





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

Dong gyu Kim,<sup>\*,1</sup> Koeun Kim <sup>\*,1</sup> Sung Hyun Bae <sup>\*,\*</sup> Hye-Ri Jung,<sup>\*</sup> Hyo Jung Kang <sup>\*,\*</sup> Yu Jin Lee,<sup>\*</sup> Kwang Won Seo,<sup>†</sup> and Young Ju Lee <sup>\*,2</sup>

<sup>\*</sup>College of Veterinary Medicine & Zoonoses Research Institute, Kyungpook National University, Daegu 41566, Republic of Korea; and <sup>†</sup>Laboratory of Veterinary Bacteriology and Infectious Diseases, College of Veterinary Medicine, Chungbuk National University, Cheongju 28644, Republic of Korea

**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  $\beta$ -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  $\beta$ -lactam-resistant isolates, 123 (29.9%) carried *bla*<sub>TEM-1</sub>, 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 *tetA* (50.0–77.0%); however, the isolates from farm A showed the highest prevalence in *tetB* (70.6%). The distribution of quinolone (*qnrB*, *qnrD*, and *qnrS*) and sulfonamide (*su12*)-resistant genes were also significantly different among the farms but that of chloramphenicol (*catA1*)- 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 (*iroN*, *ompT*, *hlyF*, 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

2022 Poultry Science 101:101571

<https://doi.org/10.1016/j.psj.2021.101571>

## 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

© 2021 The Authors. Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Received June 15, 2021.

Accepted August 26, 2021.

<sup>1</sup>These authors contributed equally to this work.

<sup>2</sup>Corresponding author: [youngju@knu.ac.kr](mailto:youngju@knu.ac.kr)

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 (Allocati 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 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–clavulanate (AMC, 20/10 µg), ampicillin (AM, 10 µg), cefazolin (30 µg), cefepime (30 µg), cefotaxime (CTX, 30 µg), cefoxitin (30 µg), ceftazidime (30 µg), cefuroxime (30 µg), cephalixin (30 µg),

cephalothin (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 *bla*<sub>TEM</sub> (Dallenne et al., 2010), *bla*<sub>SHV</sub> (Briñas et al., 2002), *bla*<sub>OXA</sub> (Briñas et al., 2002), and *bla*<sub>CTX-M</sub> (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 (Sengeløv 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*, *qnrD*, *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: *iroN*, *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%), cephalixin (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

