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Molecular characteristics of ESBL-producing *Escherichia coli* isolated from chickens with colibacillosis

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ABSTRACT

Background: Avian pathogenic *Escherichia coli* (APEC) causes colibacillosis, resulting in significant economic losses in the poultry industry.
Objectives: In this study, the molecular characteristics of two extended-spectrum beta-lactamase (ESBL)-producing APEC isolates were compared with previously reported ESBL-producing *E. coli* isolates.
Methods: The molecular characteristics of *E. coli* isolates and the genetic environments of the ESBL genes were investigated using whole genome sequencing.
Results: The two ESBL-producing APEC were classified into the phylogenetic groups C and B1 and ST410 and ST162, respectively. Moreover, the ESBL genes of the two isolates were harbored in different Inc plasmids. The EC1809182 strain, harboring the $bla_{\text{CTX-M-55}}$ gene on the plasmid, exhibited extensive homology to IncFIB (98.4%) and IncFIC(FII) (95.8%). The EC1809191 strain, harboring the $bla_{\text{CTX-M-1}}$ gene, was homologous to IncI1-I (Gamma) (99.3%). All chromosomes carried the multidrug transporter, *mdf(A)* gene. Mobile genetic elements, adjacent to CTX-M genes, facilitated the dissemination of genes in the two isolates, analogous to other ESBL-producing *E. coli* isolates.
Conclusions: This study clarifies the transmission dynamics of CTX-M genes and supports strengthened surveillance to prevent the transmission of the antimicrobial-resistant genes to humans via the food chain.

Keywords: *Escherichia coli*; *Escherichia coli* Infections; beta-lactamase CTX-M, *E coli*; beta-lactam resistance

INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) is associated with colibacillosis, resulting in significant economic losses in the poultry industry [1]. In Korea, APEC infections in broiler chickens have been continuously reported nationwide. Antimicrobial drugs, such as β-lactams, aminoglycosides, and fluoroquinolones, are used to treat colibacillosis outbreaks [2,3]. In particular, β-lactam antimicrobials are widely used for the treatment of bacterial infections in both humans and animals, leading to the emergence of extended-spectrum β-lactamase (ESBL)-producing APEC worldwide [4,5]. ESBL-producing *E. coli*

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Conflict of Interest
The authors declare no conflicts of interest.

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isolated from food animals are considered a public health problem. The antimicrobial-resistant determinants may be transferred to human bacteria, leading to third-generation cephalosporin resistance. The third-generation cephalosporins are classified as "critically important in human medicine" by the World Health Organization [6].

Mobile genetic elements, such as plasmids and transposons, play important roles in the transmission of antimicrobial-resistant genes and virulence factors [7]. In particular, the IncI1 plasmids, which have been identified in animals and humans, are associated with the dissemination of ESBL genes [8]. Recently, whole genome sequencing (WGS) has been used for surveillance and in-depth analyses of ESBL-producing *E. coli* to elucidate the genetic relationship with other bacteria [7,9]. In this study, we investigated the molecular characteristics of ESBL-producing APEC using WGS and the genetic environment of the ESBL genes in the newly isolated APEC were compared with previously reported ESBL-producing *E. coli* isolates.

MATERIALS AND METHODS

Strains

Seventy-nine *E. coli* isolates collected from 60 broiler farms suffering from colibacillosis nationwide in 2018 were previously described [10]. The phenotype confirmation for ESBLs in *E. coli* was performed using the disk diffusion method with ceftazidime (30 μg), ceftazidime-clavulanate (30/10 μg), cefotaxime (30 μg), and cefotaxime-clavulanate (30/10 μg). Isolates having differential zone diameters ≥ 5 mm in combination with clavulanate compared to ceftazidime and cefotaxime alone (e.g., ceftazidime zone = 16; ceftazidime-clavulanate zone = 21) were considered phenotypic ESBL positive [11]. The ESBL-encoding genes, $bla_{\text{CTX-M}}$ [12], bla_{TEM}, bla_{SHV}, and bla_{OXA} [13] were examined by polymerase chain reaction amplification. Subsequently, two ESBL-producing *E. coli*, named EC1809182 (GenBank accession number: JAFBIE000000000) and EC180918 (GenBank accession number JAFBIF000000000), carrying the CTX-M gene were analyzed for this study.

