Comparison of antimicrobial resistance and molecular characterization of *Escherichia coli* isolates from layer breeder farms in Korea

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**ABSTRACT** In Korea, 4 big layer companies that possess one grandparent and 3 parent stocks are in charge of 100% of the layer chicken industry. In this study, we investigated the antimicrobial resistance of commensal 578 *E. coli* isolated from 20 flocks of 4-layer breeder farms (A, B, C, and D), moreover, compared the characteristics of their resistance and virulence genes. Isolates from farms B and D showed significantly higher resistance to the *β*-lactam antimicrobials (amoxicillin, ampicillin, and 1st-, 2nd-, and 3rd-generation cephalosporins). However, resistance to ciprofloxacin, nalidixic acid, and tetracycline was significantly higher in the isolates from farm A (*P* < 0.05). Interestingly, the isolates from farm C showed significantly lower resistance to most antimicrobials tested in this study. The isolates from farms B, C, and D showed the high multiple resistance to the 3 antimicrobial classes. Furthermore, the isolates from farm A showed the highest multiple resistance against the 5 classes. Among the 412 *β*-lactam-resistant isolates, 123 (29.9%) carried *bla*TEM-1, but the distribution was significantly different among the farms from 17.5% to 51.4% (*P* < 0.05). Similarly, the most prevalent tetracycline resistance gene in the isolates from farms B, C, and D was *tet*A (50.0–77.0%); however, the isolates from farm A showed the highest prevalence in *tet*B (70.6%). The distribution of quinolone (*qnr*B, *qnr*D, and *qnr*S) and sulfonamide (*su*12)-resistant genes were also significantly different among the farms but that of chloramphenicol (*cat*A1)- and aminoglycoside (*aac* [3]*-II*, and *aac* [6]*-Ib*)-resistant genes possessed no significant difference among the farms. Moreover, the isolates from farm C showed significantly higher prevalence in virulence genes (*ira*N, *omp*T, *hly*F, and *iss*) than the other 3 farms (*P* < 0.05). Furthermore, the phenotypic and genotypic characteristics of *E. coli* isolates were significantly different among the farms, and improved management protocols are required to control of horizontal and vertical transmission of avian disease, including the dissemination of resistant bacteria in breeder flocks.

**Key words:** *Escherichia coli*, layer parent stock, antimicrobial resistance

**INTRODUCTION**

Layer breeder, which is for the purpose of producing of egg laying poultry birds, plays an important role as a reservoir because they can transfer various pathogens, antimicrobial genes, and virulence factors to commercial chickens and eggs via hatcheries and feces (Dierikx et al., 2013; Seo et al., 2019a). Therefore, breeder management systems should not only meet the basic health of the flocks but also control the transmission of potential hazards through the pyramidal structure of the egg production chain. In particular, the use of antimicrobials can help control the spread of infectious pathogens, but antimicrobial resistance at the top of the pyramidal structure poses a major public health concern because it can be widely disseminated throughout the layer-production industry. Finally, these pathogens are ultimately transmitted to humans via commercial egg consumption in the final stage.

*Escherichia coli* (*E. coli*) are common bacteria in the environment, foods, and intestines of people and animals. They are mostly harmless, except for some kind of strain, which have acquired virulent attributes that cause intestinal and extraintestinal diseases (Le Gall et al., 2007; Tenaillon et al., 2010). However, *E. coli* possesses a highly developed ability to acquire resistance
genes from the same or different species via horizontal gene transfer of mobile genetic elements such as plasmids (Poirel et al., 2018). Therefore, antimicrobial resistance in E. coli has continuously been reported in humans and food-producing animals worldwide (Alloccati et al., 2013; Krizman et al., 2017; Abo-Amer et al., 2018; Terentjeva et al., 2019).

In Korea, various antimicrobials are widely used for treating bacterial infection in layer parent flocks and a few studies have investigated the resistance to some antimicrobial classes in E. coli recovered from layer breeder farms (Kim et al., 2019; Seo et al., 2019a,b). In particular, Seo et al. (2019a) reported that 3rd-generation cephalosporin-resistant and ESBL/pAmpC-producing isolates were found at each step of the layer-production pyramid in Korea. Therefore, in this study, we compared the antimicrobial resistance and genetic characteristics of commensal E. coli from layer breeder farms and attempted to propose necessity of improved management processes at the breeder level.