**Table 1.** Antimicrobial resistance profile of 578 *E. coli* isolates from 4 layer breeder farms.

| Antimicrobials                | No. of antimicrobial resistant <i>E. coli</i> isolates in each farm (%) |                          |                        |                           | Total ( <i>n</i> = 578)    |
|-------------------------------|---|--------------------------|------------------------|---------------------------|----------------------------|
|                               | A ( <i>n</i> = 136)   | B ( <i>n</i> = 37)       | C ( <i>n</i> = 180)    | D ( <i>n</i> = 225)       |                            |
| Amoxicillin-clavulanate       | 17 (12.5) <sup>a</sup>  | 17 (45.9) <sup>b</sup>   | 25 (13.9) <sup>a</sup> | 63 (28.0) <sup>b</sup>    | 122 (21.1) <sup>B, C</sup> |
| Ampicillin                    | 59 (43.4) <sup>a</sup>  | 27 (73.0) <sup>b</sup>   | 63 (35.0) <sup>a</sup> | 86 (38.2) <sup>a</sup>    | 235 (40.7) <sup>A, B</sup> |
| Cefazoline                    | 30 (22.1) <sup>a,b,c</sup>  | 15 (40.5) <sup>c</sup>   | 21 (11.7) <sup>a</sup> | 68 (30.2) <sup>a,b</sup>  | 134 (23.2) <sup>B</sup>    |
| Cephalothin                   | 76 (55.9) <sup>a,b</sup>  | 28 (75.7) <sup>b</sup>   | 88 (48.9) <sup>a</sup> | 133 (59.1) <sup>a,b</sup> | 325 (56.2) <sup>A</sup>    |
| Cephalexin                    | 18 (13.2) <sup>a,b</sup>  | 9 (24.3) <sup>b,c</sup>  | 15 (8.3) <sup>a</sup>  | 89 (39.6) <sup>c</sup>    | 131 (22.7) <sup>B, C</sup> |
| Cefoxitin                     | 17 (12.5) <sup>a,b</sup>  | 10 (27.0) <sup>c</sup>   | 12 (6.7) <sup>a</sup>  | 54 (24.0) <sup>b,c</sup>  | 93 (16.1) <sup>B, C</sup>  |
| Cefuroxime                    | 13 (9.6) <sup>a,b</sup>   | 0 (0.0) <sup>a</sup>     | 8 (4.4) <sup>a</sup>   | 43 (19.1) <sup>b</sup>    | 64 (11.1) <sup>B, C</sup>  |
| Cefotaxime                    | 17 (12.5) <sup>a</sup>  | 1 (2.7) <sup>a</sup>     | 11 (6.1) <sup>a</sup>  | 61 (27.1) <sup>b</sup>    | 90 (15.6) <sup>B, C</sup>  |
| Ceftazidime                   | 21 (15.4) <sup>b,d</sup>  | 1 (2.7) <sup>a,b</sup>   | 10 (5.6) <sup>a</sup>  | 49 (21.8) <sup>c</sup>    | 81 (14.0) <sup>B, C</sup>  |
| Cefepime                      | 2 (1.5)   | 0 (0.0)                  | 2 (1.1)                | 0 (0.0)                   | 4 (0.7) <sup>C</sup>       |
| Chloramphenicol               | 4 (2.9)   | 3 (8.1)                  | 9 (5.0)                | 18 (8.0)                  | 34 (5.9) <sup>C</sup>      |
| Ciprofloxacin                 | 41 (30.1) <sup>b</sup>  | 2 (5.4) <sup>a</sup>     | 13 (7.2) <sup>a</sup>  | 7 (3.1) <sup>a</sup>      | 63 (10.9) <sup>B, C</sup>  |
| Gentamicin                    | 4 (2.9)   | 2 (5.4)                  | 15 (8.3)               | 9 (4.0)                   | 30 (5.2) <sup>C</sup>      |
| Nalidixic acid                | 83 (61.0) <sup>b</sup>  | 13 (35.1) <sup>a</sup>   | 41 (22.8) <sup>a</sup> | 46 (20.4) <sup>a</sup>    | 183 (31.7) <sup>A, B</sup> |
| Tetracycline                  | 68 (50.0) <sup>b</sup>  | 12 (32.4) <sup>a,b</sup> | 61 (33.9) <sup>a</sup> | 75 (33.3) <sup>a</sup>    | 216 (37.4) <sup>A, B</sup> |
| Trimethoprim-sulfamethoxazole | 12 (8.8) <sup>b</sup>   | 4 (10.8) <sup>b</sup>    | 59 (32.8) <sup>c</sup> | 4 (1.8) <sup>a</sup>      | 79 (13.7) <sup>B, C</sup>  |

Values with different lowercase superscript letters (abc) represent significant difference among farms, while different uppercase superscript letters (ABC) represent significant difference in total by each antimicrobial class ( $P < 0.05$ ).

cephalosporins, cefazolin (40.5%), CF (75.7%), and cephalixin (24.3%), including 2nd-generation cephalosporins, cefoxitin (27.0%) ( $P < 0.05$ ). Moreover, the isolates from farm D showed significantly higher resistance to 3rd-generation cephalosporins, CTX (27.1%), and ceftazidime (21.8%) ( $P < 0.05$ ). Additionally, resistance to CIP, NA, and TE was significantly higher (30.1, 61.0, and 50.0%, respectively) in the isolates from farm A than those from the other 3 farms ( $P < 0.05$ ). Interestingly, isolates from farm C showed significantly lower resistance to all antimicrobials, except for cefepime, chloramphenicol, and gentamicin ( $P < 0.05$ ).

### 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 high resistance to 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%).