WGS and analysis

Genomic DNA was extracted using the MasterPure™ DNA purification kit (Lucigen, USA) following the manufacturer's instructions. Genome sequencing was performed using an Illumina HiSeq platform according to standard Illumina protocols at Macrogen (Korea). The reads were *de novo* assembled using the hierarchical genome assembly process 3 (HGAP3). Genome annotation was performed using Rapid Annotation by Subsystem Technology version 2.0. The assembled genomes were initially screened for genes encoding antimicrobial resistance, virulence, multilocus sequence typing (MLST), and plasmid replicons using the *in silico* genomic tools, ResFinder 3.1, VirulenceFinder 1.5, MLST 2.0, and PlasmidFinder 2.1, respectively. These tools are available through the Center for Genomic Epidemiology (http://www.genomicepidemiology.org). All representative sequences for the known $bla_{\text{CTX-M}}$ genes were obtained from GenBank and compared with the two strains in this study using the BLAST algorithm. SnapGene software (GSL Biotech LLC, USA) was employed for the visualization of contigs harboring $bla_{\text{CTX-M}}$ genes.

RESULTS

Genomic features of ESBL-producing *E. coli* strains

The genomic features of two ESBL-producing *E. coli* isolated from chickens with colibacillosis are summarized in **Table 1**. The genomic sizes of the ESBL-producing EC1809182 and EC180918 ranged from 5.05 to 5.35 Mb and the GC contents were 50.0% and 50.6%, respectively. A total of 5,105 and 5,413 coding sequences, 89 and 87 tRNAs, and 22 rRNAs each were assigned. The two strains were classified into the phylogenetic groups C and B1 and ST410 and ST162 based on the *bla*ESBL genes, CTX-M-55 and CTX-M-1, respectively.

Distribution of virulence genes of ESBL-producing *E. coli* strains

The virulence genes conserved in the two ESBL-producing *E. coli* are listed in **Table 2**. The *cma, gad, lpfA,* and *iss* genes were found in both strains. The *iha, iroN, mchB, mchC*, and *mchF* genes were only found in EC1809191.

Genetic characteristics of ESBL-producing *E. coli* strains

Genetic characteristics and comparative analysis of the two ESBL-producing *E. coli* are shown in **Table 3**. The two genomes were comprised of 3 contigs each. All chromosomes carried the multidrug transporter, *mdf(A)* gene, but only the EC1809191 chromosome carried the sulfonamide resistance gene, *sul2*. The ESBL genes from the two isolates were harbored in different Inc plasmid groups. The EC1809182 strain, harboring the $bla_{\text{CTX-M-55}}$ gene on the plasmid, was homologous to IncFIB (98.4%) and IncFIC(FII) (95.8%). The EC1809191 strain harboring the $bla_{\text{CTX-M-1}}$ gene showed high identity with IncI1-I (Gamma) (99.3%). Both strains harbored antimicrobial-resistant genes for aminoglycoside (*aph(3′)-Ia*), tetracyclines (*tet(A)*), trimethoprim (*dfrA14*), and sulfonamide (*sul3* and *sul2*, respectively) on IncFIB and IncFIC group plasmids. EC1809182 harbored only one resistance gene for aminoglycosides (*aadA1*) but EC1809191 harbored three aminoglycosides resistance genes (*aac(3)-Ild, aph(3″)-Ib*, and *aph(6)-Id*), and one β-lactam resistance gene ($bla_{\text{TEM-1B}}$) on the IncFIB and IncFIC plasmid

Table 1. Genome characteristics of ESBL-producing *E. coli* isolated from colibacillosis

Strain	EC1809182	EC1809191
Genome (Mb)	5.05	5.35
%GC	50.5	50.6
No. of contigs	3	3
No. of CDS	5,105	5,413
No. of tRNA	89	87
No. of rRNAs	22	22
Phylogenetic group	C	B1
Multilocus sequence type	410	162
$bla_{\text{CTX-M}}$ gene	CTX-M-55	CTX-M-1

ESBL, extended-spectrum β-lactamase.