**MATERIALS AND METHODS**

**Bacterial Isolates**

Feces and dust samples from 20 flocks of 4-layer breeder farms from different geographic locations in Korea were collected once when the chickens were 20 wk of age (Seo et al., 2018). According to the standard set of Processing and Ingredients Specification of Livestock Products by the Ministry of Food and Drug Safety (2018), 10 g of dust were swabbed using sterile gauze moistened with sterile double strength skim milk (Fluka, Neu-Ulm, Germany). Then, approximately 10 g of feces were sampled from 15 locations of the flock house. All samples were individually inoculated in 225 mL of mEC (Merck, Darmstadt, Germany), and incubated at 37°C for 20 to 24 h. Subsequently, suspicious colonies were selected after streaking on MacConkey agar (BD Biosciences, San Jose, CA) from pre-enriched mEC broth, and were identified as E. coli by using PCR as previously described (Candrian et al., 1991). After antimicrobial susceptibility tests, if isolates from the same sample showed identical antimicrobial resistance patterns, only 1 isolate was chosen randomly. Finally, 578 E. coli isolates, including 314 isolates from the same sample showed identical antimicrobial resistance patterns, only 1 isolate was chosen randomly. Finally, 578 E. coli isolates, including 314 isolates originated from dust and 264 isolates from feces were tested in this study.

**Antimicrobial Susceptibility Test**

The antimicrobial susceptibility of all E. coli isolates was investigated through the disk diffusion method and the results were interpreted following the Clinical and Laboratory Standards Institute CLSI, 2018. The antimicrobial agents (BD Biosciences) used in this study were as follows: amoxicillin–clavulenate (AMC, 20/10 μg), ampicillin (AM, 10 μg), cefazolin (30 μg), cefepime (30 μg), cefotaxime (CTX, 30 μg), cefoxitin (30 μg), ceftriaxone (30 μg), cefuroxime (30 μg), cephalexin (30 μg), cefalothin (CF, 30 μg), chloramphenicol (30 μg), ciprofloxacin (CIP, 5 μg), gentamicin (10 μg), nalidixic acid (NA, 30 μg), tetracycline (TE, 30 μg), and trimethoprim–sulfamethoxazole (1.25/23.75 μg). In this study, E. coli ATCC 25922 served as a quality control strain. Isolates showing resistance to more than 3 antimicrobial classes were proposed to have multidrug resistance (MDR), as described by Magiorakos et al. (2012).

**Detection of Antimicrobial Resistance and Virulence Genes**

PCR amplification was conducted to detect antimicrobial resistance genes. Genes for β-lactamases were blaTEM (Dallenne et al., 2010), blaSHV (Briñàs et al., 2002), blaOXA (Briñàs et al., 2002), and blaCTX-M (Pitout et al., 2004). All amplicons were sequenced with an automatic sequencer (Cosmogenetech, Daejeon, Korea), followed by the sequence confirmation using Basic Local Alignment Search Tool program through the National Center for Biotechnology Information website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Moreover, other genes conferring resistance to each antimicrobial class were as follows: tetA, tetB, and tetC for tetracycline (Segelov et al., 2003); aac (6’)-Ib, aac (3)-II, and ant (2’)-I for aminoglycosides (Sandvang and Aarestrup, 2000; Jiang et al., 2008); qnrA, qnrB, qnrS, and qepA for quinolones (Wang et al., 2003; Cavaco et al., 2007; Jiang et al., 2008; Minarini et al., 2008); sul1 and sul2 for sulfonamides (Sandvang et al., 1998; Maynard et al., 2003); and catA1 and cmlA for chloramphenicol (Van et al., 2008). PCR was also used to confirm the 5 virulence genes: ivoN, ompT, hlyF, iss, and stx1, as previously described (Johnson et al., 2008; Kagambèga et al., 2012).

**Statistical Analysis**

Data were statistically analyzed using the SPSS 25 program (IBM Corp., Armonk, NY). Then, the significant difference (P < 0.05) in the distribution of antimicrobial resistance and virulence genes among farms was examined with the Pearson’s chi-square test. Post hoc Bonferroni correction was also performed for further multiple tests.

**RESULTS**

**Antimicrobial Resistance Analysis**

The antimicrobial resistance profile of E. coli isolates from 4-layer breeder farms is shown in Table 1. A total of 578 E. coli showed the highest resistance to CF (56.2%), followed by AM (40.7%), TE (37.4%), NA (31.7%), cefazolin (23.2%), cefepime (22.7%), and AMC (21.1%). In particular, the isolates from farms B and D showed high resistance to the β-lactam antimicrobials. Furthermore, the isolates from farm B showed a significantly higher resistance to AMC (45.9%), AM (73.0%), 1st-generation
The distribution of MDR prevalence

The distribution of MDR in E. coli isolates from the 4-layer breeder farms is shown in Figure 1. The prevalence of MDR isolates was the highest in the isolates from farm B (64.9%), followed by that from farms A (47.8%), D (41.3%), and C (38.3%; P < 0.05). However, the isolates from farms B, C, and D showed a higher prevalence of the 3 antimicrobial classes (37.8, 16.7, and 23.1%, respectively). In contrast, the isolates from farm A showed the highest resistance to the 5 classes (22.8%). Moreover, resistance to more than 7 classes was the highest in the isolates from farm B (5.4%).

