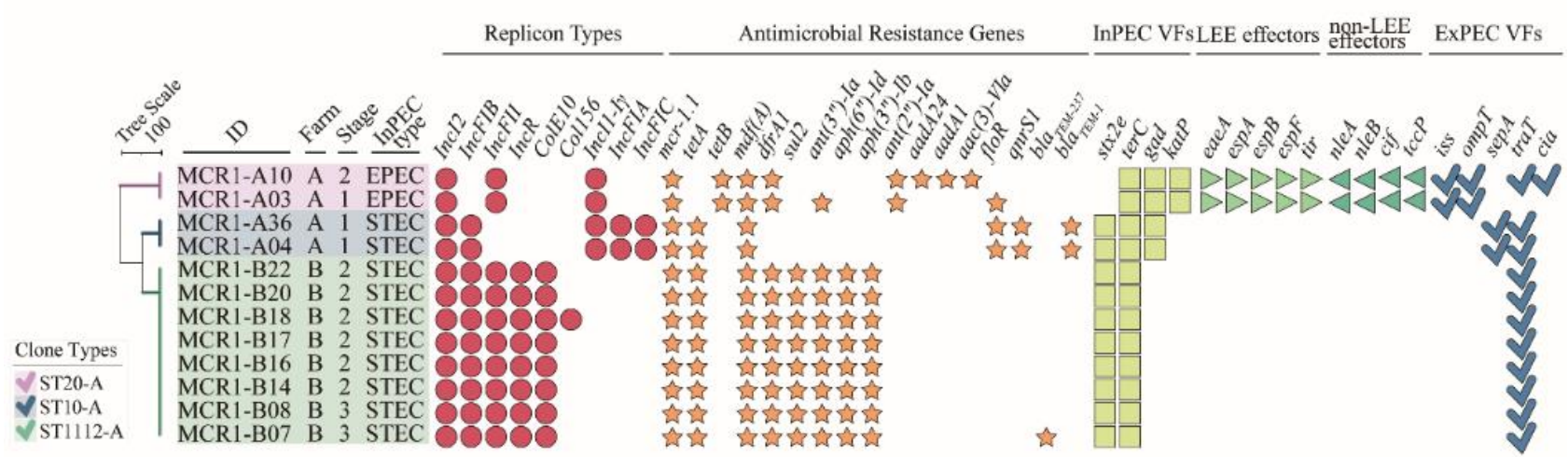


Figure 20. Whole-genome sequence (WGS)-based in-depth genetic characterization of 12 intestinal pathogenic MCR1-EC strains. The phylogenetic tree was constructed based on allele profiles of multi-locus sequence types (MLSTs) of 12 intestinal pathogenic MCR1-EC strains *via* the Unweighted Pair Group Method with Arithmetic means (UPGMA) method, calculating dice coefficients in the Bionumerics program (v6.6). Visualization was conducted using the online visualization tool iTOL (v6, https://itol.embl.de/). MCR1-EC, *Escherichia coli* carrying the mobilized colistin resistance gene *mcr-1.1*; Stage 1, weaning piglets; Stage 2, growing pigs; Stage 3, finishing pigs; Stage 4, pregnant sows; InPEC type, intestinal pathogenic *E. coli* type; EPEC, enteropathogenic *E. coli*; STEC, shiga toxin-producing *E. coli*; InPEC VFs, intestinal pathogenic *E. coli*-associated virulence factors; LEE effectors, locus of enterocyte effacement (LEE)-encoded effectors; non-LEE effectors, non-LEE-encoded effectors; and ExPEC VFs, extra-intestinal pathogenic *E. coli*-associated virulence factors.

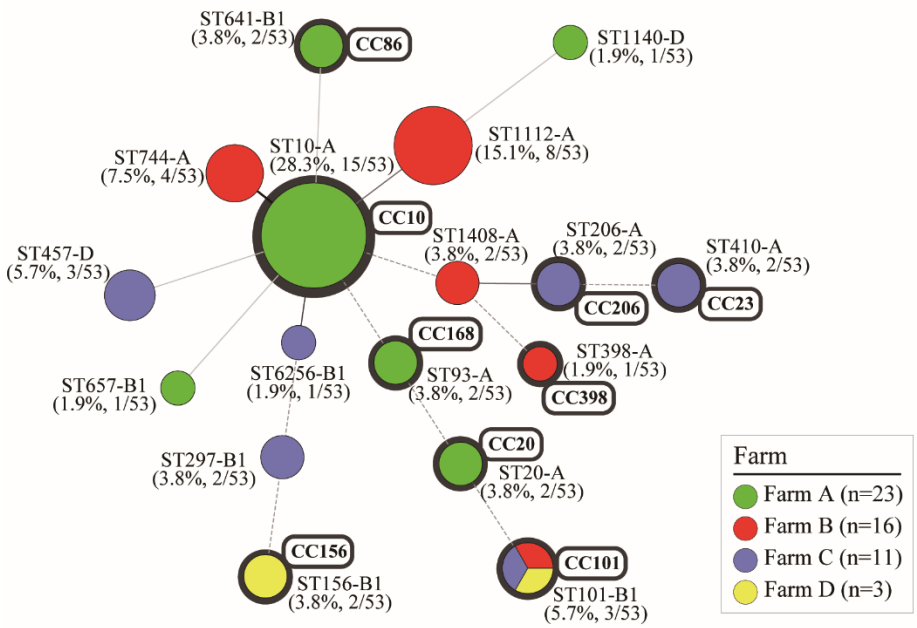


Figure 21. Clonal distribution of MCR1-EC isolates: Minimum spanning tree (MST) based on MLST allele profiles. The MST was constructed using the Bionumerics program (v6.6). The colors of nodes correspond to the four swine farms. The upper number shows the sequence type of each node, and the lower number in parentheses indicates percentages for each node. The size of the node indicates the number of strains belonging to the sequence type (ST)-phylogroup (PG) type. The gray shaded area represents the clonal complex (CC). The branch line types represent differences in the number of alleles as follows: bold solid line (one allele), thin solid line (2–3 alleles), dashed line (four alleles), and dotted line (above five alleles). MCR1-EC, *Escherichia coli* carrying the mobilized colistin resistance gene *mcr-1.1*; MLST, multi-locus sequence typing.

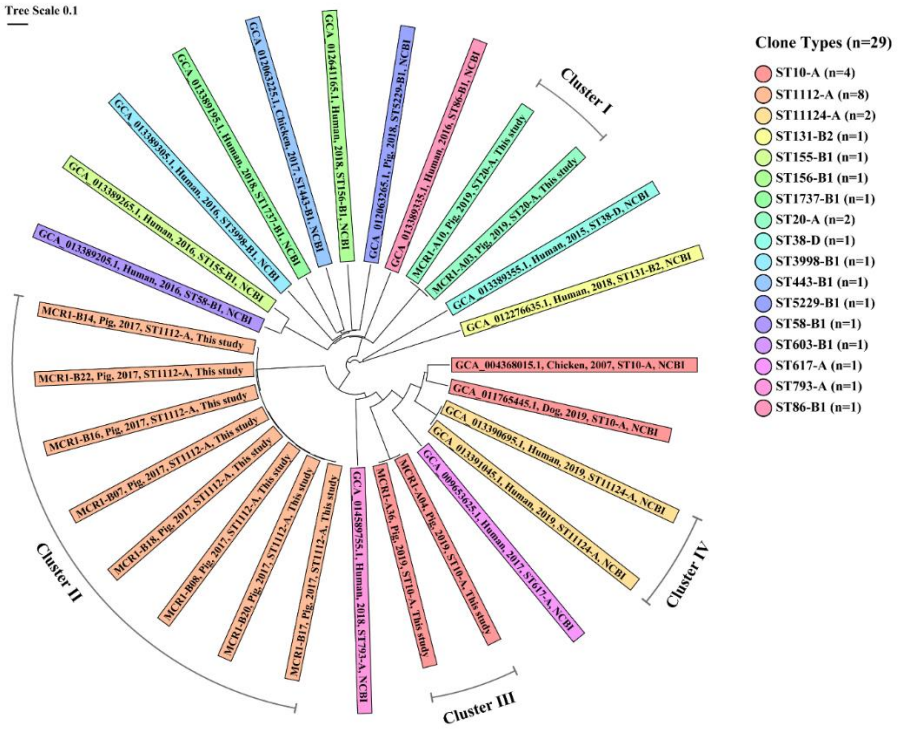


Figure 22. Core genome MLST (cgMLST)-based genetic relatedness between 29 MCR1-EC isolates from South Korea. Data comprise 12 intestinal pathogenic MCR1-EC strains isolated in this study and 17 MCR1-EC strains isolated from South Korea, published in the National Center for Biotechnology Information (NCBI) GenBank database. The phylogenetic tree based on cgMLST was constructed using the neighbor-joining algorithm with default parameters implemented in the Ridom SeqSphere+ program (v8.2.0). The color of shades corresponds to each clone type. The contents of shades include, from left to right, the assembly accession number, host, isolation date, clone type, and data source (this study or NCBI database) for each strain. Four clusters consist of MCR1-EC strains with a relatedness distance value less than 0.01. MCR1-EC, *Escherichia coli* carrying the mobilized colistin resistance gene *mcr-1.1*; MLST, multi-locus sequence typing.

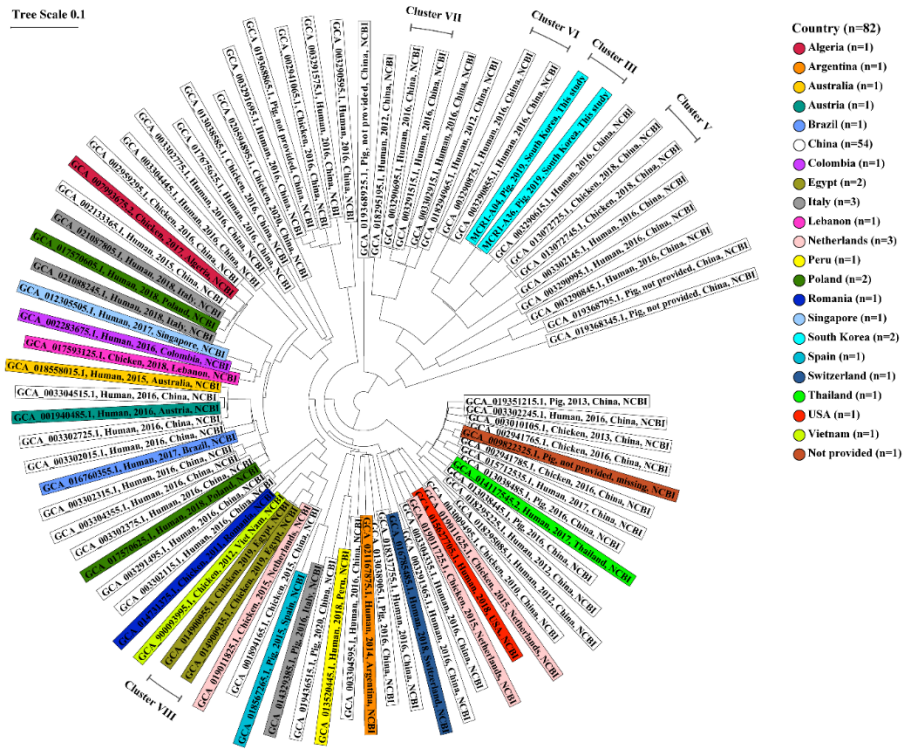


Figure 23. Core genome multi-locus sequence typing-based genetic relatedness of 82 ST10-A MCR1-EC strains isolated from humans, pigs, and chickens worldwide. Strains were derived from the NCBI GenBank database. The phylogenetic tree based on cgMLST was constructed using the neighbor-joining algorithm with default parameters implemented in the Ridom SeqSphere+ program (v8.2.0). The shaded color corresponds to the country where the strain was isolated. The contents of shades include, from left to right, the assembly accession number, host, isolation date, isolation country, and data source (this study or NCBI database) for each strain. Five clusters consist of MCR1-EC isolates with a relatedness distance value of less than 0.01. MCR1-EC, *Escherichia coli* carrying the mobilized colistin resistance gene *mcr-1.1*; cgMLST, core genome multi-locus sequence typing.

Table 4. Antimicrobial resistance rate of MCR1-EC according to the pig productions stages

Anti-microbial classes	Weaning piglets (reference)			Growing pigs			Finishing pigs			Pregnant sows		
	Rate (%)	OR (95% CI)	*P* - value	Rate (%)	OR (95% CI)	*P* - value	Rate (%)	OR (95% CI)	*P* - value	Rate (%)	OR (95% CI)	*P* - value
Pe	78.6	-	-	94.7	4.9 (0.62-39.16)	0.13	100.0	7.6 [a] (0.35-163.83)	0.19	50.0	0.3 (0.05-1.41)	0.12
3rd Cepha	28.6	-	-	10.5	0.3 (0.02-3.64)	0.34	8.3	0.2 (0.01-5.24)	0.36	25.0	0.8 (0.15-4.64)	0.84
Carba	0.0	-	-	0.0	0.7 [a] (0.01-39.73)	0.88	0.0	1.2 [a] (0.02-62.85)	0.94	0.0	1.7 [a] (0.03-94.11)	0.79
Mono	21.4	-	-	5.3	0.2 (0.01-8.01)	0.40	8.3	0.3 (0.01-16.66)	0.58	12.5	0.5 (0.23-1.20)	0.13
Bi	7.1	-	-	21.1	3.5 (0.12-104.32)	0.47	8.3	1.2 (0.05-27.38)	0.92	12.5	1.9 (1.00-3.45)	0.05
Phe	85.7	-	-	63.2	0.3 (0.01-6.71)	0.44	75.0	0.5 (0.01-19.33)	0.71	37.5	0.1 (0.04-0.28)	<0.01*
Ami	42.9	-	-	15.8	0.3 (0.12-0.51)	<0.01*	0.0	0.5 [a] (0.002-1.06)	0.05	0.0	0.1 [a] (0.003-1.59)	0.10
Te	100.0	-	-	89.5	0.2 [a] (0.01-5.44)	0.37	100.0	0.9 [a] (0.02-46.71)	0.94	50.0	0.03 [a] (0.001-0.78)	0.03†
Qui	64.3	-	-	31.6	0.3 (0.03-1.97)	0.19	16.7	0.1 (0.07-0.18)	<0.01*	50.0	0.6 (0.21-1.49)	0.24
S/T	28.6	-	-	57.9	3.4 (0.43-27.61)	0.25	33.3	1.3 (0.06-28.35)	0.89	12.5	0.4 (0.03-4.30)	0.42

The odds ratio (OR), including 95% of confidential interval (95% CI) and *P* – value, was calculated by generalized estimating equations (GEE). [a]Where zeros cause problems in calculating OR or 95% CI, Fisher's exact test was used in the calculations instead of GEE. *$P <$ 0.05, statistically significant based on GEE, †$P < 0.05$, statistically significant based on Fisher's exact test. Abbreviation. Pe, broad spectrum penicillin class; 3rd Cepha, 3rd cephalosporin class; Carba, carbapenem class; Mono, monobactam class; Bi, β-lactamase inhibitor class; Phe, phenicol class; Ami, aminoglycoside class; Te, tetracycline class; Qui, quinolone class; S/T, sulfonamide/trimethoprim; class Poly, polymyxin class.

Table 5. Analysis of pathogenic *E. coli*-associated virulence factors in MCR1-EC from swine farms

Virulence factor classes	Virulence factors	Prevalence (%)	No. of positive MCR1-EC /No. of total MCR1-EC
Intestinal pathogenic *E. coli* (InPEC)	*stx2*	18.9	10/53
	eaeA	3.8	2/53
	stx2 or *eaeA*	22.6	12/53
Extra-intestinal pathogenic *E. coli* (ExPEC)	*kpsMTII*[a]	9.4	5/53
	papC[a]	7.5	4/53
	papAH[a]	7.5	4/53
	sfa/focDE[a]	1.9	1/53
	afa/draBC[a]	0.0	0/53
	Two and more ExPEC VFs	7.5	4/53
Uropathogenic *E. coli* (UPEC)	*fyuA*[b]	7.5	4/53
	chuA[b]	7.5	4/53
	yfcV[b]	5.7	3/53
	vat[b]	3.8	2/53
	Two and more UPEC VFs	7.5	4/53
Adhesion	*fimH*	90.6	48/53
	csgA	84.9	45/53
	papC[a]	7.5	4/53
	papAH[a]	7.5	4/53
	yfcV[b]	5.7	3/53
	sfa/focDE[a]	1.9	1/53
	afa/draBC[a]	0.0	0/53
	iha	0.0	0/53
	Total adhesion (at least one)	100.0	53/53
Toxin	*hlyF*	26.4	14/53
	astA	7.5	4/53
	vat[b]	3.8	2/53
	pic	0.0	0/53
	aat	0.0	0/53
	Total toxin (at least one)	32.1	17/53
Protectin	*traT*	88.7	47/53
	ompT	26.4	14/53
	iss	13.2	7/53
	kpsMTII[a]	9.4	5/53
	Total protectin (at least one)	90.6	48/53
Siderophore	*iutA*[a]	26.4	14/53
	iroNE.coli	9.4	5/53
	fyuA[b]	7.5	4/53
	chuA[b]	7.5	4/53
	Total siderophore (at least one)	35.8	19/53

[a] Virulence factors used for criteria of ExPEC; if positive for ≥2 of five key markers including *papA* and/or *papC*, *sfa*/*focDE*, *afa*/*draBC*, *iutA*, and *kpsMTII*. [b] Virulence factors used for criteria of UPEC; if positive for ≥3 of four key markers including *vat*, *fyuA*, *chuA*, and *yfcV*.

Supplementary Table 6. Farm and sampling information for eleven swine farms included in this study and prevalence of *Escherichia coli* carrying *mcr-1* (MCR1-EC) across farms.