Table 2. Presence of virulence genes carried on ESBL-producing *E. coli* isolated from colibacillosis

Gene	Product/Function (predicted phenotype)	EC1809182	EC1809191
cma	colicin M	+	+
gad	Glutamate decarboxylase	+	+
iha	Adherence protein	–	+
lpfA	long polar fimbriae	+	+
iroN	Enterobactin siderophore receptor protein	–	+
iss	Increased serum survival	+	+
mchB	microcin H47 part of colicin H	–	+
mchC	mchC protein	–	+
mchF	ABC transporter protein MchF	–	+

ESBL, extended-spectrum β-lactamase.

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Table 3. Molecular characteristics of ESBL-producing *E. coli* isolated from colibacillosis

Strain	Contig name	Size (bp)	GenBank accession No.	Inc group		Resistance genes	Resistance found (disk diffusion test)
				Name	Identity (%)		
EC1809182	Chromosome21	4,864,566	EC18091821	-	-	*mdf(A)*	AM, C, CF, CIP, CL, CTX, CXM, CZ, GM, NA, SXT, TE
	pEC1809182-1	101,452	AP18091821	IncFIB	98.4	***bla*$_{CTX-M-55}$**, *aadA1 aph(3')-Ia*, *dfrA14*, *sul3*, *tet(A)*	
				IncFIC (FII)	95.8		
	pEC1809182-2	82,627	AP00514722	IncI1-I (Gamma)	100	-	
EC1809191	Chromosome11	5,077,215	EC18091911	-	-	*mdf(A)*, *sul2*	AM, AMC, CF, CIP, CL, CTX, CXM, CZ, NA, SXT, TE
	pEC1809191-1	173,389	AP18091811	IncFIB	98.4	***bla*$_{TEM-1B}$**, *aph(3')-Ia aac(3)-IId*, *aph(3")-Ib*, *aph(6)-Id*, *dfrA14*, *sul2*, *tet(A)*	
				IncFIC (FII)	95.8		
	pEC1809191-2	104,390	AP00514712	IncI1-I (Gamma)	99.3	***bla*$_{CTX-M-1}$**, *floR*, *sul2*	

ESBL genes are presented in bold.
ESBL, extended-spectrum β-lactamase; AM, ampicillin; AMC, amoxicillin/clavulanate; C, chloramphenicol; CF, cefalotin; CIP, ciprofloxacin; CL, colistin; CTX, cefotaxime; CXM, cefuroxime; CZ, cefazolin; NA, nalidixic acid; SXT, trimethoprim/sulfamethoxazole; TE, tetracycline.

groups. EC1809191 also harbored the resistance genes for phenicol (*floR*) and sulfonamide (*sul2*) on IncI1-I (Gamma).

Genetic environment of the *bla*ESBL gene in ESBL-producing *E. coli* strains

The genetic map of the *bla*ESBL genes for the two ESBL-producing *E. coli* and other closely related strains are shown in **Fig. 1**. In EC1809182, genes encoding for the IncI plasmid conjugative transfer proteins, tryptophan synthase, mobile elements, and shufflon-specific DNA recombinase were located near $bla_{CTX-M-55}$. In EC1809191, the tryptophan synthase gene was located adjacent to $bla_{CTX-M-1}$ and mobile element genes, analogous to other strains harboring $bla_{CTX-M-1,}$ were detected.

DISCUSSION

CTX-M is one of the most common β-lactamase genes [14] and ESBL-producing *E. coli* strains carrying the CTX-M gene have been reported not only in food animals like chickens but also in humans [14,15]. In particular, the CTX-M-1 and CTX-M-55 genes are prevalent genotypes in patients from Asian countries [14,16]. These genes have also been continuously reported in food animals, such as chicken and pork [17,18]. In this study, two ESBL-producing *E. coli* isolated from chickens with colibacillosis also carried CTX-M-1 and CTX-M-55. To understand the molecular features associated with pathogenicity, these two *E. coli* strains were compared with other *E. coli* strains harboring CTX-M using WGS analysis. A mobile element gene was identified in the downstream region of the CTX-M genes in the two *E. coli* strains; the same findings were observed in most of the *E. coli* strains harboring CTX-M-1 and CTX-M-55 genes [15]. Mobile genetic elements are associated with the capture and spread of genes related to antimicrobial resistance and virulence. The mobile genetic units facilitate the transfer of genes from one genetic location to another in the same cell or other cells [19]. The mobile element gene in the downstream region of CTX-M genes, as shown in this study, may play an important role in the spread of antimicrobial-resistant genes, such as CTX-M-1 and CTX-M-55 [20].