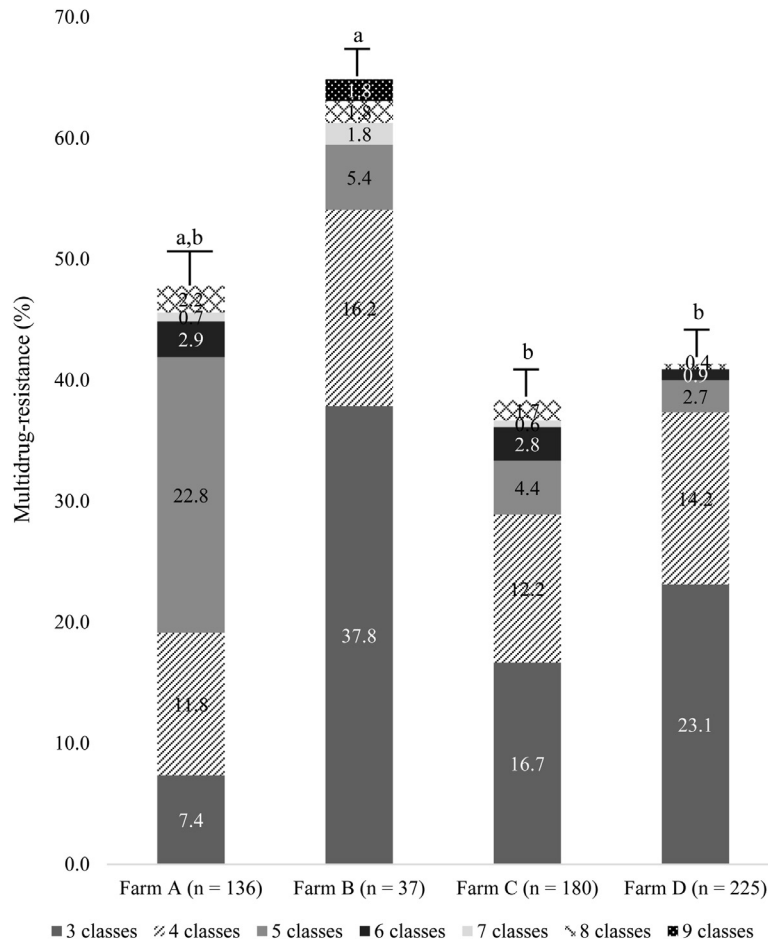
### Distribution of Antimicrobial Resistance Genes

The distribution of resistance genes in *E. coli* isolates showing resistance to each antimicrobial class is shown in Table 2. Among the 412  $\beta$ -lactam-resistant isolates, 123 (29.9%) carried *bla*<sub>TEM-1</sub>, but the distribution was significantly different among farms from 17.5 to 51.4% ( $P < 0.05$ ). Furthermore, the *bla*<sub>SHV</sub>, *bla*<sub>OXA</sub>, and *bla*<sub>CTX-M</sub> 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 were 22.8 and 24.1%, respectively, but the distribution of the *sul2* 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.

### Distribution of Virulence Genes

The distribution of virulence genes in *E. coli* isolates from 4-layer breeder farms is shown in Figure 2. The highest prevalence among the 5 virulence genes was *hlyF* (26.0%), followed by *iss* (18.2%), *ompT* (16.8%), *stx1* (16.8%), and *iroN* (13.0%). Interestingly, isolates from farm C showed significantly higher prevalence in *iroN*, *ompT*, *hlyF*, and *iss* genes than the other 3 farms ( $P < 0.05$ ). Moreover, no isolate from farm B carried *iroN* and *ompT*.