	Farm ID	Total	Farm A	Farm B	Farm C	Farm D	Farm E	Farm F	Farm G	Farm H	Farm I	Farm J	Farm K
Farm Information	Province	-	Gyeonggi-do	Gyeonggi-do	Gyeongsang-nam-do	Gyeonggi-do	Jeolla-nam-do	Jeolla-nam-do	Chungcheong-nam-do	Jeolla-buk-do	Gyeonggi-do	Chungcheong-nam-do	Chungcheong-nam-do
	Collection date	-	Jul 2019	May 2017	May 2018	Jan 2019	Jul 2017	Aug 2017	Apr 2018	May 2018	Oct 2018	Aug 2019	Aug 2019
	Prevalence of ESBL/AmpC-EC [a]	-	64.7%	82.4%	58.8%	73.1%	61.8%	23.5%	17.6%	51.5%	89.7%	36.7%	50.0%
			No. of MCR1-EC-positive samples / No. of total samples (unweighted prevalence)										
Prevalence of MCR1-EC	Weaning pigs	14/64 (21.9%)	6/6 (100.0%)	3/6 (50.0%)	5/6 (83.3%)	0/5 (0.0%)	0/6	0/6	0/6	0/6	0/5	0/6	0/6
	Growing pigs	20/117 (17.1%)	6/11 (54.5%)	6/10 (60.0%)	4/11 (36.4%)	4/10 (40.0%)	0/11	0/11	0/11	0/11	0/9	0/11	0/11
	Finishing pigs	13/117 (11.1%)	7/11 (63.6%)	4/11 (36.4%)	1/11 (9.1%)	1/8 (12.5%)	0/11	0/11	0/11	0/11	0/10	0/11	0/11
	Pregnant sows	8/62 (12.9%)	4/6 (66.7%)	3/6 (50.0%)	1/6 (16.7%)	0/3 (0.0%)	0/6	0/6	0/6	0/6	0/5	0/6	0/6
	Total	55/360 (15.3%)	23/34 (67.6%)	16/33 (48.5%)	11/34 (32.4%)	5/26 (19.2%)	0/34	0/34	0/34	0/34	0/29	0/34	0/34
Number of pigs	Weaning pigs		200	2,000	3,200	2,100	4,000	1,200	4,800	2,000	5,000	2,500	200
	Growing pigs		300	1,500	2,400	1,260	3,000	900	3,600	1,500	4,000	1,300	300
	Finishing pigs		500	1,950	3,120	1,640	3,900	1,170	4,680	1,950	15,000	4,000	500
	Pregnant sows		200	500	800	350	1,000	300	1,200	500	2,100	700	100
	Total		1,200	5,950	9,520	5,350	11,900	3,570	14,280	5950	26,100	8,500	1,100

Abbreviation. No., Number; ESBL/AmpC-EC, extended-spectrum β-lactamase-/AmpC β-lactamase-producing *Escherichia coli.* [a] Prevalence of ESBL/AmpC-EC in swine farms was investigated in our previous study [242].

Supplementary Table 7. Oligonucleotide sequences and annealing temperature of primers used for antimicrobial resistance genes and replicon typing

Function	Genes		Nucleotide sequence	Size (bp)	Temp. (°C)	Reference
Typing of *mcr-1*-carrying Plasmids Replicon	*mcr-1*	F	CCGTAATTATCCCACCGTTT	1,719	60	This study
		R	CGCCCATAATACGAATGGAG			
	mcr-1-IncI2	F	AACGGTGTCTATCTACATGGTAT	1,330	60	[212]
		R	ACTTAGCGATCTCGTTGTT			
	mcr-1-IncX4	F	AACGGTGTCTATCTACATGGTAT	1,674	60	[212]
		R	CATTGAATTTGTTCGTCCTC			
ESBL genotypes	$bla_{CTX-M-1}$ group	F	GTTACAATGTGTGAGAAGCAG	1,041	60	[197]
		R	CCGTTTCCGCTATTACAAAC			
	$bla_{CTX-M-2}$ group	F	CGACGCTACCCCTGCTATT	832	60	[197]
		R	CAGAAACCGTGGGTTACGAT			
	$bla_{CTX-M-8}$ group	F	GGCGCTGGAGAAAAGCAG	862	60	[197]
		R	GGTTTTATCCCCGACAACC			
	$bla_{CTX-M-9}$ group	F	GTGACAAAGAGAGTGCAACGG	857	60	[197]
		R	ATGATTCTCGCCGCTGAAGCC			
	$bla_{CTX-M-25}$ group	F	GCACGATGACATTCGGG	327	60	[197]
		R	AACCCACGATGTGGGTAGC			
	bla_{CMY}	F	AACACACTGATTGCGTCTGAC	1,226	60	[197]
		R	CTGGGCCTCATCGTCAGTTA			
	bla_{SHV}	F	TCGCCTGTGTATTATCTCCC	768	54	[197]
		R	CGCAGATAAATCACCACAATG			
	bla_{TEM}	F	TCCGCTCATGAGACAATAACC	1,057	58	[197]
		R	ACGCTCAGTGGAACGAAAAC			
	bla_{OXA}	F	ACACAATACATATCAACTTCGC	813	60	[197]
		R	AGTGTGTTTAGAATGGTGATC			
Phylogenetic group	*yjaA*	F	CAAACGTGAAGTGTCAGGAG	288	55	[153]
		R	AATGCGTTCCTCAACCTGTG			
	chuA	F	ATGGTACCGGACGAACCAAC	211	55	[153]
		R	TGCCGCCAGTACCAAAGACA			
	tspE4.C2	F	CACTATTCGTAAGGTCATCC	152	55	[153]
		R	AGTTTATCGCTGCGGGTCGC			
	AceK.f	F	AACGCTATTCGCCAGCTTGC	400	55	[153]
	ArpA1.r	R	TCTCCCCATACCGTACGCTA			
	ArpAgpE.f	F	GATTCCATCTTGTCAAAATATGCC	301	55	[153]
	ArpAgpE.r	R	GAAAAGAAAAAGAATTCCCAAGAG			
	trpAgpC.1	F	AGTTTTATGCCCAGTGCGAG	219	55	[153]
	trpAgpC.2	R	TCTGCGCCGGTCACGCCC			
	trpBA.f	F	CGGCGATAAAGACATCTTCAC	489	55	[153]
	trpBA.r	R	GCAACGCGGCCTGGCGGAAG			
Antimicrobial resistance	*catA*	F	AGTTGCTCAATGTACCTATAACC	547	57	[203]
		R	TTGTAATTCATTAAGCATTCTGCC			
	cmlA	F	CCGCCACGGTGTTGTTGTTATC	698	57	[203]
		R	CACCTTGCCTGCCCATCATTAG			
	floR	F	TATCTCCCTGTCGTTCCAG	399	52	[203]
		R	AGAACTCGCCGATCAATG			
	tetA	F	GCTACATCCTGCTTGCCTTC	210	58	[203]
		R	CATAGATCGCCGTGAAGAG			
	tetB	F	TTGGTTAGGGGCAAGTTTTG	659	56	[203]
		R	GTAATGGGCCAATAACACCG			

	tetD	F	AAACCATTACGGCATTCTGC	787	60	[203]
		R	GACCGGATACACCATCCATC			
	qnrA	F	ATTTCTCA CGCCAGGATTTG	516	53	[204]
		R	GATCGGCAAAGGTTAGGTCA			
	qnrB	F	GATCGTGAAAGCCAGAAAGG	469	53	[204]
		R	ACGATGCCTGGTAGTTGTCC			
	qnrC	F	GGGTTGTACATTTATTGAATC	447	50	[204]
		R	TCCACTTTACGAGGTTCT			
	qnrS1	F	ACGACATTCGTCAACTGCAA	417	53	[204]
		R	TAAATTGGCACCCTGTAGGC			
	qnrS2	F	TGGAAACCTACCGTCACACA	600	60	[243]
		R	CTGGCAATTTTGATACCTGA			
	aac(6)-Ib	F	TTGCGATGCTCTATGAGTGGCTA	482	50	[204]
		R	CTCGAATGCCTGGCGTGTTT			
	aac(3)-I	F	ACCTACTCCCAACATCAGCC	169	60	[203]
		R	ATATAGATCTCACTACGCGC			
	aac(3)-II	F	ACTGTGATGGGATACGCGTC	237	60	[203]
		R	CTCCGTCAGCGTTTCAGCTA			
	aac(3)-IV	F	CTTCAGGATGGCAAGTTGGT	286	60	[203]
		R	TCATCTCGTTCTCCGCTCAT			
	sul1	F	TGGTGACGGTGTTCGGCATTC	789	60	[203]
		R	GCGAGGGTTTCCGAGAAGGTG			
	sul2	F	CGGCATCGTCAACATAACC	722	55	[203]
		R	GTGTGCGGATGAAGTCAG			
	dfrIa	F	GTGAAACTATCACTAATGG	474	55	[203]
		R	TTAACCCTTTTGCCAGATTT			
	dfrIb	F	GAGCAGCTICTITTIAAAGC	393	60	[203]
		R	TTAGCCCTTTIICCAATTTT			
	dfrII	F	GATCACGTGCGCAAGAAATC	141	50	[203]
		R	AAGCGCAGCCACAGGATAAAT			
	dfrVII	F	TTGAAAATTTCATTGATT	474	55	[203]
		R	TTAGCCTTTTTTCCAAATCT			
	dfrXII	F	GGTGSGCAGAAGATTTTTCGC	319	60	[203]
		R	TGGGAAGAAGGCGTCACCCTC			
Replicon types	IncHI1	F	GGAGCGATGGATTACTTCAGTAC	471	60	[151]
		R	TGCCGTTTCACCTCGTGAGTA			
	IncHI2	F	TTTCTCCTGAGTCACCTGTTAACAC	644	60	[151]
		R	GGCTCACTACCGTTGTCATCCT			
	IncI1-Iγ	F	CGAAAGCCGGACGGCAGAA	139	60	[151]
		R	TCGTCGTTCCGCCAAGTTCGT			
	IncI2	F	CTGTCGGCATGTCTGTCTC	553	55	[149]
		R	CTGGCTACCAGTTGCTCTAA			
	IncX1	F	GCTTAGACTTTGTTTTATCGTT	461	62	[150]
		R	TAATGATCCTCAGCATGTGAT			
	IncX2	F	GCGAAGAAATCAAAGAAGCTA	678	63	[150]
		R	TGTTGAATGCCGTTCTTGTCCAG			
	IncX3	F	GTTTTCTCCACGCCCTTGTTCA	351	63	[150]
		R	CTTTGTGCTTGGCTATCATAA			
	IncX4	F	AGCAAACAGGGAAAGGAGAAGACT	569	62	[150]
		R	TACCCCAAATCGTAACCTG			
	IncL/M	F	GGATGAAAACTATCAGCATCTGAAG	785	60	[151]
		R	CTGCAGGGGCGATTCTTTAGG			
	IncFIA	F	CCATGCTGGTTCTAGAGAAGGTG	462	60	[151]
		R	GTATATCCTTACTGGCTTCCGCAG			
	IncFIB	F	GGAGTTCTGACACACGATTTTCTG	702	63	[151]
		R	CTCCCGTCGCTTCAGGGCATT			
	IncFIC	F	GTGAACTGGCAGATGAGGAAGG	262	60	[151]
		R	TTCTCCTCGTCGCCAAACTAGAT			
	IncFIIs	F	CTGTCGTAAGCTGATGGC	270	60	[151]
		R	CTCTGCCACAAACTTCAGC			

IncA/C	F	GAGAACCAAAGACAAAGACCTGGA	465	60	[151]
	R	ACGACAAACCTGAATTGCCTCCTT			
IncP	F	CTATGGCCCTGCAAACGCGCCAGAAA	534	60	[151]
	R	TCACGCGCCAGGGCGCAGCC			
IncK	F	GCGGTCCGGAAAGCCAGAAAAC	160	60	[151]
	R	TCTTTCACGAGCCCGCCAAA			
IncB/O	F	GCGGTCCGGAAAGCCAGAAAAC	159	60	[151]
	R	TCTGCGTTCCGCCAAGTTCGA			
IncR	F	TCGCTTCATTCCTGCTTCAGC	251	60	[244]
	R	GTGTGCTGTGGTTATGCCTCA			
IncFII	F	CACACCATCCTGCACTTA	260	60	[151]
	R	CTGATCGTTTAAGGAATTTT			
IncN	F	GTCTAACGAGCTTACCGAAG	559	55	[151]
	R	GTTTCAACTCTGCCAAGTTC			

Supplementary Table 8. Oligonucleotide sequences and annealing temperature of primers used for virulence factor typing

Function	Genes		Nucleotide sequence	Size (bp)	Temp. (°C)	Reference
Adhesion	*fimH*	F	CTGGTCATTCGCCTGTAAAACCGCCA	846	63	[198]
		R	GTCACGCCAATAATCGATTGCACATTCC			
	iha	F	CTGGCGGAGGCTCTGAGATCA	827	55	[199]
		R	TCCTTAAGCTCCCGCGGCTGA			
	papC	F	GTGGCAGTATGAGTAATGACCGTTA	200	63	[200]
		R	ATATCCTTTCTGCAGGGATGCAATA			
	csgA	F	ACTCTGACTTGACTATTACC	200	55	[198]
		R	AGATGCAGTCTGGTCAAC			
	sfa/focDE	F	CTCCGGAGAACTGGGTGCATCTTAC	410	60	[245]
		R	CGGAGGAGTAATTACAAACCTGGCA			
	afa/draBC	F	GCTGGGCAGCAAACTGATAACTCTC	794	60	[245]
		R	CATCAAGCTGTTTGTTCGTCCGCCG			
	papAH	F	ATGGCAGTGGTGTCTTTTGGTG	717	60	[246]
		R	CGTCCCACCATACGTGCTCTTC			
Toxin	*yfcV*	F	ACATGGAGACCACGTTCACC	292	60	[147]
		R	GTAATCTGGAATGTGGTCAGG			
	astA	F	TGCCATCAACACAGTATATCCG	102	65	[201]
		R	ACGGCTTTGTAGTCCTTCCAT			
	hlyF	F	GGCCACAGTCGTTTAGGGTGCTTACC	450	60	[199]
		R	GGCGGTTTAGGCATTCCGATACTCAG			
	aat	F	TCGGCTTATGAAGCAAAAATG	828	53	[202]
		R	GATAACGTCGTCTTGTCCATTC			
	pic	F	AGCCGTTTCCGCAGAAGCC	1111	63	[201]
		R	AAATGTCAGTGAACCGACGATTGG			
	vat	F	TCAGGACACGTTCAGGCATTCAGT	1100	60	[147]
		R	GGCCAGAACATTTGCTCCCTTGTT			
	stx1	F	CGATGTTACGGTTTGTTACTGTGACAGC	244	63	[201]
		R	AATGCCACGCTTCCCAGAATTG			
	stx2	F	GTTTTGACCATCTTCGTCTGATTATTGAG	324	63	[201]
		R	AGCGTAAGGCTTCTGCTGTGAC			
	aggR	F	ACGCAGAGTTGCCTGATAAAG	400	63	[201]
		R	AATACAGAATCGTCAGCATCAGC			
	elt	F	GAACAGGAGGTTTCTGCGTTAGGTG	655	63	[201]
		R	CTTTCAATGGCTTTTTTTTGGGAGTC			
	est1b	F	TGTCTTTTTCACCTTTCGCTC	171	63	[201]
		R	CGGTACAAGCAGGATTACAACAC			
	est1a	F	CCTCTTTTAGYCAGACARCTGAATCAST	157	63	[201]
		R	CAGGCAGGATTACAACAAAGTTCACAG			
	bfpB	F	GACACCTCATTGCTGAAGTCG	910	63	[201]
		R	CCAGAACACCTCCGTTATGC			
	eaeA	F	TCAATGCAGTTCCGTTATCAGTT	482	63	[201]
		R	GTAAAGTCCGTTACCCCAACCTG			
	invE	F	CGATAGATGGCGAGAAATTATATCCCG	766	63	[201]
		R	CGATCAAGAATCCCTAACAGAAGAATCA			

Protectin	*traT*	F	GGTGTGGTGCGATGAGCACAG	290	60	[198]
		R	CACGGTTCAGCCATCCCTGAG			
	ompT	F	TCATCCCGGAAGCCTCCCTCACTACTAT	496	64	[199]
		R	TAGCGTTTGCTGCACTGGCTTCTGATAC			
	kpsMTII	F	GCGCATTTGCTGATACTGTTG	272	60	[247]
		R	CATCCAGACGATAAGCATGAGC			
	iss	F	CAGCAACCCGAACCACTTGATG	323	60	[199]
		R	AGCATTGCCAGAGCGGCAGAA			
Siderophores	*fyuA*	F	TGATTAACCCCGCGACGGGAA	880	63	[198]
		R	CGCAGTAGGCACGATGTTGTA			
	iroNe.coli	F	AAGTCAAAGCAGGGGTTGCCCG	665	63	[199]
		R	GACGCCGACATTAAGACGCAG			
	iutA	F	GGCTGGACATCATGGGAACTGG	302	60	[199]
		R	CGTCGGGAACGGGTAGAATCG			
	chuA	F	CTGAAACCATGACCGTTACG	652	55	[153]
		R	TTGTAGTAACGCACTAAACC			
Phylogenetic group	*yjaA*	F	CAAACGTGAAGTGTCAGGAG	288	55	[153]
		R	AATGCGTTCCTCAACCTGTG			
	chuA	F	ATGGTACCGGACGAACCAAC	211	55	[153]
		R	TGCCGCCAGTACCAAAGACA			
	tspE4.C2	F	CACTATTCGTAAGGTCATCC	152	55	[153]
		R	AGTTTATCGCTGCGGGTCGC			
	AceK.f	F	AACGCTATTCGCCAGCTTGC	400	55	[153]
	ArpA1.r	R	TCTCCCCATACCGTACGCTA			
	ArpAgpE.f	F	GATTCCATCTTGTCAAAATATGCC	301	55	[153]
	ArpAgpE.r	R	GAAAAGAAAAAGAATTCCCAAGAG			
	trpAgpC.1	F	AGTTTTATGCCCAGTGCGAG	219	55	[153]
	trpAgpC.2	R	TCTGCGCCGGTCACGCCC			
	trpBA.f	F	CGGCGATAAAGACATCTTCAC	489	55	[153]
	trpBA.r	R	GCAACGCGGCCTGGCGGAAG			