The CTX-M and other antimicrobial-resistant genes are associated with plasmids, such as IncF and IncI1-I, which are capable of disseminating antimicrobial-resistant genes among *Enterobacteriaceae* [8,21]. In particular, IncF is the most common plasmid type detected in *E. coli* from humans and food animals [22]. In this study, the CTX-M-55 gene in EC1809182 was located on the IncFIB plasmid group with the *aadA1* gene; other resistance genes, such as

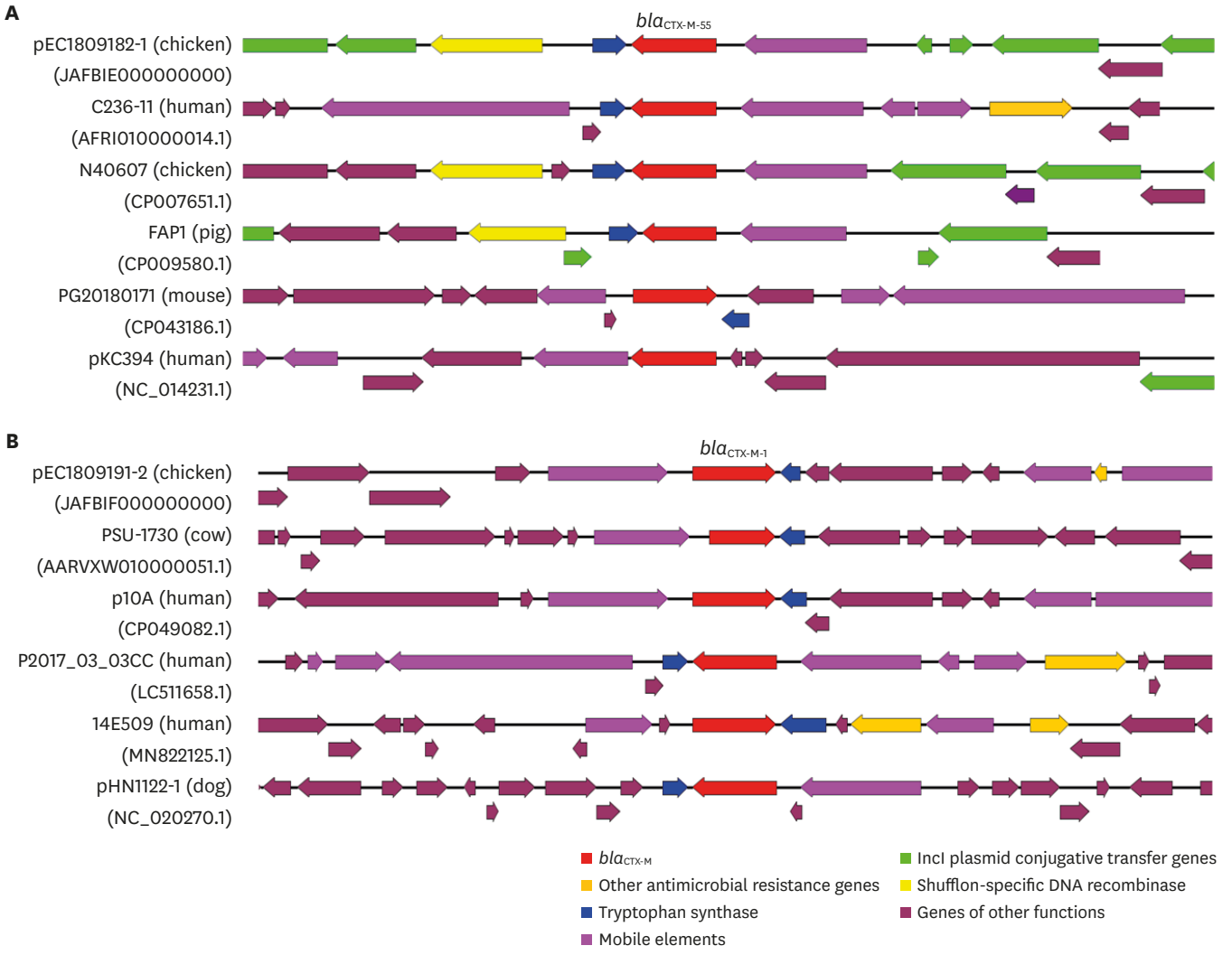

Fig. 1. Genetic environment of $bla_{CTX\text{-}M\text{-}55}$ in the EC1809182 (A) genome, $bla_{CTX\text{-}M\text{-}1}$ in the EC1809191 (B) genome and other closely related strains. GenBank accession numbers of the contigs harboring $bla_{CTX\text{-}M}$ were given below the strain names.

aph(3′)-Ia, dfrA14, sul3, and *tet(A)* genes, were located on the IncFIC(FII) plasmid. Although the CTX-M-1 gene of EC1809191 was on the IncI1-I plasmid with the *floR* and *sul2* genes, another resistance gene, TEM-1, which conveys resistance to penicillin, ampicillin, and first-generation cephalosporins, was located on the IncFIB plasmid group with the *aph(3′)-Ia* gene. Resistance genes, such as *aac(3)-Ild, aph(3″)-Ib, aph(6)-Id, dfrA14, sul2,* and *tet(A),* were on the IncFIC(FII) plasmid. Therefore, both isolates are potential reservoirs for transmission of antimicrobial resistance via human consumption, as previously described [23].

In this study, the *sul2* gene of EC1809191 was located on the IncI1-I plasmid, but also on the chromosome. This may be due to the two-stage homologous recombination process previously described [19]. When two copies of the same insertion sequence (IS) elements flank the antimicrobial-resistant gene, direct or inverted repeat gene arrangements can migrate to another site where the IS element is also found. A copy of the repeated gene can be released as a circular, double-stranded transposon and the released DNA can be rescued at the new site creating the new composite transposon.