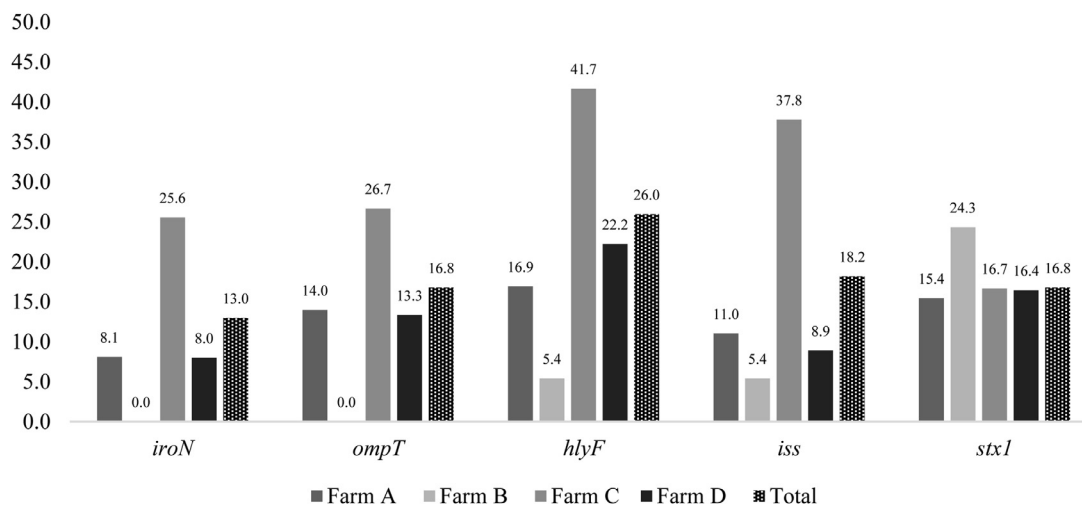


**Figure 1.** Distribution of multidrug resistant *E. coli* isolates from 4 layer breeder farms. Different subscript letters (A, B) indicates statistical difference ( $P < 0.05$ ).

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

| Antimicrobial class<br>(No. of isolates showing<br>resistance to each class) | Antimicrobial<br>resistance gene | No. of isolates carrying each gene/No. of antimicrobial resistant isolates (%) |                            |                            |                            |                             | Total |
|--|----------------------------------|--|----------------------------|----------------------------|----------------------------|-----------------------------|-------|
|  |                                  | Farm A   | Farm B                     | Farm C                     | Farm D                     |                             |       |
| $\beta$ -lactams (412)   | <i>bla</i> <sub>TEM-1</sub>      | 44/87 (50.6) <sup>a</sup>  | 19/37 (51.4) <sup>a</sup>  | 35/145 (24.1) <sup>b</sup> | 25/143 (17.5) <sup>b</sup> | 123/412 (29.9) <sup>A</sup> |       |
|  | <i>bla</i> <sub>SHV</sub>        | 0/87 (0.0)   | 0/37 (0.0)                 | 0/145 (0.0)                | 0/143 (0.0)                | 0/412 (0.0) <sup>B</sup>    |       |
|  | <i>bla</i> <sub>OXA</sub>        | 0/87 (0.0)   | 0/37 (0.0)                 | 0/145 (0.0)                | 0/143 (0.0)                | 0/412 (0.0) <sup>B</sup>    |       |
|  | <i>bla</i> <sub>CTX-M</sub>      | 0/87 (0.0)   | 0/37 (0.0)                 | 0/145 (0.0)                | 0/143 (0.0)                | 0/412 (0.0) <sup>B</sup>    |       |
| Tetracyclines (216)  | <i>tetA</i>                      | 17/68 (25.0) <sup>b</sup>  | 6/12 (50.0) <sup>a,b</sup> | 47/61 (77.0) <sup>a</sup>  | 49/75 (65.3) <sup>a</sup>  | 119/216 (55.1) <sup>A</sup> |       |
|  | <i>tetB</i>                      | 48/68 (70.6) <sup>a</sup>  | 0/12 (0.0) <sup>b</sup>    | 8/61 (13.1) <sup>b</sup>   | 13/75 (17.3) <sup>b</sup>  | 69/216 (31.9) <sup>B</sup>  |       |
|  | <i>tetC</i>                      | 1/68 (1.5)   | 0/12 (0.0)                 | 2/61 (3.3)                 | 0/75 (0.0)                 | 3/216 (1.4) <sup>C</sup>    |       |