Distribution of Antimicrobial Resistance Genes

The distribution of resistance genes in E. coli isolates showing resistance to each antimicrobial class is shown in Table 2. Among the 412 β-lactam-resistant isolates, 123 (29.9%) carried blaTEM-1, but the distribution was significantly different among farms from 17.5 to 51.4% (P < 0.05). Furthermore, the blaSHV, blaOXA, and blaCTX-M were not detected in any isolate. Moreover, the prevalences of tetA and tetB among the 216 tetracycline-resistant isolates were 55.1 and 31.9%, respectively, which showed significant differences among the farms (P < 0.05). Interestingly, the most prevalent TE resistance gene in the isolates from farms B, C, and D was tetA (50.0–77.0%), followed by tetB (0–17.3%). In contrast, the isolates from farm A showed the highest prevalence in tetB (70.6%), followed by tetA (25.0%). The tetC gene was detected only in 3 (1.4%) isolates. Among the 183 quinolone-resistant isolates, the prevalences of qnrA, qnrB, qnrD, and qnrS genes were 19.1, 6.6, 1.6, and 6.6%, respectively. However, the distribution of qnrB, qnrD, and qnrS was significantly different among the farms from 0 to 15.4%, 0 to 23.1%, and 0 to 26.8%, respectively (P < 0.05). Additionally, the qepA gene was detected only in 1 isolate from farm C. Furthermore, the prevalence of sul1 and sul2 genes among the 79 sulfonamide-resistant isolates was 22.8 and 24.1%, respectively, but the distribution of the sul12 gene only showed significant differences among the farms from 20.3 to 50.0% (P < 0.05). Additionally, 8 (23.5%) of the 34 chloramphenicol-resistant isolates carried the catA1 gene without significant differences among the farms; however, cmlA was not detected in any isolate. Finally, 10 (33.3%) and 1 (3.3%) of the 30 gentamicin-resistant isolates carried aac (3)-II gene and aac (6)-Ib genes, respectively, without significant differences among farms, but ant (2')-I gene was also not detected in any isolate.
Figure 1. Distribution of multidrug resistant E. coli isolates from 4 layer breeder farms. Different subscript letters (A, B) indicates statistical difference ($P < 0.05$).

Table 2. Distribution of antimicrobial resistance genes in E. coli isolates showing resistance against each antimicrobial class.