Supplementary Table 9. Antimicrobial susceptibility of MCR1-EC isolates according to pathogenic *E. coli* types

Antimicrobial Classes	Antimicrobial Agents	Total (n=53)	Commensal-EC (n=37)	InPEC (n=12)	ExPEC (n=4)
Broad-spectrum penicillin	ampicillin	84.9%	83.8%	83.3%	100.0%
3rd-generation Cephalosporin	cefotaxime	17.0%	16.2%	0.0%	75.0%
	ceftazidime	3.8%	5.4%	0.0%	0.0%
	ceftriaxone	17.0%	16.2%	0.0%	75.0%
β-lactamase inhibitor	amoxicillin/clavulanate	13.2%	13.5%	8.3%	25.0%
Monobactam	aztreonam	11.3%	10.8%	0.0%	50.0%
Carbapenem	imipenem	0.0%	0.0%	0.0%	0.0%
Phenicol	chloramphenicol	67.9%	78.4%	25.0%	100.0%
Aminoglycoside	amikacin	0.0%	0.0%	0.0%	0.0%
	gentamycin	15.1%	13.5%	16.7%	25.0%
Tetracycline	tetracycline	88.7%	83.8%	100.0%	100.0%
Quinolone	nalidixic acid	39.6%	45.9%	0.0%	100.0%
	ciprofloxacin	32.1%	35.1%	0.0%	100.0%
Sulfonamide/ Trimethoprim	sulfamethoxazole/ trimethoprim	37.7%	29.7%	66.7%	25.0%
Average number of classes to which strains are resistant (max=11)	-	4.8	4.8	4.0	7.0

*Abbreviation. Commensal-EC, Commensal *E. coli*; InPEC, Intestinal pathogenic *E. coli*; ExPEC, Extra-intestinal pathogenic *E. coli*

Supplementary table 10. Prevalence of antimicrobial resistance genes and their correlation coefficients with expected phenotypic resistance of MCR1-EC isolates

Antimicrobial Classes	Antimicrobial Resistance Gene	Prevalence (%)	No. of positive MCR1-EC /No. of total MCR1-EC	[a]Correlation Coefficient	*P* - value
Narrow-spectrum β-lactams	*bla*$_{TEM-1}$	47.2	25/53	0.398	<0.01*
	bla$_{TEM-215}$	5.7	3/53	0.103	0.46
	bla$_{TEM-237}$	3.8	2/53	0.083	0.55
	bla$_{TEM-20}$	1.9	1/53	0.058	0.68
	bla$_{TEM-1,215,237, or 20}$	58.8	31/53	0.377	<0.01*
Extra-spectrum β-lactams	*bla*$_{CTX-M-55}$	17.1	9/53	0.932	<0.01*
Phenicol	*catA*	3.8	2/53	0.142	0.31
	cmlA	3.8	2/53	0.142	0.31
	floR	69.8	37/53	0.917	<0.01*
	catA, cmlA, or floR	69.8	37/53	0.917	<0.01*
Aminoglycoside	*aac(3)-I*	0.0	0/53	-	-
	aac(3)-II	7.5	4/53	0.478	<0.01*
	aac(3)-IV	1.9	1/53	0.329	0.02*
	aac-(3)-I, II, or IV	9.4	5/53	0.585	<0.01*
Tetracycline	*tetA*	79.2	42/53	0.350	0.01*
	tetB	11.3	6/53	0.139	0.32
	tetD	0.0	0/53	-	-
	tetA, B, or D	90.6	48/53	0.637	<0.01*
Quinolone	*qnrA*	0.0	0/53	-	-
	qnrB	0.0	0/53	-	-
	qnrC	0.0	0/53	-	-
	qnrS1	41.5	22/53	-0.213	0.13
	qnrS2	37.7	20/53	-0.074	0.60
	aac(6)-cr-Ib	0.0	0/53	-	-
	qnrA, B, C, S1, S2 or aac(6)-cr-Ib	45.3	24/53	-0.117	0.40
Sulfonamide/ Trimethoprim	*sul1*	11.3	6/53	0.336	0.01*
	sul2	50.9	27/53	0.608	<0.01*
	dfrIa	26.4	14/53	0.593	<0.01*
	dfrIb	0.0	0/53	-	-
	dfrII	0.0	0/53	-	-
	dfrVII	0.0	0/53	-	-
	dfrXII	9.4	5/53	0.415	<0.01*
	sul1, sul2, dfrIa, Ib, II, VII, or XII	56.6	30/53	0.603	<0.01*

[a] Correlation coefficient of the carriage of antimicrobial resistance genes with expected phenotypic resistance was calculated via Spearman's correlation test

*$P < 0.05$, statistically significant

Supplementary Table 11. Prevalence of antimicrobial resistance genes and their correlation coefficients with expected phenotypic resistance of MCR1-EC isolates

Replicon types	Prevalence (%)	No. of positive MCR1-EC /No. of total MCR1-EC
IncI2	94.3	50/53
IncFIB	84.9	45/53
IncFII	67.9	36/53
IncFIC	43.4	23/53
IncR	28.3	15/53
IncI1-Iγ	28.3	15/53
IncX1	17.0	9/53
IncX4	7.5	4/53
IncFIA	7.5	4/53
IncN	1.9	1/53
IncB/O	1.9	1/53
IncHI1	0	0/53
IncHI2	0	0/53
IncA/C	0	0/53

Supplementary Table 12. Prevalence of virulence factor classes according to the four swine production stages

Virulence Factor classes	Weaning pigs (Reference)			Growing pigs			Finishing pigs			Pregnant sows		
	Prevalence (%)	OR (95% CI)	*P* - value	Prevalence (%)	OR (95% CI)	*P* - value	Prevalence (%)	OR (95% CI)	*P* - value	Prevalence (%)	OR (95% CI)	*P* - value
Adhesion	100.0	-	-	100.0	1.3 [a] (003-71.86)	0.88	100.0	0.9 [a] (0.02-46.71)	0.94	100.0	0.6 [a] (0.01-32.34)	0.79
Toxin	35.7	-	-	31.6	1.2 (0.11-13.77)	0.88	33.3	1.1 (0.18-6.79)	0.91	25.0	1.7 (0.50-5.54)	0.41
Protectin	85.7	-	-	100.0	7.8 [a] (0.35-176.35)	0.20	91.7	1.8 [a] (0.15-23.16)	0.64	75.0	0.5 [a] (0.06-4.47)	0.54
Sidero-phore	42.9	-	-	31.6	0.62 (0.04-8.75)	0.72	16.7	0.27 (0.04-2.02)	0.20	62.5	2.2 (0.57-8.67)	0.25

[a] Where zeros cause problems in calculating OR or 95% CI, Fisher's exact test was used in the calculations instead of GEE.

[*] $P < 0.05$, statistically significant based on GEE

[†] $P < 0.05$, statistically significant based on Fisher's exact test

Chapter 3.

Different threats posed by two major mobilized colistin resistance genes –*mcr-1.1* and *mcr-3.1*– revealed through comparative genomic analysis

Abstract

Global spread of mobilized colistin resistance gene (*mcr*)-carrying *Escherichia coli* (MCR-EC) poses serious threats to public health. This study aimed to determine the different threats posed by two major mobilized colistin resistance genes –*mcr-1.1* and *mcr-3.1*– based on the comparative genomic analysis. Genetic backgrounds and characteristics of mobile genetic elements carrying *mcr-1.1* or *mcr-3.1* in 74 MCR-EC isolated from swine farms were analyzed, and comparative genomic analysis was performed with the public sequence database. The *mcr-1.1* showed high horizontal transferability (6.30 logCFU/ml), with simple gene cassette without insertion sequences (ISs), "*mcr-1.1-pap2*". The *mcr-1.1* cassettes were highly shared across strains and transferred without MDR. Whereas, *mcr-3.1* exhibited relatively lower conjugation frequency (0.97 logCFU/ml). The *mcr-3.1*-cassette was flanked by IS26 and was highly variable across strains due to insertion, deletion, or truncation of IS6100, IS4321 or IS5075. Nearby *mcr-3.1*-cassette, MDR regions consisting of antimicrobial/heavy metal resistance genes were identified and co-transferred with *mcr-3.1*. From this study, it was first reported that *mcr-3.1*-carrying IncHI2-fragment may be co-integrated into the bacterial chromosome via IS26, implying it's possibility of dual lifestyle including horizontal- and vertical transmission. Our study showed the different threat by *mcr-1.1* and *mcr-3.1* through culture-based and comparative genomic analysis, highlighting the need for suitable strategies based on their differences to control colistin resistance.

Keywords: *mcr-1.1*, *mcr-3.1*, MGE, MDR, comparative genomic analysis

3.1. Introduction

Colistin has been prescribed as the last treatment option for multi-drug resistant bacterial infections such as extended-spectrum β-lactams or carbapenem-resistant *Enterobacteriaceae,* and was classified as a critically important antimicrobial agent by the World Health Organization [17]. Since mobilized colistin resistance gene-1, *mcr-1*, was first described in the *Escherichia coli* strain isolated from pig farms in China in 2016, *mcr* variant-mediated colistin resistance has been reported worldwide in a variety of hosts, including humans, livestock, and companion animals, posing a serious threat to public health [18, 248]. Colistin was generally used to treat swine diseases, making swine farms an important reservoir of *E. coli* strains carrying the *mcr* gene (MCR-EC) [206-209]. To date, 10 *mcr* variants, *mcr-1* to *mcr-10*, have been reported, with *mcr-1* and *mcr-3* being the most prevalent variants [19]. *Mcr-3*, which exhibits 45.0% nucleotide sequence identity with *mcr-1,* was first described in a plasmid carried by an *E. coli* strain isolated from a swine farm in 2017 [95].

Mcr variants are mobilized in the form of gene-cassettes containing insertion sequences (IS) or transposons (Tn), and are transferred horizontally using mobile genetic element (MGE) vectors, mainly plasmids [19, 24, 95, 239-241]. Recent studies have shown that the major types of MGE vectors encoding *mcr*-carrying gene cassettes are dependent on the *mcr* types. *Mcr-1* is mobilized by the ISApl1-mediated composite transposon Tn6330 (ISApl1-*mcr-1-pap2-ISApl1*) [19, 239]. In contrast, IS26 is responsible for the mobilization of *mcr-3* [24, 95]. In general, the characteristics of MGE vectors used by mobile resistance genes may affect the gene expression or

transferability of mobile resistance genes [19, 24, 95, 239-241]. Understanding the differences in genetic background and transfer characteristics between *mcr* variants could be an invaluable cornerstone in controlling the spread of *mcr*-mediated colistin resistance.

This study aimed to provide insights into the different threats posed by colistin resistance mediated by two major *mcr* variants, *mcr-1.1* and *mcr-3.1*, based on the comparative genomic analysis. To this end, first, the horizontal transfer characteristics of *mcr-1.1* and *mcr-3.1* were analyzed in 74 MCR-EC strains isolated from swine farms. Second, the composition and diversity of the *mcr-1.1*- and *mcr-3.1*-carrying gene cassettes were analyzed in MCR-EC strains isolated in the present study and compared with the public whole genome database. Finally, whole genome sequences (WGS) of *E. coli* strains carrying *mcr-1.1* (MCR1-EC) and *E. coli* strains carrying *mcr-3.1* (MCR3-EC) were aligned and analyzed to understand the genetic environments of MGE vectors encoding *mcr*-carrying gene-cassettes.

3.2. Materials and Methods

Bacterial strains

A total of 53 MCR1-EC and 21 MCR3-EC strains isolated from five swine farms (Farm A to E) in South Korea between May-2017 and July-2019 were included in this study (Supplementary Table 13).

Conjugation assay

To evaluate the conjugation frequencies of *mcr-1.1* and *mcr-3.1* as

well as their co-transferability with antimicrobial resistance genes, conjugation experiments were performed using 74 MCR-EC isolates as doner cells and *E. coli* J53-Azi^{R} strain as recipient cell. Conjugation assays were performed as previously described [249], using Luria-Bertani agar plates containing colistin (2 mg/L) and sodium azide (100 mg/L). Conjugation assay was performed in triplicate and the mean value of conjugation frequency was displayed with 95% confidence interval. The presence of *mcr*-cassettes, plasmids, and antimicrobial resistance genes in the *mcr*-transconjugant strains was confirmed using polymerase chain reaction (PCR). Primer sequences and reaction conditions are summarized in Supplementary Table 14.

Comparative genomic analysis of *mcr-1.1*- and *mcr-3.1*-carrying gene-cassettes

Mcr-1.1- and *mcr-3.1*-carrying gene-cassettes of 74 MCR-EC isolates were analyzed using PCR mapping and sanger sequencing as previously described [212], with slight modifications. Nucleotide sequences of *mcr-1.1*-cassettes of pHNSHP45 (KP347127.1) and pHNSHP49 (MF774188.1), and *mcr-3.1*-cassettes of pWJ1 (KY924928.1) and pHN8 (MG780294.1) were used as references. Amplicon sequences were aligned and compared with sequences submitted to the National Center for Biotechnology Information (NCBI) GenBank database using BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Primer sequences and reaction conditions are summarized in Supplementary Table 15.

Mcr-1.1-carrying MGE vectors of 35 MCR1-EC isolates were identified using PCR mapping and sanger sequencing [212], same as the analysis of *mcr-1.1*-cassettes. To identify the *mcr-3.1*-carrying MGE vector,

long-read whole genome sequencing was conducted for nine representative strains with different multi-locus sequence types (MLST) among 21 MCR3-ECs: MCR3-A19, MCR3-E05, MCR3-E07, MCR3-E08, MCR3-E09, MCR3-E11, MCR3-E13, MCR3-E15, and MCR3-E17. Long-read sequencing was conducted via the MinION platform (Oxford Nanopore Technologies, UK), and raw long-reads were assembled using Unicycler (v0.5.0) [250].

For comparative genomic analysis of *mcr*-cassettes, we analyzed 100 of 500 *mcr-1.1*-carrying plasmids and 27 *mcr-3.1*-encoding plasmids whose complete WGS data were available in the NCBI database (accessed on 05-DEC-2021). To select 100 of 500 *mcr-1.1*-carrying plasmids, we used simple random sampling procedure in Social Sciences (SPSS) program, v27.0 (IBM SPSS Statistics for Windows, USA). Sequences were compared using easyfig (v2.2.3) [125]. Resistance genes and replicons were analyzed using ResFinder (v4.1) [251] and PlasmidFinder (v2.1) [252], respectively. The NCBI accession numbers of all strains analyzed in this study are summarized in Supplementary Table 16.

Comparative genomic analysis of MGE vectors encoding *mcr*-carrying gene-cassettes

To understand the genetic environment of *mcr*-carrying MGE vectors, we conducted whole genome sequencing and comparative genomic analysis of five MCR-ECs: MCR1-A03, MCR1-04, MCR1-B18, MCR3-A19 and MCR3-E13. Whole genome sequencing was performed using a combination of NextSeq® 500 technology (Illumina, Inc., USA) and MinION platforms (Oxford Nanopore Technologies). Raw short-read and long-read data were assembled using hybrid-assembly strategy in Unicycler (v0.5.0) [250]. Genome

annotations were generated using prokka (v1.14.5) [253]. Comparative genomic visualization was performed using easyfig (v2.2.3) [125] and pangenome analysis of GView Server [254]. The WGS data presented in this study are deposited in the NCBI sequence read archive repository (PRJNA757225 and PRJNA875028).

Finally, to investigate the genomic re-arrangement of *mcr-3.1*-carrying MGE vectors between MCR3-EC isolates from the same swine farm (farm E), comparative genomic analysis of MCR3-E13 chromosome and seven *mcr-3.1*-encoding plasmids (pMCR3-E05, pMCR3-E07, pMCR3-E08, pMCR3-E09, pMCR3-E11, pMCR3-E15, and pMCR3-E17) was performed. In addition, circular intermediates of antimicrobial resistance cassette (AR-cassette) and heavy metal resistance cassette (MR-cassette) were investigated using inverse PCR and sanger sequencing. Primer sequences and reaction conditions are summarized in Supplementary Table 17.

3.3. Results

Horizontal transfer characteristics of *mcr-1.1* and *mcr-3.1*

Conjugation frequency of *mcr-1.1* was 6.30 logCFU/ml, while that of *mcr-3.1* was 0.97 logCFU/ml (Supplementary Figure 4). Identical *mcr-1.1* and *mcr-3.1*-carrying cassettes of donors were identified in their conjugants. Plasmid-mediated antimicrobial resistance genes were rarely transferred in the *mcr-1.1* conjugation assay, with co-transfer rates of 4.2% (2/48) for $bla_{CTX\text{-}M\text{-}55}$, *floR,* and *tetA*. In contrast, various resistance genes were co-transferred with *mcr-3.1* in the conjugation assay, including *floR* (94.1%, 16/17), *sul2* (76.5%,

13/17), and *bla*$_{TEM-1}$ (35.3%, 6/17).