| Quinolone (183)  | <i>qnrA</i>                      | 17/83 (20.5)   | 1/13 (7.7)                 | 6/41 (14.6)                | 11/46 (23.9)               | 35/183 (19.1) <sup>A</sup>  |       |
|  | <i>qnrB</i>                      | 9/83 (10.8) <sup>a,b</sup>   | 2/13 (15.4) <sup>a</sup>   | 1/41 (2.4) <sup>a,b</sup>  | 0/46 (0.0) <sup>b</sup>    | 12/183 (6.6) <sup>B</sup>   |       |
|  | <i>qnrD</i>                      | 0/83 (0.0) <sup>b</sup>  | 3/13 (23.1) <sup>a</sup>   | 0/41 (0.0) <sup>b</sup>    | 0/46 (0.0) <sup>b</sup>    | 3/183 (1.6) <sup>B,C</sup>  |       |
|  | <i>qnrS</i>                      | 1/83 (1.2) <sup>b</sup>  | 0/13 (0.0) <sup>b</sup>    | 11/41 (26.8) <sup>a</sup>  | 0/46 (0.0) <sup>b</sup>    | 12/183 (6.6) <sup>B</sup>   |       |
| Sulfonamide (79)   | <i>qepA</i>                      | 0/83 (0.0)   | 0/13 (0.0)                 | 1/41 (2.1)                 | 0/46 (0.0)                 | 1/183 (0.5) <sup>C</sup>    |       |
|  | <i>sul1</i>                      | 5/12 (41.7)  | 2/4 (50.0)                 | 9/59 (15.3)                | 2/4 (50.0)                 | 18/79 (22.8)                |       |
| Phenicolns (34)  | <i>sul2</i>                      | 4/12 (33.3) <sup>a,b</sup>   | 2/4 (50.0) <sup>a</sup>    | 12/59 (20.3) <sup>b</sup>  | 1/4 (25.0) <sup>a,b</sup>  | 19/79 (24.1)                |       |
|  | <i>catA1</i>                     | 2/4 (50.0) <sup>a</sup>  | 2/3 (100.0) <sup>a</sup>   | 4/9 (44.4) <sup>a</sup>    | 0/18 (0.0) <sup>b</sup>    | 8/34 (23.5) <sup>A</sup>    |       |
| Aminoglycosides (30)   | <i>cmlA</i>                      | 0/4 (0.0)  | 0/3 (0.0)                  | 0/9 (0.0)                  | 0/18 (0.0)                 | 0/34 (0.0) <sup>B</sup>     |       |
|  | <i>aac(6')-Ib</i>                | 0/4 (0.0)  | 0/2 (0.0)                  | 1/15 (6.7)                 | 0/9 (0.0)                  | 1/30 (3.3) <sup>B</sup>     |       |
|  | <i>aac(3)-II</i>                 | 2/4 (50.0)   | 2/2 (100.0)                | 5/15 (33.3)                | 1/9 (11.1)                 | 10/30 (33.3) <sup>A</sup>   |       |
|  | <i>ant(2'')-I</i>                | 0/4 (0.0)  | 0/2 (0.0)                  | 0/15 (0.0)                 | 0/9 (0.0)                  | 0/30 (0.0) <sup>B</sup>     |       |

