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

Sunghyun Yoon 1,2, Young Ju Lee 1,*

1College of Veterinary Medicine & Zoonoses Research Institute, Kyungpook National University, Daegu 41566, Korea
2Division of Microbiology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, USA

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 \( \text{bla}_{CTX-M-55} \) gene on the plasmid, exhibited extensive homology to IncFIB (98.4%) and IncFIC(FII) (95.8%). The EC1809191 strain, harboring the \( \text{bla}_{CTX-M-1} \) gene, was homologous to IncI1-I (Gamma) (99.3%). All chromosomes carried the multidrug transporter, \( \text{mdf}(A) \) 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 \( \beta \)-lactams, aminoglycosides, and fluoroquinolones, are used to treat colibacillosis outbreaks [2,3]. In particular, \( \beta \)-lactam antimicrobials are widely used for the treatment of bacterial infections in both humans and animals, leading to the emergence of extended-spectrum \( \beta \)-lactamase (ESBL)-producing APEC worldwide [4,5]. ESBL-producing E. coli

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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, blaCTX-M [12], blaTEM, blaSHV, and blaOXA [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 replications 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 blaCTX-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 blaCTX-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 *blaCTX-M-55* gene on the plasmid, was homologous to IncFIB (98.4%) and IncFIC(FII) (95.8%). The EC1809191 strain harboring the *blaCTX-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 (*blaTEM-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 |
| *blaCTX-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.
The genetic map of the blaESBL genes for the two ESBL-producing E. coli and other closely related strains are shown in Fig. 1. In EC1809182, genes encoding for the IncF plasmid conjugative transfer proteins, tryptophan synthase, mobile elements, and shufflon-specific DNA recombinase were located near blaCTX-M-55. In EC1809191, the tryptophan synthase gene was located adjacent to blaCTX-M-1 and mobile element genes, analogous to other strains harboring blaCTX-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 IncI-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

| Strain | Contig name | Size (bp) | Genbank accession No. | Inc group | Resistance genes | Resistance found (disk diffusion test) |
|--------|-------------|-----------|-----------------------|-----------|------------------|---------------------------------------|
| EC1809182 Chromosome | - | 4,864,566 | EC1809182 | - | mdf(A) | AM, C, CF, CIP, CL, CTX, CXM, CZ, GM, NA, SXT, TE |
| pEC1809182-1 | IncFIB | 101,452 | AP1809181 | IncFIB | blaCTX-M-55, aadA1 aph(3′)-Ia, dfrA14, sul3, tet(A) | AM, C, CF, CIP, CL, CTX, CXM, CZ, GM, NA, SXT, TE |
| pEC1809182-2 | Incl-I (Gamma) | 82,627 | AP00514722 | Incl-I (Gamma) | 100 | - |
| EC1809191 Chromosome | - | 5,077,215 | EC1809191 | - | mdf(A), sul2 | AM, AMC, CF, CIP, CL, CTX, CXM, CZ, NA, SXT, TE |
| pEC1809191-1 | IncFIB | 173,389 | AP18091811 | IncFIB | blaTEM-1B, aph(3′)-Ia aac(3)-Id, aph(3′)-lb, aph(6)-Id, dfrA14, sul2, tet(A) | AM, AMC, CF, CIP, CL, CTX, CXM, CZ, NA, SXT, TE |
| pEC1809191-2 | Incl-I (Gamma) | 104,390 | AP00514712 | Incl-I (Gamma) | 99.3 | blaCTX-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.
ESBL-producing *E. coli* from chickens

Some resistance genes, such as *aac(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)-IId, 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.

**Fig. 1.** Genetic environment of *bla*<sub>CTX-M</sub>-1 in the EC1809182 (A) genome, *bla*<sub>CTX-M</sub>-55 in the EC1809191 (B) genome and other closely related strains. GenBank accession numbers of the contigs harboring *bla*<sub>CTX-M</sub> were given below the strain names.
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 blaNDM-5, blaOX A-1, blaCTX-M-15, 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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