| Antimicrobial class (No. of isolates showing resistance to each class) | Antimicrobial resistance gene | No. of isolates carrying each gene/No. of antimicrobial resistant isolates (%) | Farm A | Farm B | Farm C | Farm D | Total |
|---|---|---|---|---|---|---|---|
| β-lactams (412) | blaTEM-1 | 44/87 (50.6)a | 19/37 (51.4)a | 35/145 (24.1)b | 25/143 (17.5)b | 123/412 (29.9)A |
|  | blaSHV | 0/87 (0.0) | 0/37 (0.0) | 0/145 (0.0) | 0/143 (0.0) | 0/412 (0.0)B |
|  | blaOXA | 0/87 (0.0) | 0/37 (0.0) | 0/145 (0.0) | 0/143 (0.0) | 0/412 (0.0)B |
|  | blaCTX-M | 0/87 (0.0) | 0/37 (0.0) | 0/145 (0.0) | 0/143 (0.0) | 0/412 (0.0)B |
| Tetracyclines (216) | tetA | 17/68 (25.0)b | 6/12 (50.0)a,b | 47/61 (77.0)a | 49/75 (65.3)a | 119/216 (55.1)A |
|  | tetB | 48/68 (70.6)a | 0/12 (0.0)b | 8/61 (13.1)b | 13/75 (17.3)b | 69/216 (31.9)B |
|  | tetC | 1/68 (1.5) | 0/12 (0.0) | 2/61 (3.3) | 0/75 (0.0) | 3/216 (1.4)C |
| Quinolone (183) | qnrA | 17/83 (20.5) | 1/13 (7.7) | 6/41 (14.6) | 11/46 (23.9) | 35/183 (19.1)A |
|  | qnrB | 9/83 (10.8)a,b | 2/13 (15.4)a | 1/41 (2.4)b | 0/46 (0.0)b | 12/183 (6.6)B |
|  | qnrD | 0/83 (0.0)a | 3/13 (23.1)a,b | 0/41 (0.0)b | 0/46 (0.0)b | 3/183 (1.6)B,C |
|  | qnrS | 1/83 (1.2)b | 0/13 (0.0)b | 11/41 (26.8)a | 0/46 (0.0)b | 12/183 (6.6)B |
|  | qepA | 0/83 (0.0) | 0/13 (0.0) | 1/21 (2.1) | 0/46 (0.0) | 1/183 (0.5)C |
| Sulfonamide (79) | sulI | 5/12 (41.7) | 2/4 (50.0) | 9/59 (15.3) | 2/50 (0.0) | 18/79 (22.8) |
|  | sulB | 4/12 (33.3)a,b | 2/4 (50.0)a | 12/59 (20.3)b | 1/25 (4.0)b | 19/79 (24.1) |
| Phenolics (34) | catA1 | 2/4 (50.0)a | 2/3 (100.0)a | 4/9 (44.4)a | 0/18 (0.0)b | 8/34 (23.5)A |
|  | catB | 0/4 (0.0) | 0/3 (0.0) | 0/9 (0.0) | 0/18 (0.0) | 0/34 (0.0)B |
| Aminoglycosides (30) | aac(6’)-Ib | 0/4 (0.0) | 0/2 (0.0) | 1/15 (6.7) | 0/9 (0.0) | 1/30 (3.3)B |
|  | aac(3)-IId | 2/4 (50.0) | 2/2 (100.0) | 5/15 (33.3) | 1/9 (11.1) | 10/30 (33.3)A |
|  | ant(2’)-I | 0/4 (0.0) | 0/2 (0.0) | 0/15 (0.0) | 0/9 (0.0) | 0/30 (0.0)B |

Values with different lowercase superscript letters (ab) represent significant difference among farms, while different uppercase superscript letters (ABC) represent significant difference in total by each antimicrobial class ($P < 0.05$).
Figure 2. Distribution of five virulence genes in 578 E. coli isolates from 4 layer breeder farms. *The asterisk means that virulence genes were distributed significantly different among four farms (P < 0.05).

DISCUSSION

The pyramid structure not only attributes to the vertical transmission of antimicrobial-resistant bacteria but also spreads the bacteria to humans through the food chain, which poses a serious risk to public health. In Korea, four big layer companies that possessed one grandparent stock, Hy-Line Brown, and 3 parent stocks, Lohmans Brown Lite, Tetra Brown, and Isa Brown, are in charge of 100% of the layer chicken industry. Additionally, each vertical-operation system supports different biosecures, sanitation practices, housing technologies, feeding regimens, vaccination programs, and antimicrobial applications. Therefore, the characteristics of antimicrobial-resistant bacteria from layer parent stocks are variable by companies, as previously described in isolates from broiler breeders in Korea (Noh et al., 2020; Yoon et al., 2020).

In this study, E. coli isolates showed the highest resistance to CF (56.2%), but the prevalence was significantly different among the 4-layer breeder farms (P < 0.05). In particular, isolates from farm B showed high resistance to 1st- and 2nd-generation cephalosporins, whereas, isolates from farm D showed high resistance to 1st-, 2nd-, and 3rd-generation cephalosporins. Furthermore, ceftiofur, which is a 3rd-generation cephalosporin, has been administered in ovo or via subcutaneous injection to day-old chicks in grandparent and parent flocks, with the vaccine for Mareks disease in Korea. But, vertical transmission from grandparent or parent flocks or contamination of the hatchery environment of ceftiofur-resistant isolates were considered as a public hazard because 3rd-generation cephalosporins were listed as “critically important antibacterial agents for human medicine” by the World Health Organization (World Health Organization, 2018). Hiroi et al. (2011) have already reported that the prevalence of cephalosporin-resistant E. coli in commercial broiler chickens decreased after the withdrawal of ceftiofur use in hatcheries in Japan; therefore, ceftiofur is also no longer used in the poultry industry since 2020 in Korea. Moreover, isolates from farms B and D also showed high resistance to other β-lactams; AMC and AM. Otherwise, isolates from farm A showed a significantly higher resistance rate to NA, TE, and CIP, whereas isolates from farm C showed significantly lower resistance to most antimicrobials tested in this study. Furthermore, the prevalence of MDR showed significant differences among farms, and particularly, only isolates from farm B showed multiple resistance to 9 antimicrobial classes. Therefore, these results support that the different resistance rates to antimicrobial agents are due to differences in the number and frequency of the antimicrobial agents used for disease prevention or therapeutic purposes in each farm (Rizzotti et al., 2005).