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 biosecurity, 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, had been administered in ovo or via subcutaneous injection to day-old chicks in grandparent and parent flocks, with the vaccine for Marek’s 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  $\beta$ -lactams; AMC and AM. Otherwise, isolates from farm A showed a significantly higher resistance 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  $\beta$ -lactamases (TEM, SHV, OXA, and CTX-M), which hydrolyze  $\beta$ -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  $\beta$ -lactams, the *bla*<sub>TEM-1</sub> gene appeared significantly higher in the isolates from farms A and B than farms C and D. Furthermore, Szmolka and Nagy (2013) reported that *bla*<sub>TEM-1</sub> 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 *bla*<sub>TEM-1</sub> 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; Drugdová and Kmeť, 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-Schouleir 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.

## ACKNOWLEDGMENTS

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through Agriculture, Food and Rural Affairs Research Center Support Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (716002-7).

## 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.

## REFERENCES

- Abo-Amer, A. E., M. Y. Shobrak, and A. D. Altalhi. 2018. Isolation and antimicrobial resistance of *Escherichia coli* isolated from farm chickens in Taif, Saudi Arabia. *J. Glob. Antimicrob. Resist.* 15:65–68.
- Allocati, N., M. Masulli, M. F. Alexeyev, and C. Di Ilio. 2013. *Escherichia coli* in Europe: an overview. *Int. J. Environ. Res. Public Health* 10:6235–6254.
- Briñas, L., M. Zarazaga, Y. Sáenz, F. Ruiz-Larrea, and C. Torres. 2002.  $\beta$ -lactamases in ampicillin-resistant *Escherichia coli* isolates from foods, humans, and healthy animals. *Antimicrob. Agents Chemother.* 46:3156–3163.
- Byrne-Bailey, K. G., W. H. Gaze, P. Kay, A. B. A. Boxall, P. M. Hawkey, and E. M. H. Wellington. 2009. Prevalence of sulfonamide resistance genes in bacterial isolates from manured agricultural soils and pig slurry in the United Kingdom. *Antimicrob. Agents Chemother.* 53:696–702.
- Candrian, U., B. Furrer, C. Höfelein, R. Meyer, M. Jermini, and J. Lüthy. 1991. Detection of *Escherichia coli* and identification of enterotoxigenic strains by primer-directed enzymatic amplification of specific DNA sequences. *Int. J. Food Microbiol.* 12:339–351.
- Cavaco, L. M., D. S. Hansen, A. Friis-Møller, F. M. Aarestrup, and H. Hasman. 2007. First detection of plasmid-mediated quinolone resistance (*qnrA* and *qnrS*). *Escherichia Coli Strains Isolated from Humans in Scandinavia. J. Antimicrob. Chemother.* 59:804–805.
- Clinical and Laboratory Standards Institute (CLSI). 2018. Performance Standards for Antimicrobial Susceptibility Testing, M100-S27. Clinical and Laboratory Standards Institute, Wayne, PA.
- Dallenne, C., A. da Costa, D. Decré, C. Favier, and G. Arlet. 2010. Development of a set of multiplex PCR assays for the detection of genes encoding important  $\beta$ -lactamases in Enterobacteriaceae. *J. Antimicrob. Chemother.* 65:490–495.
- Dierikx, C. M., J. A. Van Der Goot, H. E. Smith, A. Kant, and D. J. Mevius. 2013. Presence of ESBL/AmpC-producing *Escherichia coli* in the broiler production pyramid: a descriptive study. *PLoS One* 8:e79005.
- Drugdová, Z., and V. Kmeť. 2013. Prevalence of  $\beta$ -lactam and fluoroquinolone resistance, and virulence factors in *Escherichia coli* isolated from chickens in Slovakia. *Biol* 68:11–17.
- Le Gall, T., O. Clermont, S. Gouriou, B. Picard, X. Nassif, E. Denamur, and O. Tenaillon. 2007. Extraintestinal virulence is a coincidental by-product of commensalism in b2 phylogenetic group *Escherichia coli* strains. *Mol. Biol. Evol.* 24:2373–2384.
- Ghafourian, S., N. Sadeghifard, S. Soheili, and Z. Sekawi. 2015. Extended spectrum beta-lactamases: definition, classification and epidemiology. *Curr. Issues Mol. Biol.* 17:11–22.
- Guerra, B., E. Junker, A. Schroeter, B. Malorny, S. Lehmann, and R. Helmuth. 2003. Phenotypic and genotypic characterization of