Mcr-1.1- and *mcr-3.1*-carrying gene-cassettes of 74 MCR-EC strains isolated from swine farms

Three types of *mcr-1.1*-cassettes were identified among the 53 MCR1-EC isolates from swine farms (Figure 24A). The most predominant type was "ISApl1-*mcr-1.1-pap2*" (26/53, 49.1%), encoded on IncI2. The second major type was "*mcr-1.1-pap2*" (25/53, 47.2%), encoded on either IncI2 (22/25, 86.4%) or IncX4 (3/25, 13.6%). The least common type was "ISApl1-IS1-*mcr-1.1-pap2"* (2/53, 3.8%), encoded on IncI2. Comparison of swine farms showed that the major *mcr-1.1*-carrying gene-cassette types for each farm were present, accounting for 60.0%–100% of total MCR1-EC strains in each farm.

Five *mcr-3.1*-cassette types were identified from the 21 MCR3-EC isolates from swine farms (Figure 24B). The two major types were "IS26-IS6100-IS4321-ΔTnAs2-Δ*nimC-mcr3.1-dgkA*-ISKpn40-Δ*nimC*-Δ*ble*-IS15DI" (7/21, 33.3%), encoded on IncHI2 (pMCR3-E05 and pMCR3-E17) or chromosome (MCR3-E13), and "IS26-IS6100-ΔTnAs2-Δ*nimC-mcr-3.1-dgkA*-ISKpn40-Δ*nimC*-Δ*ble*-IS15DI" (7/21, 33.3%), encoded on IncHI2 (pMCR3-E07, pMCR3-E09, and pMCR3-E15). The two minor types were "IS26-ΔTnAs2-Δ*nimC-mcr-3.1-dgkA*-ISKpn40-Δ*ble*-IS15DI" (3/21, 14.3%), encoded on IncHI2 (pMCR3-E11) and "IS26-IS6100-ΔTnAs2-Δ*nimC-mcr-3.1-dgkA*-ISKpn40" (2/21, 9.5%), encoded on IncHI2 (pMCR3-E08). The least common type was "ΔIS26-ΔTnAs2-*mcr-3.1-dgkA*-ISKpn40-Δ*nimC*-IS15DI" (2/21, 9.5%), encoded on IncX1 (pMCR3-A19). Comparison of swine farms showed that 19 of 21 MCR3-EC strains were isolated from Farm E, while the remaining two strains were isolated from Farm A. Two MCR3-EC isolates from

Farm A shared an identical *mcr-3*-carrying gene cassette. In contrast, four types of *mcr-3*-carrying gene cassettes were identified in Farm E-derived MCR3-EC strains, accounting for 10.5–36.8% of the total MCR3-EC strains from Farm E for each gene cassette type.

Comparative genomic analysis of *mcr-1.1*-cassettes in MCR1-EC isolates from swine farms and those published in NCBI database

Whole genome sequencing of MCR1-A03, MCR1-A04, and MCR1-A10 identified three *mcr-1.1*-carrying IncI2 plasmids: pMCR1-A03, pMCR1-A04, and pMCR1-B18 with 152 736 bp, 65 724 bp, and 64 207 bp lengths, respectively (Figure 25). These three *mcr-1.1*-carrying plasmids carried no other resistance genes. The 13 137-bp genetic background of the *mcr-1.1*-cassette in MCR1-B18, ranging from *traL* to *topB*, shared 99.9% nucleotide sequence identity with the corresponding region in pNHSHP45 (Figure 26A). The 12 067-bp genetic background of the *mcr-1.1*-cassette in MCR1-A03 and MCR1-A04, ranging from *traL* to *topB*, also shared high sequence similarity (>99.9%) with pNHSHP45, apart from the loss of ISApl1.

Comparative genomic analysis of 100 *mcr-1.1*-cassettes, whose WGS data were uploaded on the NCBI database, showed that *mcr-1.1* was encoded on six replicon types (Figure 26B). The three predominant replicon types were IncI2 (40/100, 40.0%), IncX4 (27/100, 27.0%), and IncHI2 (25/100, 25.0%), accounting for 92.0% (92/100) of all strains. Analysis of *mcr-1.1*-cassette identified 10 cassette types. The two dominant types were "*mcr-1.1-pap2*" (54/100, 54.0%) and "ISApl1-*mcr-1.1-pap2*" (29/100, 29.0%), which accounted for 83.0% (83/100) of all strains. The other eight cassette types accounted for only 1.0–8.0% of all strains and showed insertions or deletions

of truncated ISApl1, other IS types, or *pap2* in the *mcr-1.1*-cassette. The most major cassette type 01 "*mcr-1.1-pap2*" was mainly encoded on IncX4 (27/54, 50.0%) or IncI2 (24/54, 44.4%), and rarely encoded on IncHI2 (2/54, 5.7%).

Next, we analyzed antimicrobial resistance genes of 100 *mcr-1.1*-carrying plasmids uploaded to the NCBI database (Supplementary Figure 5A). Typing of resistance genes based on the replicon types showed that 96.3% (26/27) of *mcr-1.1*-carrying IncX4 and 82.5% (33/40) of *mcr-1.1*-carrying IncI2 harbored only *mcr-1.1* without any other resistance genes. In contrast, 92.0% (23/25) of *mcr-1.1*-carrying IncHI2 carried three or more resistance genes.

Comparative genomic analysis of *mcr-3.1*-cassettes in MCR3-EC isolates from swine farms and those published in the NCBI database

The *mcr-3.1*-cassette in MCR3-A19 was "ΔIS26-ΔTnAs2-*mcr3.1-dgkA*-ΔISKpn40-Δ*ble*-IS15DI", which had a "Δ*ble*" deletion between "Δ*nimC*" and "IS15DI" compared with the corresponding region in the reference plasmid pHN8 (Figure 27A). The *mcr-3.1*-cassette in MCR3-E13 was "IS26-IS6100-IS4321-ΔTnAs2-Δ*nimC-mcr3.1-dgkA*-ΔISKpn40-Δ*nimC*-Δ*ble*-IS15DI", which had two insertion genes: "IS4321" inserted between "IS26" and "IS6100" and "Δ*nimC*" inserted between "ISKpn40" and "Δ*ble*" compared with the corresponding regions in the reference plasmid pWJ1.

Comparative genomic analysis of 27 *mcr-3.1*-cassettes, whose WGS data were uploaded to the NCBI database, showed that *mcr-3.1* was encoded on nine replicon types (Figure 27B). Three replicon types, IncA/C2 (9/27, 33.3%), IncF (5/27, 18.5%), and IncHI2 (4/27, 14.8%), accounted for 66.7% (18/27) of all strains. A total of 17 *mcr-3.1*-cassette types were identified. Type

17, "ΔIS26-ΔTnAs2-*mcr3.1-dgkA*-ΔISKpn40-Δ*nimC*-Δ*ble*-IS15DI", accounted for 25.9% (7/27) of all strains, while the other types accounted for only 3.7–7.4% of total strains. The *mcr-3.1*-cassettes were multifarious between strains due to insertion, deletion, or truncation of genes encoding mobile elements (IS26, IS6100, TnAs2, IS15DI, IS4321, or IS5075).

Typing of resistance genes in the 27 *mcr-3.1*-carrying plasmids showed that all replicon types, including IncA/C2, IncF, and IncHI2, harbored three or more resistance genes, apart from IncP1, which carried only *mcr-3.1* (Supplementary Figure 5B).

Comparative genomic analysis of MGE vectors encoding *mcr-3.1*-cassette

The WGS analysis of MCR3-A19 and MCR3-E13 showed that *mcr-3.1* was encoded on the IncX1-type plasmid, pMCR3-A19, and on the MCR3-E13 chromosome, respectively. Plasmid pMCR3-A19, which is 45 354 bp long, carried four antimicrobial resistance genes: *aac(3")-IId*, *qnrS2*, bla_{TEM-1}, and *floR* (Figure 28). The resistance genes were clustered in a MDR region near ΔIS26, located upstream of the *mcr-3* cassette: "DUF3363-*floR*-IS26-*qnrS2*-IS26-*hp*-Tn3-bla_{TEM-1}-TnAs1-IS26-*aac(3")-IId-tmrB*-ΔIS26-ΔTnAs2-*mcr-3.1*".

The *mcr-3.1*-carrying gene cassette in MCR3-E13 was integrated in the chromosomal phosphonate ABC transporter gene, *phnE*, resulting in the cleavage of *phnE* (Figure 29A). The integration was a co-integrated form of a Mu-like prophage (35 896 bp) and *mcr-3.1*-carrying IncHI2-type plasmid fragment (203 107 bp). The co-integration site of the two elements was the DNA invertase encoding gene, *gin*, of Mu-like prophage and IS26 of IncHI2-type plasmid fragment, respectively (Figure 29B). At this site, 14-bp terminal

inverted repeats (IRL: inverted repeat left; IRR: inverted repeat right) of IS26 and 8-bp target site duplication (TSD) of *gin* of Mu-like prophage were identified. In the comparative genetic analysis with pWJ1, the *mcr-3.1*-carrying IncHI2-type plasmid fragment shared high sequence homology with the region of replication and stability (*repA*, *parA*, *topB,* and *dnaB*) and conjugation (*traD*, *traG*, resolvase gene, and *xerD*). In contrast, the structure of the MDR region was multifarious between two strains.

Next, we conducted comparative genomic analysis of the eight IncHI2-type plasmid fragments from pig farm E (Figure 30). Among seven IncHI2 plasmids, the WGS of pMCR3-E17 showed the highest similarity with that of IncHI2-type plasmids fragment of MCR3-E13. The only difference between two strains was a 26 963-bp region in pMCR3-E05, which consisted of DNA replication terminus site-binding protein, tyrosine-type recombinase. Hemolysin expression-modulating protein, Hha, etc., was deleted from the IncHI2-type plasmid fragment of MCR3-E13 compared with pMCR3-E17.

The WGS of IncHI2-type plasmid fragment of MCR3-E13 and seven IncHI2 plasmids carrying *mcr-3.1* shared high sequence homology in the regions of replication, stability, and conjugation. In contrast, the MDR region was multifarious between strains due to insertion and/or deletion of gene cassettes containing antimicrobial/heavy metal resistance genes. Four AR-cassettes and two MR-cassettes were identified in eight strains. To investigate the possibility of genetic re-arrangement based on the intermediate circular form, we conducted inverse PCR and amplicon sequencing of the six gene cassettes. Of the six cassettes, the IS26-bracketed intermediate circular form was identified in AR1, but not in five cassettes (Supplementary Figure 6).

3.4. Discussion and Conclusion

This study aimed to provide insights into the different threats associated with colistin resistance mediated by *mcr-1.1* and *mcr-3.1*, based on comparative genomic analysis. The most dominant *mcr-1.1*-cassette type was "*mcr-1.1-pap2*" in this study. *Mcr-1.1* has been reported to be mainly mediated by plasmids and the reported major replicon types encoding *mcr-1.1* are IncI2, IncX4, and IncHI2 [19, 209, 239]. Interestingly our results showed that "*mcr-1.1-pap2*", which lost all ISs, was mainly encoded on IncI2 or IncX4-type plasmids, but not IncHI2. In contrast, *mcr-3.1* was encoded on the variable replicon types, including IncHI2, IncA/C2, and IncX1, without a dominant type. *Mcr-3.1*-cassette was bracketed by IS26 and IS15DI and flanked by multiple ISs elements (IS26, IS6100, TnAs2, or ISKpn40). IS15DI belongs to the same IS6 family as IS26 and shares 99.6% nucleotide sequence similarity with IS26 [24]. The *mcr-3.1*-cassettes were highly multifarious between strains due to insertion, deletion or truncation of MGEs (IS26, IS6100, TnAs2, IS15DI, IS4321, or IS5075), without a dominant cassette-type. Several *mcr-3.1*-cassette types had lost or truncated IS15DI, ΔTnAs2, and IS26. Loss of the MGE in AR-cassettes may lead to the stabilization of their genomic background without genetic rearrangements such as intra- or intermolecular transposition [19]. Epidemiological studies have proposed that *mcr-1* was initially captured and mobilized by the composite transposon Tn6330 (ISApl1-*mcr-1.1-pap2*-ISApl1), followed by the loss of ISApl1 over time, leading to the formation of stable genetic background of *mcr-1.1*, "*mcr-1.1-pap2*" [19, 239]. Considering that the loss of ISApl1 over time was proposed as stabilization step in the formation of the *mcr-1.1*-cassette [19, 239], the loss of several mobile elements

in *mcr-3.1*-carrying gene cassette may be proposed as evidence that *mcr-3.1*-carrying gene cassettes are progressing through the stabilization process.

The *mcr* variants could be transferred from commensal *E. coli* to various *Enterobacteriaceae*, including pathogenic bacteria [219]. Noteworthy, the reported conjugation rate was different depending on the *mcr* variant types. Compared to other variant types, the conjugation rates of reported two major *mcr* variants, *mcr-1* and *mcr-3*, were confirmed to be 100.0% [255-257]. Whereas, the *mcr-4* and *mcr-5* were reported to be encoded on the non-conjugative plasmid types, such as plasmid ColE, and not horizontally transferred [96, 258-262]. For *mcr-7*, *8*, *9*, and *10*, the conjugation may or may not occur depending on the studies, implying a low conjugation rate [231, 249, 261, 263-265]. For *mcr-2* and *mcr-6*, isolation was rarely reported worldwide, and information on conjugation rates was lacking [108]. One hypothesis for the cause of the different conjugation rates could be the different fitness costs driven by expression for each *mcr* gene. According to previously reported studies on fitness cost of mcr, *mcr-1* and *mcr-3* exhibited a lower fitness cost compared to expression of other *mcr* variants [266]. In particular, it has been reported that *mcr-1* has the lowest fitness cost even compared to *mcr-3* [266, 267].

Another hypothesis for different conjugation rates could be the different plasmid types favored by *mcr* variants. The horizontal transferability of plasmid-mediated resistance genes may differ based on replicon type [240, 241, 268]. Noteworthy, conjugation frequencies of *mcr-1.1* and *mcr-3.1* differed in this study: *mcr-1.1* showed a significantly higher conjugation rate compared to *mcr-3.1*. And the preferred plasmid types were different for *mcr-*

1.1 and *mcr-3.1*. The favored replicon types in MCR-EC differed between *mcr-1.1* and *mcr-3.1*: *mcr-1.1* was encoded by IncI2 or IncX4, whereas *mcr-3.1* was mainly encoded by IncHI2 in this study. The ProQ/FinO protein, a transcriptional regulator encoded by IncI2, may favor the fitness of bacteria harboring *mcr*-bearing IncI2, making IncI2 a successful vector for *mcr-1.1* compared with other replicon types [240]. Lu et al (2019) reported that IncI2 and IncX4 showed significantly higher *mcr-1.1* transferability than IncHI2 [241]. Considering that the replicon types may play an important role in the horizontal transferability of plasmid-mediated resistance genes [240, 241, 268], our results imply that differences in their vector types could be one of possible reasons which caused the observed differences in conjugation rates between *mcr-1.1* and *mcr-3.1*.

Carrying multiple plasmid-mediated resistance genes has been reported to be a significant risk factor affecting fitness cost in bacterial hosts [269-271]. The number of antimicrobial resistance genes carried differed depending on the replicon type of the *mcr*-carrying plasmid. Analysis of antimicrobial resistance genes in WGS data of *mcr*-carrying plasmids from the NCBI database showed that majority of *mcr-1.1*-carrying IncX4 and IncI2 harbored only *mcr-1.1*, with no other resistance gene, while *mcr-1.1*-carrying IncHI2 carried three or more resistance genes. All *mcr-3.1*-carrying plasmids, including IncHI2, IncA/C2, and IncF-type replicons, apart from IncP1, carried three or more resistance genes. Although additional research on the fitness cost and conjugation of *mcr* variants are needed, our results suggest that the higher proportion of antimicrobial resistance genes identified in *mcr-3.1*-carrying IncHI2-type plasmids could be one of the possible reasons for the lower conjugation rate of *mcr-3.1.* However, possessing various antibiotic resistance

genes together also acts as a selection pressure in an environment where it can be exposed to various antibiotics such as pig farms, which could increase the possibility to survival of MCR3-EC through natural selection. And it may have helped *mcr-3*.1 become a global epidemic.

Comparative genomic analysis of *mcr-1.1*-cassette and its genetic environment showed high sequence homology between strains not only from swine farms in this study, but also from various countries, livestock breeds, and bacterial strains published on the NCBI database. Similarly, genetic environment of *mcr-3.1*-carrying plasmids shared high sequence homology in replication, stability, and conjugation regions. However, MDR regions, including the IS26-bracketed *mcr-3.1*-cassette, were highly variable between strains due to deletion/insertion of resistance gene-cassettes between plasmids. A shared feature of MDR regions in *mcr-3.1*-carrying plasmids was that multiple IS26 and IS15DI were present within the MDR regions, implying that these IS elements may play an essential role in the formation of the MDR clusters.

To investigate the possibility of genetic re-arrangement based on the intermediate circular form of IS26-bracketed AR gene cassette, we conducted inverse PCR and amplicon sequencing of the eight *mcr-3.1*-carrying IncHI2 type fragments isolated from Farm E. The intermediate circular form of IS26-bracketed AR1 (*sul1-qacEΔ1-aadA2-dfrA12*-Intl1) was identified. The intermediate circular form of IS26-bracketed *mcr-3.1*-cassette was recently reported [24], although it was not identified in this study. IS26 is a highly active IS element that favors both intramolecular and intermolecular transpositions [50]. Inter-/intramolecular transposition-based genomic re-arrangement is an

important driving force for bacterial genome evolution [272, 273]. IS26 is frequently associated with resistance genes and is found more frequently on plasmids than on chromosomes [273, 274]. IS26 may form a circular intermediate of AR cassettes and transfer it through "copy-out-paste-in" mechanism [272]. IS26-mediated intramolecular translocation events bring several antibiotic resistance determinants closer to one another to form resistance clusters [273]. Our results imply that the use of IS26 with a high potential for intramolecular translocation may be responsible for their higher diversity and carriage of multiple resistance genes in the *mcr-3.1*-cassette.