Gram-negative bacteria produce various types of β-lactamases (TEM, SHV, OXA, and CTX-M), which hydrolyze β-lactam antimicrobials. Among these, TEM-1 is the most prevalent type (Ghafourian et al., 2015). In this study, although the isolates from farms B and D showed high phenotypic resistance to β-lactams, the blaTEM-1 gene appeared significantly higher in the isolates from farms A and B than farms C and D. Furthermore, Szmolka and Nagy (2013) reported that blaTEM-1 was associated with multiple resistant phenotypes in E. coli from animals. Significantly high prevalence of MDR has been identified in the isolates from farms A and B, which is proposed to be related to the existence of the blaTEM-1 gene in this strain. Additionally, although many isolates from farms B and D showed high resistance to cephalosporins, the CTX-M type, which confers resistance to extended-spectrum cephalosporins, was not detected in this study.

TE is a broad-spectrum antimicrobial, which prevents bacterial protein synthesis (Roberts, 1996), and resistance genes (tetA, tetB, and tetC) encode efflux pumps (Poirel et al., 2018). In this study, the prevalence of tetA (55.1%) was significantly higher than tetB (31.9%) and tetC (1.4%). However, tetB is known as the main gene conferring TE resistance (Seifi and Khoshbakht, 2016). Therefore, the isolates from farm A, which carried tetB were significantly high in prevalence, and showed the highest phenotypic resistance to TE.
Fluoroquinolone is also a critically important antimicrobial medicine to both humans and animals (World Organisation for Animal Health OIE, 2018; World Health Organization, 2018). In Korea, although the use of CIP was banned since 2008, enrofloxacin that is metabolized to CIP has been used in the poultry industry till now. In particular, isolates from farm A showed significantly higher resistance to NA (61.0%) and CIP (30.1%) than the other 3 farms, and the prevalence of resistance genes were also in accordance with phenotypic characteristics.

Resistance to sulfonamide is primarily mediated by the sulfonamide resistance genes, sul1 and sul2 (Poirel et al., 2018), and these genes are mainly located in mobile genetic elements, making the bacteria develop multiple resistance to antimicrobials that are co-selected by sulfonamide (Phuong Hoa et al., 2008; Byrne-Bailey et al., 2009). However, a more frequent presence of sul2 than sul1 has been reported in previous studies on E. coli from the poultry industry (Guerra et al., 2003; Drugová and Knet, 2013). In this study, the frequency of sul1 and sul2 was 22.8 and 24.1%, respectively. Results also showed that sul2 was significantly distributed among the different farms. In particular, the isolates from farm C showed the highest phenotypic resistance to trimethoprim–sulfamethoxazole, and the distribution of the sul2 gene was also significantly higher in the isolates from farm C. Sulfonamides are majorly used as veterinary antimicrobials worldwide, including Korea, due to their affordability and low costs (Kools et al., 2008). Therefore, sulfonamide-resistant isolates and their resistance genes led to the increasing prevalence of multiple resistance to antimicrobials in food-producing livestock and farm environments.

Screening of virulence genes in E. coli isolated from breeder farms would help identify potential zoonotic reservoirs because virulence factors, which are associated with human extraintestinal pathogenic E. coli (Moulin-Schouleur et al., 2007), are also easily transmitted to human consumption through commercial layer chickens and eggs in the pyramidal production structure. In this study, 5 virulence genes; iroN (siderophore), ompT (outer membrane protease), hlyF (hemolysin), iss (increased serum survival), and stx1 (shiga-toxin), were detected, as previous reports (De Oliveira et al., 2015; Ramadan et al., 2016). Certain E. coli serotypes, which develop diseases in humans acquire virulence genes through horizontal gene transfers, can also cause public health problems. Especially, all farms included E. coli isolates carrying stx1, which can seriously exert harmful influence on public health. Moreover, Szmolka et al. (2012) reported that tetA expresses an extensive correlation with iroN and iss in avian originated E. coli because they were co-located in the same plasmid. Interestingly, the isolates from farm C showed a significantly high prevalence in tetA as well as in iroN, ompT, hlyF, and iss genes. Our findings indicate that the phenotypic and genotypic characteristics related to antimicrobial resistance and virulence factors were significantly different among the farms. Moreover, improved management protocols are required to control of horizontal and vertical transmission of avian disease, including the dissemination of resistant bacteria in breeder flocks.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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