- antimicrobial resistance in German *Escherichia coli* isolates from cattle, swine and poultry. *J. Antimicrob. Chemother.* 52:489–492.
- Hiroi, M., T. Harada, F. Kawamori, N. Takahashi, T. Kanda, K. Sugiyama, T. Masuda, Y. Yoshikawa, and N. Ohashi. 2011. A survey of  $\beta$ -lactamase-producing *Escherichia coli* in farm animals and raw retail meat in Shizuoka Prefecture. *Japan. J. Infect. Dis.* 64:153–155.
- Jiang, Y., Z. Zhou, Y. Qian, Z. Wei, Y. Yu, S. Hu, and L. Li. 2008. Plasmid-mediated quinolone resistance determinants qnr and aac (6')-Ib-cr in extended-spectrum  $\beta$ -lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in China. *J. Antimicrob. Chemother.* 61:1003–1006.
- Johnson, T. J., Y. Wannemuehler, C. Doetkott, S. J. Johnson, S. C. Rosenberger, and L. K. Nolan. 2008. Identification of minimal predictors of avian pathogenic *Escherichia coli* virulence for use as a rapid diagnostic tool. *J. Clin. Microbiol.* 46:3987–3996.
- Kagambèga, A., O. Martikainen, T. Lienemann, A. Siitonen, A. S. Traoré, N. Barro, and K. Haukka. 2012. Diarrheagenic *Escherichia coli* detected by 16-plex PCR in raw meat and beef intestines sold at local markets in Ouagadougou, Burkina Faso. *Int. J. Food Microbiol.* 153:154–158.
- Kim, Y. B., K. W. Seo, J. B. Shim, S. Hyun Son, E. B. Noh, and Y. J. Lee. 2019. Molecular characterization of antimicrobial-resistant *Enterococcus faecalis* and *Enterococcus faecium* isolated from layer parent stock. *Poult. Sci.* 98:5892–5899.
- Kools, S. A. E., J. F. Moltmann, and T. Knacker. 2008. Estimating the use of veterinary medicines in the European union. *Regul. Toxicol. Pharmacol.* 50:59–65.
- Krizman, M., J. A. Avgustin, I. Zdovc, M. Golob, M. Trkov, U. J. Ciglenecki, M. Biasizzo, and A. Kirbis. 2017. Antimicrobial resistance and molecular characterization of extended-spectrum  $\beta$ -lactamases and other *Escherichia coli* isolated from food of animal origin and human intestinal isolates. *J. Food Prot.* 80:113–120.
- Magiorakos, A. P., A. Srinivasan, R. B. Carey, Y. Carmeli, M. E. Falagas, C. G. Giske, S. Harbarth, J. F. Hindler, G. Kahlmeter, B. Olsson-Liljequist, D. L. Paterson, L. B. Rice, J. Stelling, M. J. Struelens, A. Vatopoulos, J. T. Weber, and D. L. Monnet. 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 18:268–281.
- Maynard, C., J. M. Fairbrother, S. Bekal, F. Sanschagrín, R. C. Levesque, R. Brousseau, L. Masson, S. Larivière, and J. Harel. 2003. Antimicrobial resistance genes in enterotoxigenic *Escherichia coli* O149: K91 isolates obtained over a 23-year period from pigs. *Antimicrob. Agents Chemother.* 47:3214–3221.
- Minarini, L. A. R., L. Poirel, V. Cattoir, A. L. C. Darini, and P. Nordmann. 2008. Plasmid-mediated quinolone resistance determinants among enterobacterial isolates from outpatients in Brazil. *J. Antimicrob. Chemother.* 62:474–478.
- Ministry of Food and Drug Safety (MFDS). 2018. Processing standards and ingredient specifications for livestock products. Cheongju, Republic of Korea.
- Moulin-Schouleur, M., M. Répérant, S. Laurent, A. Brée, S. Mignon-Grasteau, P. Germon, D. Rasschaert, and C. Schouleur. 2007. Extraintestinal pathogenic *Escherichia coli* strains of avian and human origin: link between phylogenetic relationships and common virulence patterns. *J. Clin. Microbiol.* 45:3366–3376.
- Noh, E. B., Y. Bin Kim, K. W. Seo, S. H. Son, J. S. Ha, and Y. J. Lee. 2020. Antimicrobial resistance monitoring of commensal *Enterococcus faecalis* in broiler breeders. *Poult. Sci.* 99:2675–2683.
- De Oliveira, A. L., D. A. Rocha, F. Finkler, L. B. De Moraes, N. L. Barbieri, D. B. Pavanelo, C. Winkler, T. T. Grassotti, K. C. T. De Brito, B. G. De Brito, and F. Horn. 2015. Prevalence of ColV plasmid-linked genes and in vivo pathogenicity of avian strains of *Escherichia coli*. *Foodborne Pathog. Dis.* 12:679–685.
- Puong Hoa, P. T., L. Nonaka, P. Hung Viet, and S. Suzuki. 2008. Detection of the sul1, sul2, and sul3 genes in sulfonamide-resistant bacteria from wastewater and shrimp ponds of north Vietnam. *Sci. Total Environ.* 405:377–384.
- Pitout, J. D. D., A. Hossain, and N. D. Hanson. 2004. Phenotypic and molecular detection of CTX-M-beta-Lactamases produced by *Escherichia coli* and *Klebsiella* spp. *J. Clin. Microbiol.* 42:5715–5721.