Virulence factors contribute to different environmental adaptations and enable sharing of various genes, such as virulence determinants and antimicrobial-resistant genes [24,25]. In this study, both isolates carried genes related to cytotoxicity (*cma*), colonization (*lpfA*), glutamate decarboxylase (*gad*), and increased serum survival (*iss*). One isolate also carried genes related to adherence (*iha*), enterobactin siderophore receptor protein (*iroN*), microcin H47 part of colicin H (*mchB*), mchC protein (*mchC*), and the ABC transporter protein MchF (*mcfF*). These virulence factors do not necessarily induce colibacillosis, but can facilitate pathogenicity. Moreover, *E. coli* isolates from avian colibacillosis showed a variety of virulence factors that impede the differentiation between pathogenic and non-pathogenic strains [26]. Since the pathogenic mechanisms of colibacillosis are poorly understood, further investigation into the pathophysiology of *E. coli* from colibacillosis is needed to understand the disease [26].

In the multilocus sequence type, many researchers reported that *E. coli* ST410 was associated with both humans and animals [27,28] and various antimicrobial-resistant genes, such as bla_{NDM-5}, bla_{OXA-1}, $bla_{CTX-M-15}$, bla_{CMY-2}, *aac(3)-IIa,* and *aac(6′)-Ib-cr* and various virulence genes were conserved [27]. Roer et al. [28] reported that the *E. coli* ST410 strains are emerging high-risk clones, since they have been reported over the past two decades in Europe, showing accumulated multidrug resistance. Both *E. coli* ST162 and ST410 with ESBL genes were detected in ready-to-eat food in Ecuador [29]. The dissemination of antimicrobial-resistant genes in multiple clonal lineages, such as ST162 and ST410, is a public health problem because they are globally distributed with various antimicrobial-resistant genes and are easily transmitted between hosts [28,30].

The present study clarifies the transmission dynamics of CTX-M genes. Moreover, continuous monitoring of ESBL-producing bacteria from food animals, which pose a potential risk to public health, is necessary to surveil the transmission to humans through the food chain.

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