Genome sequence analysis of the MCR3-E13 strain isolated in this study showed that *mcr-3.1* may be integrated to the bacterial chromosome as a co-integrated form of Mu-like prophage and *mcr-3.1*-carrying IncHI2-type plasmid fragment. In the co-integration site, 14-bp terminal inverted repeats (IRL and IRR) of IS26 and 8-bp duplication of *gin* gene of Mu-like prophage were identified. IS26-mediated replicon fusions or co-integration between replicons and phage genome has been reported from several studies [50, 274-276]. When the IS26 and the target site are in two different replicons/chromosomes (intermolecular transposition), subsequent DNA replication at the intermediate branch fuses the two replicons/chromosomes, duplicating both the IS26 and a short nucleotide sequence flanking the insertion site (TSD) [273]. Bacteriophage Mu, 36 717 bp in length, is a temperate phage of *E. coli* and several other enteric bacteria [276]. It has the remarkable ability to insert its DNA in apparently random *E. coli* chromosomal sites [277]. Consistently, multiple insertions of the Mu-like prophage were confirmed in the MCR3-E13 chromosome. Of the several inserted Mu-like prophages in the MCR3-E13 chromosome, the *mcr-3.1*-carrying IncHI2-type plasmid fragment

was inserted into the *gin* gene region in one prophage, resulting in prophage cleaved. The remaining Mu-like prophages were intact, without cleavage. This result implies that the random insertion of Mu-like prophages into the MCR3-E13 chromosome could be followed by insertion of the *mcr-3.1*-carrying IncHI2-type plasmid fragment.

Gao et al (2021) recently reported a novel integrative and conjugative element (ICE) carrying *mcr-1*, ICEAsp1, in an *Actinobacillus* GY-402 strain of swine origin [278]. ICEs encode their own excision from the host chromosome, transfer by conjugation to a recipient bacterium, as well as site-specific integration into the chromosome of the new host [279]. ICEs share similarities in functional components with conjugative and mobilizable plasmids: ICEs normally consist of three functional components: recombination, conjugation, and fitness region within which various antimicrobial/heavy metal resistance genes and IS elements are clustered [280]. Diverse ICEs have been identified from bacterial species, with many of them recognizing $tRNA^{Leu}$ as their insertion target [281]. The *mcr-3.1*-carrying plasmid fragment of MCR3-E13 exhibited two major differences compared to ICEAsp1: it carried the IncHI2-type replication initiation protein, *repA*, and used IS26 and *gin* of Mu-like prophage as its integration site, unlike ICEAsp1, which used $tRNA^{Leu}$ as its insertion target. To the best our knowledge, our study is the first to describe that *mcr-3.1*-carrying plasmid fragment could be inserted into the bacterial chromosome. Once integrated into the bacterial host chromosome, the IS26-mediated resistance plasmids are replicated as part of the chromosome during segregation of cell division, likely improving its stability compared with plasmids in bacterial lineages [279]. Furthermore, the cointegrate can be subsequently resolved into a plasmid identical to the original donor plasmid and

conjugated into recipient cells through horizontal transfer system [273, 279], enabling dual pathways of both vertical and horizontal transmission. Our results suggest that usage of IS26, which actively favors inter- and intramolecular transposition, as a vector in the *mcr-3.1* cassette may be one of the possible reasons for the global success of *mcr-3.1* propagation.

In conclusion, to the best our knowledge, this is the first study to analyze the different transfer characteristics of two *mcr* variants, mcr-1.1 and mcr-3.1, based on the combination of culture-based and comparative genomic analysis. The *mcr-1.1* was often transferred based on the simple genetic cassette/plasmid without MDR and showed high horizontal transferability, implying a major role on colistin resistance propagation. In contrast, *mcr-3.1* had dual pathways mediated by plasmid transfer (horizontal transmission) and chromosomal insertion (vertical transmission), enabling it to proliferate stably despite of its relatively lower horizontal transferability. Furthermore, the *mcr-3.1* was mainly transferred with MDR, suggesting a significant challenge on public health. Our study showed the different threat by *mcr-1.1* and *mcr-3.1*, highlighting the need for suitable strategies based on their differences to control colistin resistance.

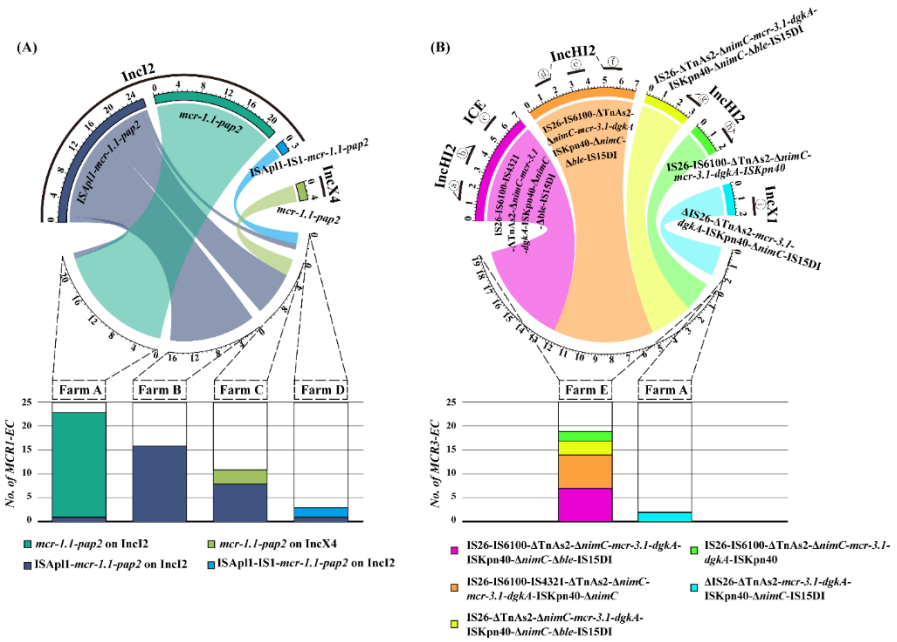


Figure 24. Distribution of the genetic backgrounds of *mcr-1* (A) and *mcr-3* (B) in *Escherichia coli* isolates from swine farms. Chord diagrams and bar charts were generated using R statistical software (ver. 4.3.2). In the chord diagrams, the size of segments on the top represents the number of *Escherichia coli* isolates carrying *mcr* (MCR-EC) with the *mcr-1.1* and *mcr-3.1* genetic backgrounds. The size of segments on the bottom represents the number of MCR-EC isolates detected in different swine farms. Ribbons connecting the top and bottom segments represent the number of MCR-EC isolates with a specific type of *mcr-1.1* or *mcr-3.1* genetic background found in the respective farms. The connected bar charts show the composition of *mcr-1* and *mcr-3* genetic backgrounds based on the number of MCR-EC isolates from different farms. ⓐ, MCR3-E05; ⓑ, MCR3-E17; ⓒ, MCR3-E13; ⓓ, MCR3-E07; ⓔ, MCR3-E09; ⓕ, MCR3-E15; ⓖ, MCR3-E11; ⓗ, MCR3-E08; ⓘ, MCR3-A19.

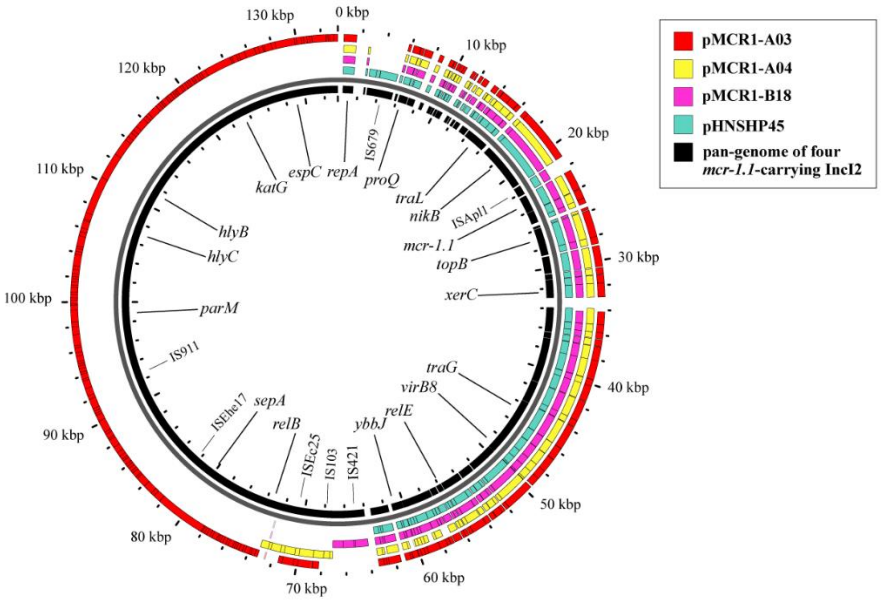


Figure 25. Whole genome configuration of four *mcr-1.1*-carrying plasmids. The comparative circular genome visualization was constructed using GView server, with an identity threshold of 70–90%.

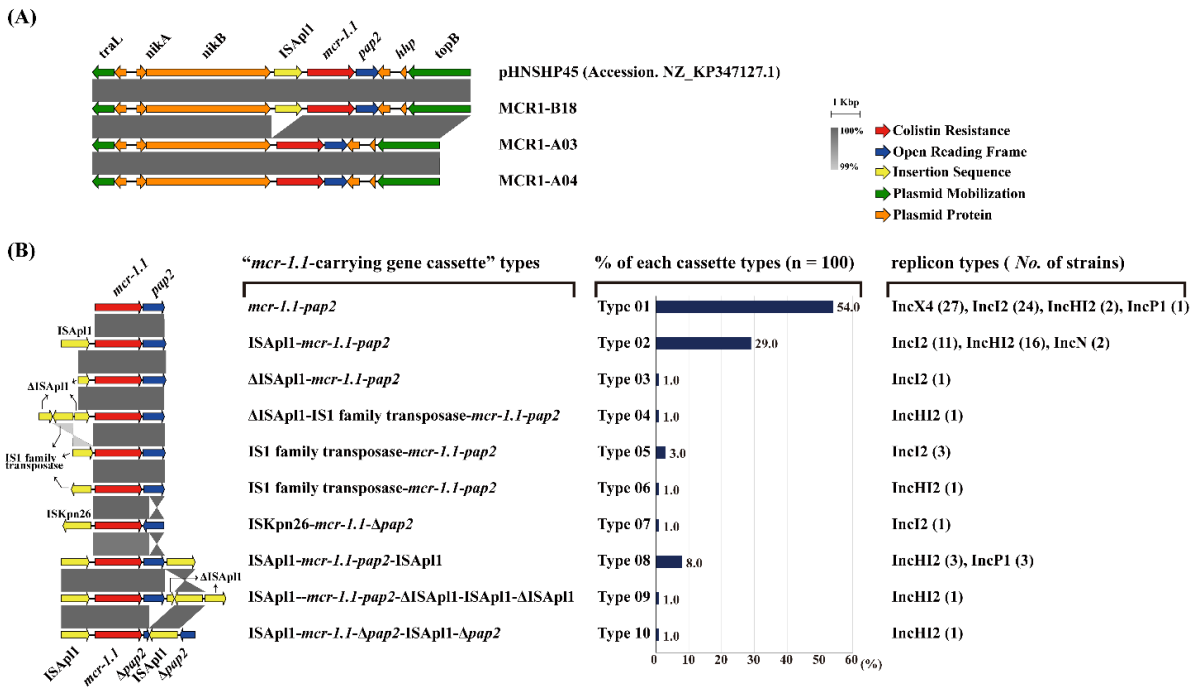


Figure 26. Comparative genomic analysis of *mcr-1.1*-carrying cassettes using (A) three *mcr-1.1*-carrying plasmids (pMCR1-A03, pMCR1-A04, and pMCR1-B18) isolated in this study compared with the reference strain pHNSHP45, and (B) 100 plasmids whose whole genome sequences were uploaded to the NCBI database. The nucleotide sequences of *mcr*-carrying segments were aligned and compared using Easyfig (v2.2.3).

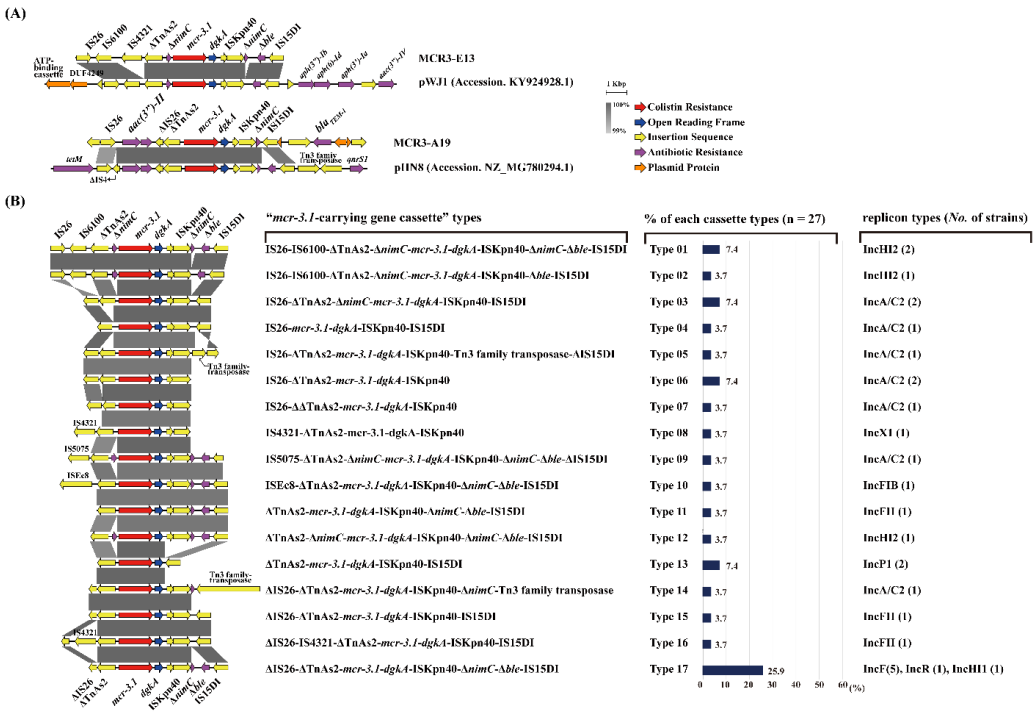


Figure 27. Comparative genomic analysis of *mcr-3.1*-carrying cassette using (A) two *mcr-3.1*-carrying plasmids (MCR3-A19 and MCR3-E13) isolated in this study compared with the reference strains pHN8 and pWJ1, and (B) 27 plasmids whose whole genome sequences were uploaded to the NCBI database. The nucleotide sequences of *mcr*-carrying segments were aligned and compared using easyfig (v2.2.3).

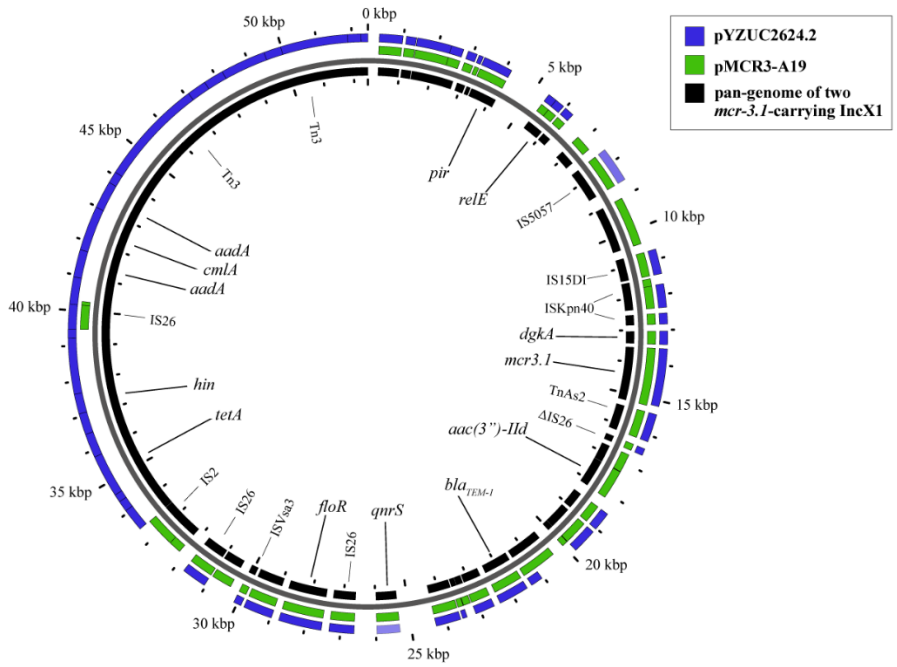


Figure 28. Whole genome configuration of pMCR3-A19. The comparative circular genome visualization was constructed using GView server, with an identity threshold of 70–90%.