- Poirel, L., J.-Y. Madec, A. Lupo, A.-K. Schink, N. Kieffer, P. Nordmann, and S. Schwarz. 2018. Antimicrobial resistance in *Escherichia coli*. *Microbiol. Spectr.* 6.
- Ramadan, H., A. Awad, and A. Ateya. 2016. Detection of phenotypes, virulence genes and phylotypes of avian pathogenic and human diarrheagenic *Escherichia coli* in Egypt. *J. Infect. Dev. Ctries.* 10:584–591.
- Rizzotti, L., D. Simeoni, P. Cocconcelli, S. Gazzola, F. Dellaglio, and S. Torriani. 2005. Contribution of enterococci to the spread of antibiotic resistance in the production chain of swine meat commodities. *J. Food Prot.* 68:955–965.
- Roberts, M. C. 1996. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. *FEMS Microbiol. Rev.* 19:1–24.
- Sandvang, D., and F. M. Aarestrup. 2000. Characterization of aminoglycoside resistance genes and class 1 integrons in porcine and bovine gentamicin-resistant *Escherichia coli*. *Microb. Drug Resist.* 6:19–27.
- Sandvang, D., F. M. Aarestrup, and L. B. Jensen. 1998. Characterisation of integrons and antibiotic resistance genes in Danish multiresistant *Salmonella enterica* Typhimurium DT104. *FEMS Microbiol. Lett.* 160:37–41.
- Seifi, S., and R. Khoshbakht. 2016. Prevalence of tetracycline resistance determinants in broiler isolated *Escherichia coli* in Iran. *Br. Poult. Sci.* 57:729–733.
- Sengeløv, G., B. Halling-Sørensen, and F. M. Aarestrup. 2003. Susceptibility of *Escherichia coli* and *Enterococcus faecium* isolated from pigs and broiler chickens to tetracycline degradation products and distribution of tetracycline resistance determinants in *E. coli* from food animals. *Vet. Microbiol.* 95:91–101.
- Seo, K. W., Y. Bin Kim, H. Y. Jeon, S. K. Lim, and Y. J. Lee. 2018. Comparative genetic characterization of third-generation cephalosporin-resistant *Escherichia coli* from chicken meat produced by integrated broiler operations in South Korea. *Poult. Sci.* 97:2871–2879.
- Seo, K. W., J. B. Shim, and Y. J. Lee. 2019a. Comparative genetic characterization of third-generation cephalosporin-resistant *Escherichia coli* isolated from a layer operation system in Korea. *Poult. Sci.* 98:1472–1479.
- Seo, K. W., J. B. Shim, and Y. J. Lee. 2019b. Emergence of CMY-2-producing *Escherichia coli* in Korean layer parent stock. *Microb. Drug Resist.* 25:462–468.
- Szmlolka, A., M. F. Anjum, R. M. La Ragione, É. J. Kaszanyitzky, and B. Nagy. 2012. Microarray based comparative genotyping of gentamicin resistant *Escherichia coli* strains from food animals and humans. *Vet. Microbiol.* 156:110–118.
- Szmlolka, A., and B. Nagy. 2013. Multidrug resistant commensal *Escherichia coli* in animals and its impact for public health. *Front. Microbiol.* 4:1–13.
- Tenaillon, O., D. Skurnik, B. Picard, and E. Denamur. 2010. The population genetics of commensal *Escherichia coli*. *Nat. Rev. Microbiol.* 8:207–217.
- Terentjeva, M., M. Streikiša, J. Avsejenko, J. Trofimova, K. Kovajenko, D. Elferts, and A. Berziņš. 2019. Prevalence and antimicrobial resistance of *Escherichia coli*, *Enterococcus* spp. and the major foodborne pathogens in calves in Latvia. *Foodborne Pathog. Dis.* 16:35–41.
- Van, T. T. H., J. Chin, T. Chapman, L. T. Tran, and P. J. Coloe. 2008. Safety of raw meat and shellfish in Vietnam: an analysis of *Escherichia coli* isolations for antibiotic resistance and virulence genes. *Int. J. Food Microbiol.* 124:217–223.
- Wang, M., J. H. Tran, G. A. Jacoby, Y. Zhang, F. Wang, and D. C. Hooper. 2003. Plasmid-mediated quinolone resistance in clinical isolates of *Escherichia coli* from Shanghai, China. *Antimicrob. Agents Chemother.* 47:2242–2248.
- World Health Organization. 2018. Critically Important Antimicrobials (CIA), 6th Revision. World Health Organization, Geneva.
- Yoon, S., Y. Bin Kim, K. W. Seo, J. S. Ha, E. B. Noh, and Y. J. Lee. 2020. Characteristics of linezolid-resistant *Enterococcus faecalis* isolates from broiler breeder farms. *Poult. Sci.* 99:6055–6061.
- World Organisation for Animal Health (OIE). 2018. Pages 1–10 in OIE list of antimicrobial agents of veterinary importance. Accessed April 2021. [http://www.oie.int/fileadmin/Home/eng/Our\\_scientific\\_expertise/docs/pdf/AMR/A\\_OIE\\_List\\_antimicrobials\\_May2018.pdf](http://www.oie.int/fileadmin/Home/eng/Our_scientific_expertise/docs/pdf/AMR/A_OIE_List_antimicrobials_May2018.pdf).