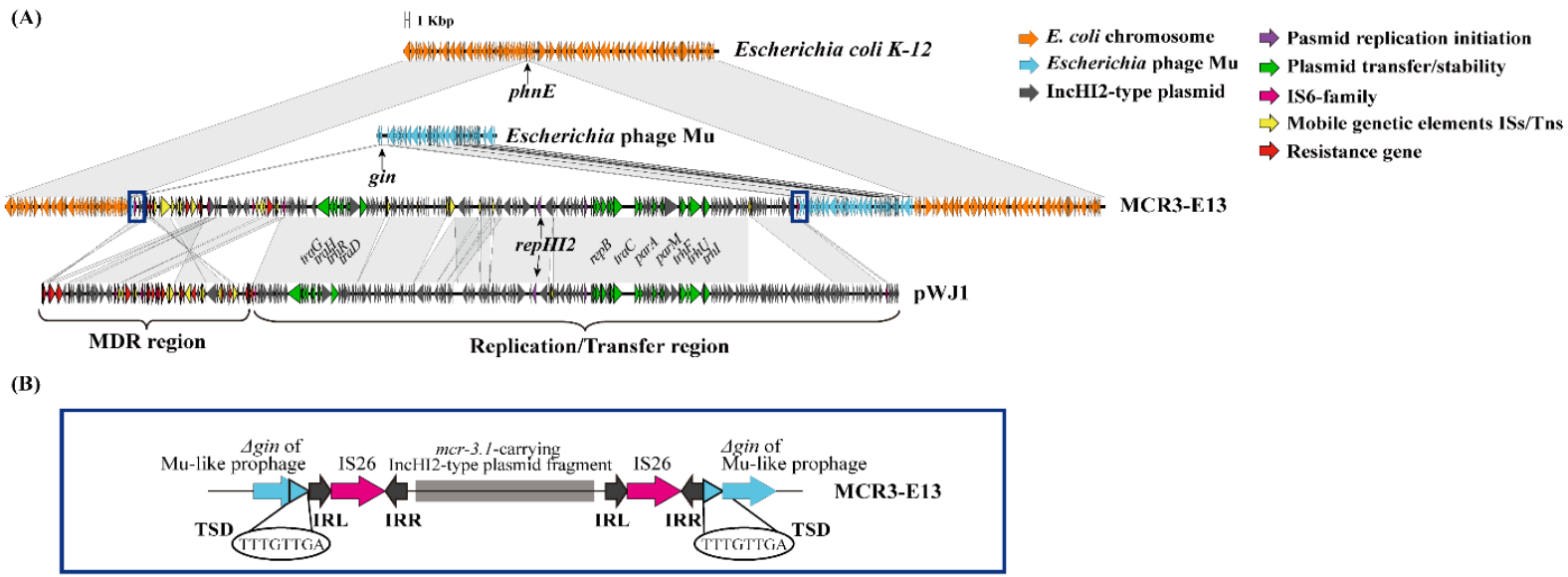


Figure 29. Co-integration of Mu-like prophage and *mcr-3.1*-carrying IncHI2-type fragment into the chromosome of MCR3-E13 (A) and the co-integration site (B). The nucleotide sequences were aligned and compared using Easyfig (v2.2.3).

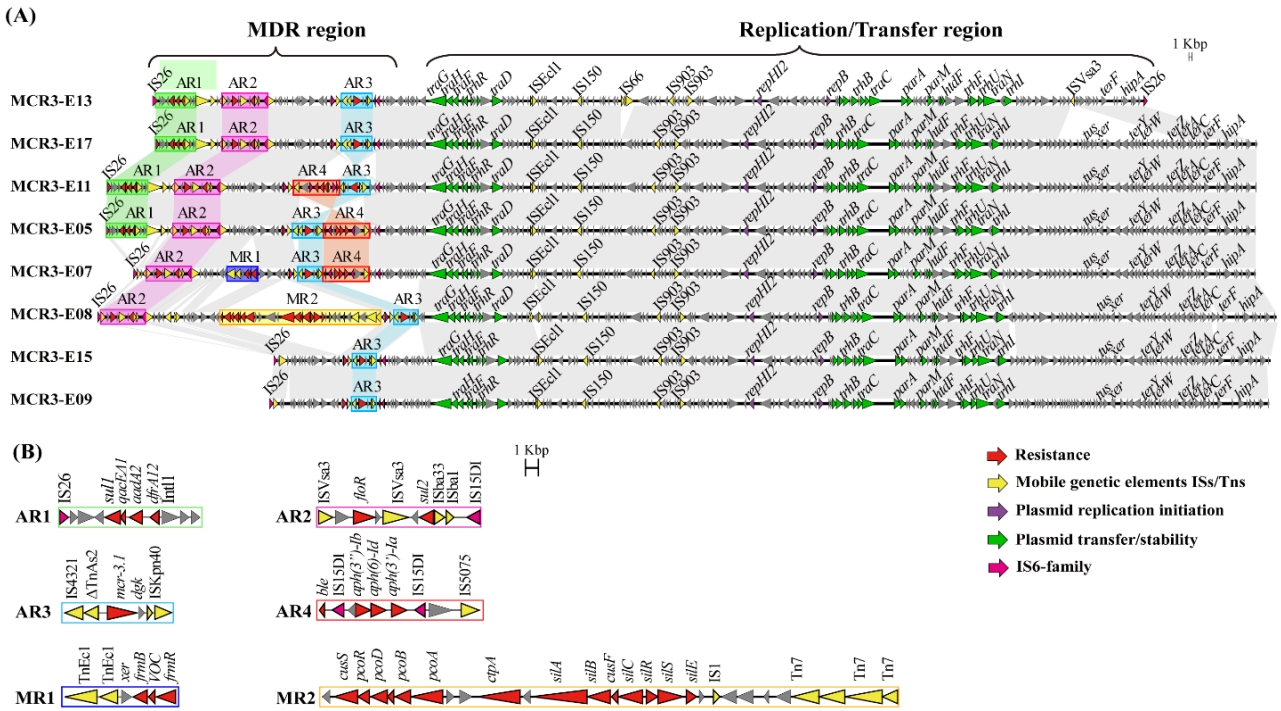
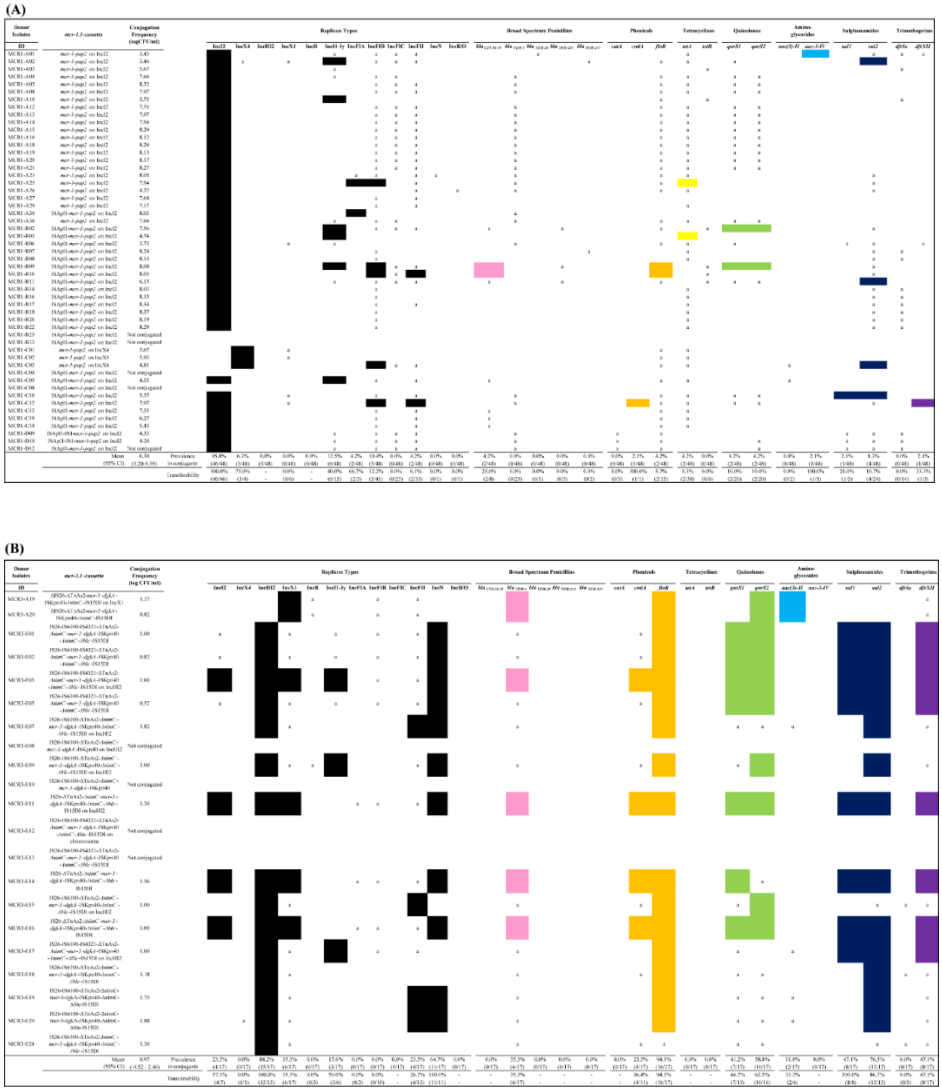
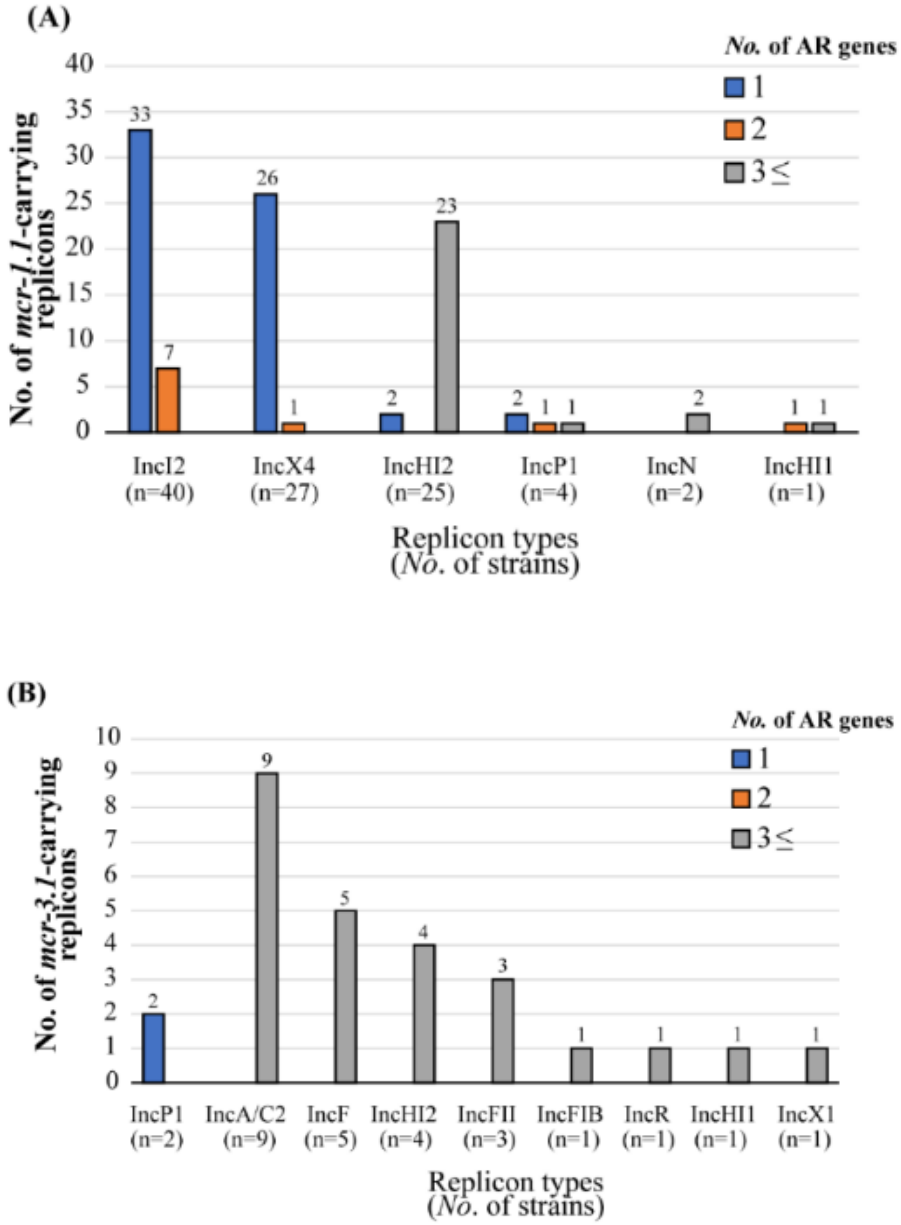


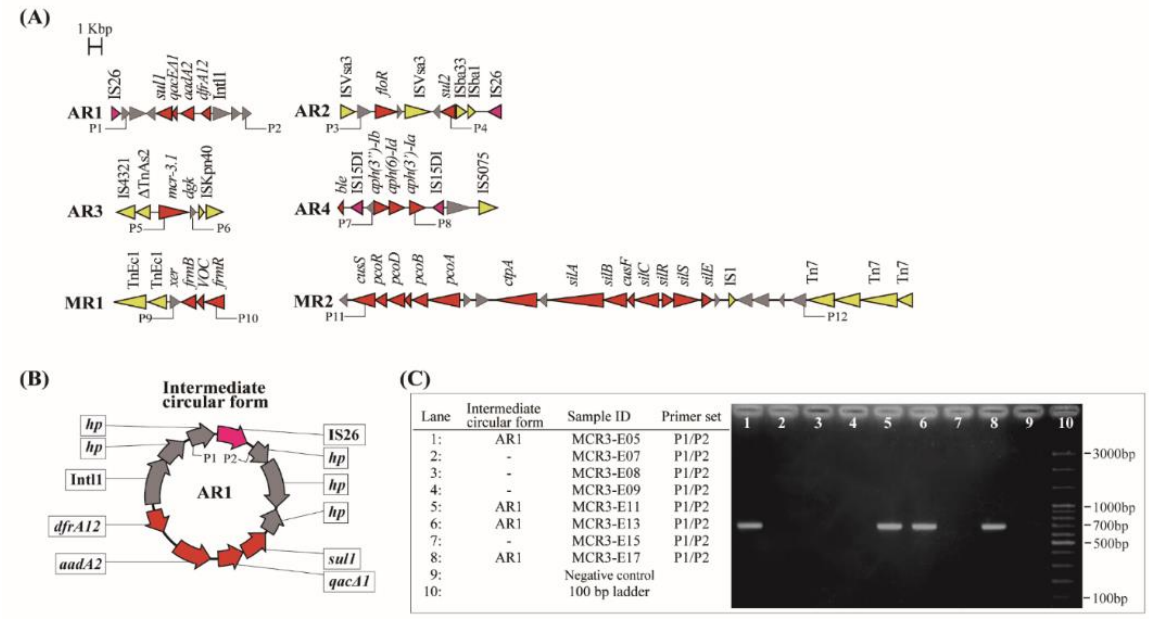
Figure 30. Comparative genomic analysis of eight *mcr-3.1*-carrying IncHI2-type plasmid fragments isolated from swine farm E (A) and antimicrobial/heavy metal resistance gene cassettes (B). The nucleotide sequences were aligned and compared using Easyfig (v2.2.3). AR, Antimicrobial resistance gene cassette; MR, heavy metal resistance gene cassette.



Supplementary Figure 4. Horizontal Genetic Transferability of *mcr*-carrying cassette, resistance genes, and replicon types of MCR-EC in the conjugation assay of *mcr-1.1* (A) and *mcr-3.1* (B). Colored cells represent the transfer of genes/plasmids (indicated in each column) to the *E. coli* J53-Azi^{R} strain in the conjugation assay. The transferability of genes was confirmed via PCR. [a] detected in donor strains but were not transferred. Blank = plasmid replicon or antimicrobial resistance genes were not detected in donor strains.



Supplementary Figure 5. Numbers of antimicrobial resistance (AR) genes encoded in the 100 plasmid-carrying *mcr-1.1* isolates (A) and the 27 plasmid-carrying *mcr-3.1* isolates (B) following the replicon types.



Supplementary Figure 6. Analysis of intermediate circular form of antimicrobial/heavy metal resistance gene cassettes. The primer site used for inverse PCR (A). Identified intermediate circular form of AR1 (B). Gel electrophoresis of the PCR amplicon for detecting the intermediate circular form of AR1. AR, Antimicrobial resistance gene cassette; MR, heavy metal resistance gene cassette.

Supplementary Table 13. Bacterial strains included in this study

Isolate ID	Isolate information		Meta information			
	mcr variant type	Clone type[a]	Pig Stage	Farm	Province	Year
MCR1-A01	*mcr-1.1*	ST1140-D	Weaning piglet	A	GG	2019
MCR1-A02	*mcr-1.1*	ST10-A	Weaning piglet	A	GG	2019
MCR1-A03	*mcr-1.1*	ST20-A	Weaning piglet	A	GG	2019
MCR1-A04	*mcr-1.1*	ST10-A	Weaning piglet	A	GG	2019
MCR1-A05	*mcr-1.1*	ST10-A	Weaning piglet	A	GG	2019
MCR1-A08	*mcr-1.1*	ST10-A	Growing pig	A	GG	2019
MCR1-A10	*mcr-1.1*	ST20-A	Growing pig	A	GG	2019
MCR1-A12	*mcr-1.1*	ST10-A	Growing pig	A	GG	2019
MCR1-A13	*mcr-1.1*	ST10-A	Growing pig	A	GG	2019
MCR1-A14	*mcr-1.1*	ST10-A	Growing pig	A	GG	2019
MCR1-A15	*mcr-1.1*	ST10-A	Growing pig	A	GG	2019
MCR1-A16	*mcr-1.1*	ST10-A	Finishing pig	A	GG	2019
MCR1-A18	*mcr-1.1*	ST10-A	Finishing pig	A	GG	2019
MCR1-A19	*mcr-1.1*	ST10-A	Finishing pig	A	GG	2019
MCR1-A20	*mcr-1.1*	ST10-A	Finishing pig	A	GG	2019
MCR1-A21	*mcr-1.1*	ST10-A	Finishing pig	A	GG	2019
MCR1-A23	*mcr-1.1*	ST93-A	Finishing pig	A	GG	2019
MCR1-A25	*mcr-1.1*	ST93-A	Finishing pig	A	GG	2019
MCR1-A26	*mcr-1.1*	ST657-B1	Sow	A	GG	2019
MCR1-A27	*mcr-1.1*	ST10-A	Sow	A	GG	2019
MCR1-A29	*mcr-1.1*	ST641-B1	Sow	A	GG	2019
MCR1-A30	*mcr-1.1*	ST641-B1	Sow	A	GG	2019
MCR1-A36	*mcr-1.1*	ST10-A	Weaning piglet	A	GG	2019
MCR1-B02	*mcr-1.1*	ST744-A	Sow	B	GG	2017
MCR1-B03	*mcr-1.1*	ST1408-A	Sow	B	GG	2017
MCR1-B06	*mcr-1.1*	ST1408-A	Finishing pig	B	GG	2017
MCR1-B07	*mcr-1.1*	ST1112-A	Finishing pig	B	GG	2017
MCR1-B08	*mcr-1.1*	ST1112-A	Finishing pig	B	GG	2017
MCR1-B09	*mcr-1.1*	ST744-A	Weaning piglet	B	GG	2017
MCR1-B10	*mcr-1.1*	ST744-A	Weaning piglet	B	GG	2017
MCR1-B11	*mcr-1.1*	ST744-A	Weaning piglet	B	GG	2017
MCR1-B14	*mcr-1.1*	ST1112-A	Growing pig	B	GG	2017
MCR1-B16	*mcr-1.1*	ST1112-A	Growing pig	B	GG	2017
MCR1-B17	*mcr-1.1*	ST1112-A	Growing pig	B	GG	2017
MCR1-B18	*mcr-1.1*	ST1112-A	Growing pig	B	GG	2017
MCR1-B20	*mcr-1.1*	ST1112-A	Growing pig	B	GG	2017
MCR1-B22	*mcr-1.1*	ST1112-A	Growing pig	B	GG	2017

MCR1-B23	*mcr-1.1*	ST101-B1	Finishing pig	B	GG	2017
MCR1-B33	*mcr-1.1*	ST398-A	Sow	B	GG	2017
MCR1-C01	*mcr-1.1*	ST206-A	Weaning piglet	C	GN	2018
MCR1-C02	*mcr-1.1*	ST206-A	Weaning piglet	C	GN	2018
MCR1-C03	*mcr-1.1*	ST101-B1	Weaning piglet	C	GN	2018
MCR1-C04	*mcr-1.1*	ST410-A	Weaning piglet	C	GN	2018
MCR1-C05	*mcr-1.1*	ST410-A	Weaning piglet	C	GN	2018
MCR1-C08	*mcr-1.1*	ST297-B1	Growing pig	C	GN	2018
MCR1-C10	*mcr-1.1*	ST297-B1	Growing pig	C	GN	2018
MCR1-C12	*mcr-1.1*	ST6256-B1	Growing pig	C	GN	2018
MCR1-C13	*mcr-1.1*	ST457-D	Growing pig	C	GN	2018
MCR1-C19	*mcr-1.1*	ST457-D	Finishing pig	C	GN	2018
MCR1-C34	*mcr-1.1*	ST457-D	Sow	C	GN	2018
MCR1-D09	*mcr-1.1*	ST156-B1	Growing pig	D	GG	2019
MCR1-D10	*mcr-1.1*	ST156-B1	Growing pig	D	GG	2019
MCR1-D12	*mcr-1.1*	ST101-B1	Growing pig	D	GG	2019
MCR3-A19	*mcr-3.1*	STNT1-A	Finishing pig	A	GG	2019
MCR3-A20	*mcr-3.1*	STNT1-A	Finishing pig	A	GG	2019
MCR3-E01	*mcr-3.1*	ST2951-E	Weaning piglet	E	GG	2018
MCR3-E02	*mcr-3.1*	ST2951-E	Weaning piglet	E	GG	2018
MCR3-E03	*mcr-3.1*	ST2951-E	Weaning piglet	E	GG	2018
MCR3-E05	*mcr-3.1*	ST2951-E	Weaning piglet	E	GG	2018
MCR3-E07	*mcr-3.1*	ST4762-A	Growing pig	E	GG	2018
MCR3-E08	*mcr-3.1*	ST5229-B1	Growing pig	E	GG	2018
MCR3-E09	*mcr-3.1*	STNT2-A	Growing pig	E	GG	2018
MCR3-E10	*mcr-3.1*	ST5229-B1	Growing pig	E	GG	2018
MCR3-E11	*mcr-3.1*	ST93-A	Growing pig	E	GG	2018
MCR3-E12	*mcr-3.1*	STNT3-A	Growing pig	E	GG	2018
MCR3-E13	*mcr-3.1*	STNT3-A	Growing pig	E	GG	2018
MCR3-E14	*mcr-3.1*	ST93-A	Growing pig	E	GG	2018
MCR3-E15	*mcr-3.1*	ST10-A	Finishing pig	E	GG	2018
MCR3-E16	*mcr-3.1*	ST93-A	Finishing pig	E	GG	2018
MCR3-E17	*mcr-3.1*	ST156-B1	Finishing pig	E	GG	2018
MCR3-E18	*mcr-3.1*	ST10-A	Finishing pig	E	GG	2018
MCR3-E19	*mcr-3.1*	ST4762-A	Finishing pig	E	GG	2018
MCR3-E20	*mcr-3.1*	ST4762-A	Finishing pig	E	GG	2018
MCR3-E24	*mcr-3.1*	ST10-A	Finishing pig	E	GG	2018

Clone type[a] is the combination of multi-locus sequence type and *E. coli* phylogenetic group. ST, multi-locus sequence type; NT, non-typable; GG, Gyeonggi-do; GN, Gyeongsang-nam-do.

Supplementary Table 14. Oligonucleotide sequences and annealing temperatures for primers used in the analysis of the genetic backgrounds of *mcr-1* and *mcr-3*

Func-tion	Target site		Nucleotide sequence	Size (bp)	Temp. (℃)	Refe-rence
Genetic background of *mcr-1*	*nikB* (IncI2)-ISApl1	F	AGTGGATGTTACGGAGCAG	851	60	This study
		R	GGAGCTACGTCCCAGTGC			
	nikB (IncI2)-*mcr-1*	F	AGTGGATGTTACGGAGCAG	894	60	[212]
		R	CCACAAGAACAAACGGACT			
	ISApl1-*mcr-1*	F	CGAAGCACCAAGACATCA	393	60	[212]
		R	CCACAAGAACAAACGGACT			
	mcr-pap2	F	AACGGTGTCTATCTACATGGTAT	462	60	This study
		R	ACAGTAGCAAATCTGGCAA			
	pap2-ISApl1	F	TTGCCAGATTTGCTACTGT	696	60	[212]
		R	TTTCTCGCTCGTTTATTGTA			
	pap2-hp (IncI2)	F	GTATCTGGTGCTGACTTTGA	723	60	[212]
		R	ACTTAGCGATCTCGTTGTT			
	DUF2806 (IncX4)-*mcr-1*	F	AGAGCTTGAGGGAATAGAA	879	60	[212]
		R	CACAGGCTTTAGCACATAG			
	mcr-1-DUF2726 (IncX4)	F	AACGGTGTCTATCTACATGGTAT	1,674	60	[212]
		R	CATTGAATTTGTTCGTCCTC			
Genetic background of *mcr-3*	IS26-IS6100	F	TGGTACTGGCGTAACCCTTC	820	60	This study
		R	GAGAGAGCTTTTGGCATTGG			
	IS6100-TnAs2	F	GACTTCGAATCCCTTGATCG	2000	60	This study
		R	CTGACCGGCGATTACCTATG			
	TnAs2-*mcr-3*	F	AGCCTGTCGCTGCTAATCAT	946	60	This study
		R	ATGAAATGGCGAAACCAAAC			
	mcr-3-dgkA	F	ATCGTCAGTTCACCCCTGAC	871	60	This study
		R	GCAGAGCCCATGTCTTTAGC			
	dgkA-ISKpn40	F	GCTAAAGACATGGGCTCTGC	1019	60	This study
		R	GGGTATGGCAATCAATCACC			
	ISKpn40-*ble*	F	CTGGAGCAAGCCTTAATTGC	1000	60	This study
		R	AGTGGTGGGATGAACGAGAC			
	ble-IS15DI	F	ACTCCGCACCTGTACAAACC	1000	60	This study
		R	TGGTACTGGCGTAACCCTTC			

Supplementary Table 15. Oligonucleotide sequences and annealing temperatures for primers used in the typing of resistance genes and plasmid types

Function	Genes		Nucleotide sequence	Size (bp)	Temp. (℃)	Reference
ESBL genotypes	*bla*$_{CTX\text{-}M\text{-}1\text{-}family}$	F	GTTACAATGTGTGAGAAGCAG	1,041	60	[197]
		R	CCGTTTCCGCTATTACAAAC			
	bla$_{CTX\text{-}M\text{-}2\text{-}family}$	F	CGACGCTACCCCTGCTATT	832	60	[197]
		R	CAGAAACCGTGGGTTACGAT			
	bla$_{CTX\text{-}M\text{-}8\text{-}family}$	F	GGCGCTGGAGAAAAGCAG	862	60	[197]
		R	GGTTTTATCCCCGACAACC			
	bla$_{CTX\text{-}M\text{-}9\text{-}family}$	F	GTGACAAAGAGAGTGCAACGG	857	60	[197]
		R	ATGATTCTCGCCGCTGAAGCC			
	bla$_{CTX\text{-}M\text{-}25\text{-}family}$	F	GCACGATGACATTCGGG	327	60	[197]
		R	AACCCACGATGTGGGTAGC			
	bla$_{CMY}$	F	AACACACTGATTGCGTCTGAC	1,226	60	[197]
		R	CTGGGCCTCATCGTCAGTTA			
	bla$_{SHV}$	F	TCGCCTGTGTATTATCTCCC	768	54	[197]
		R	CGCAGATAAATCACCACAATG			
	bla$_{TEM\text{-}family}$	F	TCCGCTCATGAGACAATAACC	1,057	58	[197]
		R	ACGCTCAGTGGAACGAAAAC			
	bla$_{OXA}$	F	ACACAATACATATCAACTTCGC	813	60	[197]
		R	AGTGTGTTTAGAATGGTGATC			
Antimicrobial resistance	*catA*	F	AGTTGCTCAATGTACCTATAACC	547	57	[203]
		R	TTGTAATTCATTAAGCATTCTGCC			
	cmlA	F	CCGCCACGGTGTTGTTGTTATC	698	57	[203]
		R	CACCTTGCCTGCCCATCATTAG			
	floR	F	TATCTCCCTGTCGTTCCAG	399	52	[203]
		R	AGAACTCGCCGATCAATG			
	tetA	F	GCTACATCCTGCTTGCCTTC	210	58	[203]
		R	CATAGATCGCCGTGAAGAG			
	tetB	F	TTGGTTAGGGGCAAGTTTTG	659	56	[203]
		R	GTAATGGGCCAATAACACCG			
	tetD	F	AAACCATTACGGCATTCTGC	787	60	[203]
		R	GACCGGATACACCATCCATC			
	qnrA	F	ATTTCTCA CGCCAGGATTTG	516	53	[204]
		R	GATCGGCAAAGGTTAGGTCA			
	qnrB	F	GATCGTGAAAGCCAGAAAGG	469	53	[204]
		R	ACGATGCCTGGTAGTTGTCC			
	qnrC	F	GGGTTGTACATTTATTGAATC	447	50	[204]
		R	TCCACTTTACGAGGTTCT			
	qnrS1	F	ACGACATTCGTCAACTGCAA	417	53	[204]
		R	TAAATTGGCACCCTGTAGGC			
	qnrS2	F	TGGAAACCTACCGTCACACA	600	60	[243]
		R	CTGGCAATTTTGATACCTGA			
	aac(6)-Ib-cr	F	TTGCGATGCTCTATGAGTGGCTA	482	50	[204]
		R	CTCGAATGCCTGGCGTGTTT			

	aac(3)-I	F	ACCTACTCCCAACATCAGCC	169	60	[203]
		R	ATATAGATCTCACTACGCGC			
	aac(3)-II	F	ACTGTGATGGGATACGCGTC	237	60	[203]
		R	CTCCGTCAGCGTTTCAGCTA			
	aac(3)-IV	F	CTTCAGGATGGCAAGTTGGT	286	60	[203]
		R	TCATCTCGTTCTCCGCTCAT			
	sul1	F	TGGTGACGGTGTTCGGCATTC	789	60	[203]
		R	GCGAGGGTTTCCGAGAAGGTG			
	sul2	F	CGGCATCGTCAACATAACC	722	55	[203]
		R	GTGTGCGGATGAAGTCAG			
	dfrIa	F	GTGAAACTATCACTAATGG	474	55	[203]
		R	TTAACCCTTTTGCCAGATTT			
	dfrIb	F	GAGCAGCTICTITTIAAAGC	393	60	[203]
		R	TTAGCCCTTTIICCAATTTT			
	dfrII	F	GATCACGTGCGCAAGAAATC	141	50	[203]
		R	AAGCGCAGCCACAGGATAAAT			
	dfrVII	F	TTGAAAATTTCATTGATT	474	55	[203]
		R	TTAGCCTTTTTTCCAAATCT			
	dfrXII	F	GGTGSGCAGAAGATTTTTCGC	319	60	[203]
		R	TGGGAAGAAGGCGTCACCCTC			
Replicon types	IncHI1	F	GGAGCGATGGATTACTTCAGTAC	471	60	[151]
		R	TGCCGTTTCACCTCGTGAGTA			
	IncHI2	F	TTTCTCCTGAGTCACCTGTTAACAC	644	60	[151]
		R	GGCTCACTACCGTTGTCATCCT			
	IncR	F	TCGCTTCATTCCTGCTTCAGC	251	60	[244]
		R	GTGTGCTGTGGTTATGCCTCA			
	IncI1-Iγ	F	CGAAAGCCGGACGGCAGAA	139	60	[151]
		R	TCGTCGTTCCGCCAAGTTCGT			
	IncI2	F	CTGTCGGCATGTCTGTCTC	553	55	[149]
		R	CTGGCTACCAGTTGCTCTAA			
	IncX1	F	GCTTAGACTTTGTTTTATCGTT	461	62	[150]
		R	TAATGATCCTCAGCATGTGAT			
	IncX2	F	GCGAAGAAATCAAAGAAGCTA	678	63	[150]
		R	TGTTGAATGCCGTTCTTGTCCAG			
	IncX3	F	GTTTTCTCCACGCCCTTGTTCA	351	63	[150]
		R	CTTTGTGCTTGGCTATCATAA			
	IncX4	F	AGCAAACAGGGAAAGGAGAAGACT	569	62	[150]
		R	TACCCCAAATCGTAACCTG			
	IncFII	F	CACACCATCCTGCACTTA	260	60	[151]
		R	CTGATCGTTTAAGGAATTTT			
	IncL/M	F	GGATGAAAACTATCAGCATCTGAAG	785	60	[151]
		R	CTGCAGGGGCGATTCTTTAGG			
	IncFIA	F	CCATGCTGGTTCTAGAGAAGGTG	462	60	[151]
		R	GTATATCCTTACTGGCTTCCGCAG			
	IncFIB	F	GGAGTTCTGACACACGATTTTCTG	702	63	[151]
		R	CTCCCGTCGCTTCAGGGCATT			
	IncFIC	F	GTGAACTGGCAGATGAGGAAGG	262	60	[151]
		R	TTCTCCTCGTCGCCAAACTAGAT			

IncFIIs	F	CTGTCGTAAGCTGATGGC	270	60	[151]
	R	CTCTGCCACAAACTTCAGC			
IncA/C	F	GAGAACCAAAGACAAAGACCTGGA	465	60	[151]
	R	ACGACAAACCTGAATTGCCTCCTT			
IncP	F	CTATGGCCCTGCAAACGCGCCAGAAA	534	60	[151]
	R	TCACGCGCCAGGGCGCAGCC			
IncK	F	GCGGTCCGGAAAGCCAGAAAAC	160	60	[151]
	R	TCTTTCACGAGCCCGCCAAA			
IncB/O	F	GCGGTCCGGAAAGCCAGAAAAC	159	60	[151]
	R	TCTGCGTTCCGCCAAGTTCGA			
IncN	F	GTCTAACGAGCTTACCGAAG	559	55	[151]
	R	GTTTCAACTCTGCCAAGTTC			

Supplementary Table 16. Accession numbers of strains analyzed in this study

100 plasmids used in the analysis of the *mcr-1.1*-carrying cassette				
CP042644.1	NZ_CP060519.1	NZ_KY120364.1	NZ_MG489944.1	NZ_MK477619.1
MW264508.1	NZ_CP061123.1	NZ_KY471313.1	NZ_MG515249.1	NZ_MK477620.1
NZ_CP016187.1	NZ_CP061186.1	NZ_KY471314.1	NZ_MG552133.1	NZ_MK571810.1
NZ_CP019052.1	NZ_CP069661.1	NZ_KY795977.1	NZ_MG557851.1	NZ_MK574665.1
NZ_CP020493.1	NZ_CP069680.1	NZ_KY802014.1	NZ_MG591702.1	NZ_MK875281.1
NZ_CP022735.1	NZ_CP069685.1	NZ_LC511660.1	NZ_MG747472.1	NZ_MK875287.1
NZ_CP024139.1	NZ_CP069705.1	NZ_MF083142.1	NZ_MG825373.1	NZ_MN200943.1
NZ_CP029748.1	NZ_CP070915.1	NZ_MF135534.1	NZ_MG825374.1	NZ_MN232194.1
NZ_CP032076.1	NZ_CP080136.1	NZ_MF175190.1	NZ_MH208235.1	NZ_MN232195.1
NZ_CP032987.1	NZ_KP347127.1	NZ_MF175191.1	NZ_MH213346.1	NZ_MN232196.1
NZ_CP034400.1	NZ_KX084393.1	NZ_MF381176.1	NZ_MH522409.1	NZ_MN232206.1
NZ_CP035916.1	NZ_KX377410.1	NZ_MF774188.1	NZ_MH522410.1	NZ_MN476093.1
NZ_CP038181.1	NZ_KX505142.1	NZ_MF990207.1	NZ_MH522420.1	NZ_MN689940.1
NZ_CP041113.1	NZ_KX570748.1	NZ_MG210937.1	NZ_MH522422.1	NZ_MN746290.1
NZ_CP041997.1	NZ_KX856065.1	NZ_MG210940.1	NZ_MH733010.1	NZ_MT499884.1
NZ_CP042587.1	NZ_KY012275.1	NZ_MG257881.1	NZ_MK477603.1	NZ_MT929285.1
NZ_CP046418.1	NZ_KY012276.1	NZ_MG299136.1	NZ_MK477605.1	NZ_MT929286.1
NZ_CP047664.1	NZ_KY075655.1	NZ_MG299138.1	NZ_MK477606.1	NZ_MW495059.1
NZ_CP049356.1	NZ_KY075660.1	NZ_MG299140.1	NZ_MK477611.1	NZ_MW999352.1
NZ_CP055260.1	NZ_KY075662.1	NZ_MG372114.1	NZ_MK477618.1	NZ_MZ062605.1
27 plasmids used in the analysis of the *mcr-3.1*-carrying cassette				
AP023301.1	NZ_CP053729.1	NZ_AP018939.1	CP049300.1	NZ_MK770642.1
NZ_CP045953.1	CP042628.1	NZ_MN647787.1	NZ_CP053721.1	NZ_MN647789.1
NZ_CP039562.1	NZ_MT449719.1	NZ_MT449720.1	AP023314.1	MK962306.2
NZ_CP050727.1	NZ_MT449718.1	NZ_MT449722.1	AP023307.1	
NZ_MN647788.1	NZ_MG780294.1	NZ_AP018354.1	NZ_CP063506.1	
NZ_CP050732.1	KY924928.1	NZ_OU015324.1	NZ_MH077952.1	

Supplementary Table 17. Oligonucleotide sequences and annealing temperatures for primers used in the analysis of the intermediate circular form of antimicrobial/heavy metal resistance gene cassette

Target site		Nucleotide sequence	Temp. (°C)	Reference
AR1	P1	CGACAAGGTACGGTAGGGAA	60	This study
	P2	TACCGTGGCAGGAAGAAATC		
AR2	P3	GTCCGATCCGATCTGTTTGT	60	
	P4	CGGCATCGTCAACATAACC		
AR3	P5	ATGAAATGGCGAAACCAAAC	60	
	P6	GCTAAAGACATGGGCTCTGC		
AR4	P7	AAAAATGATCACGGCGGTAG	60	
	P8	CGATACCAGGATCTTGCCAT		
MR1	P9	TTTGTACCGCTGATGTTCTCC	60	
	P10	AAGAATGGAACGGCATTCAG		
MR2	P11	TTTGTACCGCTGATGTTCTCC	60	
	P12	AAGAATGGAACGGCATTCAG		

General Discussion and Conclusion

Concerns have been raised regarding the impacts of antibiotic use in food animals on the health of people on farms and, ultimately, of consumers via the food chain. Swine farms are an important reservoir of CIA-resistant bacteria, and antibiotic resistance in pig farms may be not limited to pig farms, but spread to various ecosystems including animals, people, food, and the environment. The present study aimed to investigate the risks of ESBL/AmpC/MCR-EC strains according to swine production stages, an important reservoir of CIA-resistant bacteria, and to evaluate the potential threat of swine farm-derived strains to humans by understanding molecular epidemiological dynamics and resistance mechanisms.

The ESBL/AmpC/MCR-EC strains, carrying MDR and virulence potential, were distributed throughout the pig farms, with the high prevalence. The swine farm-derived ESBL/AmpC/MCR-EC strains shared ESBL/AmpC types, multi-drug resistance, and clone types with strains from pork meats and humans in South Korea, providing an indirect scientific evidence that swine farm-derived ESBL/AmpC/MCR-EC strains could be transmitted to humans through food-chains. To strengthen the possibility of transmission of resistant bacteria and/or genes via food-chains, further studies on WGS-based genetic relatedness analysis and metagenomic analysis of ESBL/AmpC/MCR-EC in slaughterhouses producing pork meats from pigs of swine farms included in this study would be interesting.

High-risk ExPEC and InPEC clones which shared with human-derived strains were widely distributed in swine farms. In pig farms, a variety of bacterial pathogens and antimicrobial resistances co-exist, which making

swine farms as a melting pot of pathogens and antimicrobial resistances. The InPEC or ExPEC in pig farms acquiring MDR through a horizontal transfer mechanism may contribute the occurrence of super-bacteria that are difficult to treat even with last-resort antibiotic prescriptions. In this situation, the misuse and abuse of antimicrobial agents may simply impose selection pressure, leading to disease treatment failure and the spread of colistin resistance in swine farms. To control these potentially high-risk clones of ESBL/AmpC/MCR-EC in swine farms, efforts to use antibiotic substitutes are needed rather than indiscriminate prescription of antibiotics for disease control.

The ESBL/AmpC/MCR-EC strains from different swine stages exhibited highly closed genetic distance and shared antimicrobial gene types within farm, implying a high possibility of cross-infection of antimicrobial resistance within swine farms. This result suggests that ESBL/AmpC/MCR-EC could spread into other swine at different stages and could continue to exist within swine farms, through the repeated cycle with shedding from pig feces, survival in the farm environment, and reintroduction to pigs. In order to reduce cross-contamination in pig farms, it can be helpful to introduce a comprehensive policy, improve quarantine, manage farm staff movement, and manage personal hygiene.

In swine industry, different antimicrobial agents are prescribed according to swine stages, which may cause the distribution of antibiotic-resistant bacteria to vary according to stages. Therefore, establishing a monitoring strategy and control plan without considering swine stages may cause errors in the analysis results by acting as a confounding factor in understanding the distribution and characteristics of antibiotic resistance in pig

farms. Furthermore, antimicrobial prescription without considering the characteristics of each breeding stage within the pig farm could cause not only treatment failure but also spread of antimicrobial resistant bacteria in swine farms. Taken together, the present study suggest that it is necessary to establish a resistance management strategy based on the periodic monitoring considering the different characteristics ESBL/AmpC/MCR-EC according to swine production stages.

To manage residual antibiotics in pork meats at slaughterhouses, the antimicrobial prescription in finishing pigs is regularly controlled. But relatively little attention is paid to antibiotic prescriptions in weaning piglets, which causing antibiotic abuse. In this study, the prevalence of ESBL/AmpC/MCR-EC was distribution of resistant bacteria in weaning piglets was significantly higher compared to other breeding stages. Given that antibiotic-resistant bacteria colonized in weaning pigs can persist in the pig intestine for more than 6 months without exposure to antibiotics, suggesting that ESBL/AmpC/MCR-EC colonized in the intestine of weaning pigs could persist until the slaughterhouse stages. Therefore, the present study proposes the need to pay attention from weaning stage to control ESBL/AmpC/MCR-EC strains in swine farms, an important reservoir of CIA-resistant bacteria.

The major two mobilized colistin resistance gene –*mcr-1.1* and *mcr-3.1*– exhibited a remarkably high horizontal genetic transferability, suggesting that they played an important role in the global spread of colistin resistance. Two major *mcr* variants showed different horizontal transfer characteristics. In particular, *mcr-3.1* was co-transferred with various antimicrobial resistance genes, implying a high risk of MDR transmission by *mcr-3*-mediated horizontal

genetic transfer. This study showed that even the same resistance genes can be transferred by different MGE vectors depending on the variant types, thereby showing different transfer characteristics. In order to control antimicrobial resistance, a customized approach for each variant seems to be needed.

To analyze the overall characteristics of pig farms in South Korea, multi-stage stratified random sampling was conducted targeting large-scale farms with more than 1,000 pigs and located in the provinces with the largest number of pig farms in South Korea. The optimal sampling numbers were calculated based on the expected prevalence and population size. In addition, a generalized estimating equation-based analysis was performed to manage potential farm/local-derived differences. However, this study included eleven swine farms in South Korea, which implies there is a possibility that this result may not reflect the national characteristics of ESBL/AmpC/MCR-EC, but regional characteristics. Further studies based on the national antimicrobial monitoring system by expanding the target farms and sampling size may help further describe the nationwide prevalence and characteristics of ESBL/AmpC/MCR-EC in swine farms in South Korea.

The present study could serve as a cornerstone for further studies, such as evaluation of antibiotic resistance transfer mechanism, potential for clonal expansion of MDR bacteria and other transfer properties of antimicrobial resistance variants. This study is expected to contribute to the improvement of antimicrobial resistance management strategies by presenting scientific evidence and epidemiological models for an in-depth approach to different antibiotic resistance in the livestock industry.

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국문초록

한국 돼지농장 유래 ESBL 생성 및 콜리스틴 내성 대장균의 분자역학과 내성 기전 연구

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3세대 세팔로스포린 및 콜리스틴 항생제는 인간의 다제내성세균 감염 질환 치료에 있어 최후의 항생제로 언급된다. 그러나 양돈산업에서는 이러한 항생제들이 돼지의 질병 치료 및 예방을 위하여 지속적으로 처방되어 왔고, 이러한 추세로 인해 돼지농장에서 ESBL 및 AmpC β-lactamase을 생성하는 대장균 (ESBL/AmpC 생성 대장균) 과 콜리스틴 내성 유전자인 mobilized colistin resistance gene (*mcr*)을 보유하는 대장균 (*mcr* 보유 대장균)의 분리율이 급격히 증가하였다.

돼지의 사육단계는 일령에 따라 이유자돈, 육성돈, 비육돈으로 나뉘며, 일반적으로 다른 사육단계의 돼지들은 분만사, 자돈사, 육성사, 비육사로 구분되어 사육된다. 돼지의 사육단계에 따라 다발하는 질병이 다르기 때문에 처방되는 항생제의 종류 및 양은 돼지 사육단계 따라 차이가 있으며, 이는 돼지의 사육단계별 항생제 내성균의 분포 및 특성을 다르게 하는 주된 요인으로 작용할 수 있다. ESBL/AmpC 생성 및 *mcr* 보유 대장균의 중요한 보균원인 돼지농장에서 이들 균주의 돼지의 사육단계별 분포 및 특성을 이해하는 것은 항생제 내성균을 제어하고 관리를 위한 중요한 초석으로 작용할 수 있다.

본 연구는 분자역학 및 내성 전달기전 분석을 기반으로 ESBL/AmpC 생성 및 *mcr* 보유 대장균에 대하여 돼지의 사육단계별 유병률과 특성의 차이를 분석하였다. 또한 공개 데이터베이스를 활용하여 사람, 돼지고기 등 다양한 유래 균주들과의 유전적 근연관계를 분석함으로써, 돼지농장 유래 균주의 공중보건학적 위해를 분석하고자 하였다. 마지막으로, 돼지농장 유래 균주들과 더불어 공개데이터베이스를 활용하여, 두 가지 주요 콜리스틴 내성 유전자인 *mcr-1.1* 및 *mcr-3.1*의 전달 기전에 대하여 비교유전체분석을 기반으로 분석하였다. 본 연구를 위하여 2017년 5월부터 2020년 3월까지 국내 돼지 농가수가 가장 많은 지역인 경기, 경북, 충남, 전남, 전북에 위치해 있는 11개의 돼지 농장을 대상으로 다단계 계층화 무작위 샘플링 (이유자돈, 육성돈, 비육돈, 임신모돈)을 실시하였고, 분리된 ESBL/AmpC 생산 및 *mcr* 보유 대장균이 분석에 포함되었다.

국내 양돈장에서의 ESBL/AmpC 생성 대장균의 유병률은 55.1%로 확인되었으며, 돼지의 사육단계별 균주의 유병률 및 특성이 다른 것으로 확인되었다. 이유자돈에서의 ESBL/AmpC 생성 대장균의 유병률은 86.3%로, 다른 사육단계 (육성돈 58.3%, 비육돈 48.4%, 임신모돈 43.1%)에서의 유병률과 비교하여 통계적으로 유의한 수준으로 높았다. 돼지의 사육단계별 비교에서 ESBL 생성 대장균은 모든 돼지 생산 단계에 분포해 있었으나, AmpC 생성 대장균은 육성돈 및 비육돈에서만 확인되었다. *K*-평균 군집 분석 기반 ESBL/AmpC 생성 대장균의 클론 분포 유사성 분석에서는, 같은 양돈장내 다른 돼지 생산 단계 유래 균주 간 높은 클론분포 유사성이 확인되었으며, 이는 농장내에서 사육단계간 교차 감염 가능성이 높음을 시사한다. 공개 데이터베이스(National Center for Biotechnology Information, NCBI)에 등록되어 있는 한국의 다양한 유래 균주와 비교분석 결과, 본 연구에서 분리된 돼지농장 유래 균주들은 인체, 돈육 유래 균주와 ESBL/AmpC 유형 및 클론유형을 공유하는 것이 확인되었으며, 특히 돼지농장 유래 균주 중 특히 ST101-B1, ST648-F, 그리고 ST457-

F 등 장외 병원성 대장균 클론 타입이 공유되는 것이 확인되었다. 이는 돼지농장 유래 다제내성 장외 병원성 대장균 균주가 도축장, 돈육 등의 식품유통경로를 통해 인간에게 전염될 수 있다는 간접적인 과학적 증거를 제시한다.

국내 돼지농장에서 *mcr-1* 보유 대장균의 가중 유병률은 8.4%였다. 다른 사육단계와 비교하여 이유기(13.0%)에서 가장 높은 유병률을 보였으며, 이유자돈 유래 균주는 다른 사육단계 유래 균주와 비교하여 다제내성률이 통계적으로 유의한 수준으로 높았다. 전장유전체기반 분석에서 다제내성 및 병원성 이점을 가진 *mcr-1* 보유 대장균이 농장 내 돼지 단계 간 공유되는 것이 확인되었다. 반면, NCBI에 등록되어 있는 한국의 사람, 돈육, 돼지농장에서 분리된 균주 간에는 클론 타입이 전혀 공유되지 않는 것으로 확인되었으며, 이는 *mcr-1*의 환경 간 전파에 있어서 클론전파가 상대적으로 낮은 영향력을 가지고 있음을 시사한다. 한편, *mcr-1* 보유 대장균은 장외 및 장내 대장균 병원성 유전자와 바이오필름 형성과 같은, 균주의 생존에 이점을 주는 병원성을 보유하고 있는 것으로 확인되었다. 이러한 병원성 이점은 food-chain 환경 등 생존에 불리한 환경에서 *mcr-1* 보유 대장균의 생존 가능성을 높일 수 있으며, 수평전이 등을 통해 *mcr-1*을 다른 병원성 박테리아 등에 전달하는 중요한 공급원 역할을 할 수 있도록 도울 수 있음을 암시한다.

*mcr-1.1*은 다른 내성 유전자나 삽입유전자 (Insertion sequence)이 없는 단순한 유전적 카세트 "*mcr-1.1-pap2*"를 기반으로 전달되었으며, 높은 수평전이빈도 (6.30 logCFU/ml)를 보였다. 이는 콜리스틴 내성 전파에 있어 *mcr-1.1*의 수평 전이가 주된 역할을 할 수 있음을 시사한다. 반면, *mcr-3.1*은 *mcr-1.1*와 비교하여 낮은 수평전이빈도 (0.97 logCFU/ml)를 보였으나, 다양한 항생제 및 중금속 내성 유전자 및 삽입유전자로 구성된 유전자 카세트의 형태로 전달되는 것이 확인되었다. 이는 *mcr-3.1*의 전파가

콜리스틴 내성뿐만 아니라 다제내성을 같이 전파함으로써 공중보건학적인 위해를 가져올 수 있음을 시사한다. 이 연구에서는 세계최초로 *mcr-3.1* 플라즈미드가 IS26을 매개로 하여 박테리아의 염색체 (Chromosome)에 통합될 수 있는 가능성을 보고하였다. 이 결과는 *mcr-3.1*가 수평 및 수직 전이를 통해 전달될 수 있음을 암시하며, *mcr-3.1*이 전세계적으로 전파될 수 있었던 성공이유 중 하나로 제안될 수 있다. 본 연구는 배양기법 기반 및 비교유전체분석을 통해 *mcr-1.1* 및 *mcr-3.1*에 의한 서로 다른 전달특성을 제시하였으며, 콜리스틴 내성을 제어하기 위하여 이러한 차이점을 고려한 적절한 전략의 필요성을 시사한다.

결론적으로, 본 논문은 돼지농장이 ESBL/AmpC 생성 및 *mcr* 보유 대장균의 중요한 보균원이며, 이들 균주가 food-chain을 통해 사람에게 전달되어 공중보건학적 위해가 될 수 있는 가능성에 대한 간접적인 과학적인 증거를 제시하였다. 돼지의 사육 단계에 따라 ESBL/AmpC 생성 및 *mcr* 보유 대장균의 유병률과 특성이 다르다는 것을 제시하였으며, 이들 균주의 중요한 저장소인 돼지농장에서 돼지의 사육단계별 다른 균주 특성을 고려한 다단계 체계적 전략의 필요성을 강조하였다. 또한 돼지 농장내에서 사육단계 간 ESBL/AmpC 생성 및 *mcr* 보유 대장균의 교차오염 가능성이 높음을 제시하였으며, 이러한 다제내성균을 제어하기 위하여 농장 내 교차오염을 줄이기 위한 노력이 필수적임을 시사하였다. 본 연구는 축산업계의 다양한 항생제 내성에 대한 심층적 접근을 위한 과학적 근거와 역학 모델을 제시함으로써 항생제 내성 관리 전략의 개선에 기여할 것으로 기대된다.

키워드 : ESBL β-lactamase, AmpC β-lactamase, *mcr,* 돼지농장, 돼지사육단계, 전장유전체, 비교유전체

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