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«Research Note»

Distribution of Fluoroquinolone-Resistant *Escherichia coli* in Parent Flocks Treated with Fluoroquinolones on Chick Stage and their Broiler Offspring

Takeshi Kawasaki

Research Office Concerning the Health of Humans and Birds, Abashiri, 099-3119, Japan

This study investigated the distribution of ofloxacin-resistant *Escherichia coli* (OFXR-EC) in broiler parent flocks (PS) treated with ofloxacin for 5 days from the time the chicks arrived at the poultry house, and their broiler offspring. OFXR-EC was detected in 22.95% of neonatal parent stock chicks (PSN) arriving at the poultry house. The detection rate of OFXR-EC in PS rearing was 72.49%, which was significantly higher than that detected in PSN. In addition, the detection rate of OFXR-EC was significantly lower in neonatal chicks of their offspring broilers (CSN) at 7.06% than that of PS, but was 24.62% in offspring broiler flocks (CS) at approximately 6 weeks of age. The OFXR-EC detection rate was significantly higher in CS than that in CSN, even though no therapeutic antimicrobials, including ofloxacin, were used from CSN to CS. In addition, the proportions of OFXR-ECs in *E. coli* isolated from samples in which OFXR-ECs were detected were 63.85% for PSN, 10.52% for PS, 62.00% for CSN, and 8.25% for CS. There was little difference in the composition ratio of OFXR-EC between PSN and CSN, or between PS and CS.

Key words: broiler, *Escherichia coli*, fluoroquinolone-resistant, ofloxacin, parent stock

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Introduction

Neonatal chicks develop *Escherichia coli* infections due to vertical transmission from their parents, often leading to significant chick losses[1]. Additionally, antimicrobial-resistant and pathogenic *E. coli* derived from parent birds may be horizontally transmitted to other chicks in an incubator immediately after hatching via feathers, thereby affecting chick hygiene[2]. If numerous chicks die or become obsolete within the first week of rearing, offspring production is severely affected, particularly in commercial parent flocks. Therefore, in situations where there is a high risk of bacterially-infected chicks arriving at poultry houses, antibacterial agents may be used to prevent these issues. However, the possibility of a selective increase in antimicrobial-resistant bacteria in chick intestinal flora is a severe hygiene concern in breeding.

This study determined the distribution of fluoroquinolone-resistant *E. coli* (FQR-EC) in broiler parent flocks treated with fluoroquinolones and their offspring.

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Correspondence: Takeshi Kawasaki, DVM, Ph.D. Research Office Concerning the Health of Humans and Birds, 2-7-1, Masu-ura, Abashiri, 099-3119, Japan. (E-mail: takeshi@kawavet.com)

Materials and Methods

Collection of E. coli isolate samples

At the request of poultry farms, this study was conducted to scientifically evaluate veterinary measures.

Fluoroquinolones inhibit the activity of bacterial DNA gyrase, are highly active against gram-negative aerobes, including *Enterobacteriaceae*, and have low, but useful activity against gram-positive aerobes[3]. Ofloxacin and enrofloxacin are fluoroquinolones that are used to rear chickens in Japan to the extent necessary for veterinary measures. This study investigated the parents of broilers treated with ofloxacin and their offspring.

The samples for detecting *E. coli* were collected from ofloxacin-treated broiler parent stock flocks (approximately 2,500–5,000 birds/flock) and their offspring (approximately 8,000–9,000 birds/flock) between October 2022 and December 2023. The parent stock flocks were treated orally with ofloxacin (Meiji Animal Health, Kumamoto, Japan) at 10 mg/kg body weight for five days when the neonatal chicks arrived at the poultry house. None of the poultry houses in which the parent stock flocks were reared had a history of antibiotic use other than ofloxacin over the past 10 years. No therapeutic antimicrobial preparations containing ofloxacin were used in the broiler flocks. None of the broiler farms had a history of antibiotic use, including ofloxa-

Table 1. **The detection rate of ofloxacin-resistant *Escherichia coli* (OFXR-EC) in each category.**

	PSN [ab]	PS [c]	CSN [a]	CS [b]
OFXR-EC detection	14 (22.95%)	311 (72.49%)	6 (7.06%)	129 (24.62%)
OFXR-EC non-detection	47	118	79	395
E.coli detection samples	61	429	85	524
Number of samples	72	431	107	531

PSN: Container mats collected when PS chicks arrived at the poultry houses. PS: Fecal swabs collected from the ofloxacin-treated PS flocks. CSN: Container mats collected when the offspring broiler chicks arrived at the poultry houses. CS: Fecal swabs collected from the offspring broiler flocks. OFXR-EC detection: Number of detected OFXR-EC samples in the EC samples. OFXR-EC non-detection: Number of non-detected OFXR-EC samples in the EC detection samples. Significant differences (p <0.05) in OFXR-EC detection rate have been found between categories with different superscript letters. PSN, neonatal parent stock chicks; PS, broiler parent flocks; CSN, neonatal broiler chicks; CS, broilers at approximately 6 weeks of age.

cin, for more than 10 years. Diets fed to broiler flocks included ionophores and polypeptide antimicrobial agents during the early rearing stages. The shortest distance between parent and broiler flocks was >10 km.

Samples from the neonatal chicks of parent stocks (PSN) and neonatal chicks of the offspring broilers (CSN) were collected from container mats when the chicks arrived at the poultry house. Samples from the parent stock flocks (PS) and offspring broiler flocks (CS) were collected from excreted feces on the floor using sterilized swabs. Samples collected from the parent flocks included those collected at different ages from the same flock. PS samples were collected at 5–59 weeks, and CS samples were collected at approximately 6 weeks.

Evaluation of the accuracy of E. coli selection based on citric acid utilization in suspected E. coli colonies grown on X-MG agar plates (XMG) or X-MG agar plates supplemented with 10 mg/L ofloxacin (OFX-XMG)

One hundred and three putative *E. coli* colonies grown on XMG or OFX-XMG were inoculated into heart infusion broth and Simmons citric acid medium, respectively. The heart infusion broth was cultured at 37 °C overnight, and after culturing in Mueller-Hinton medium, the bacterial species were identified using an API20E test (Biomerieux, Marcy-l'Étoile, France). Samples were cultured in Simmons citric acid medium at 37 °C for 2 days, and the estimated ability of *E. coli* to utilize citric acid was determined.

Detection of ofloxacin-resistant E. coli (OFXR-EC)

Chicken feces samples collected from PS or CS were inoculated into novobiocin-supplemented mEC broth and incubated overnight at 42 °C. The incubated broth was streaked onto XMG and OFX-XMG and cultured at 37 °C overnight. Container mat samples collected from PSN and CSN were cultured overnight at 37 °C in phosphate-buffered peptone water and then treated in the same manner as fecal samples. After confirming the presence or absence of the growth of putative *E. coli* colonies on OFX-XMG and XMG, for samples in which suspected *E. coli* colonies grew on OFX-XMG, 10 estimated *E. coli* colonies from XMG were inoculated into heart infusion broth and Simmons citric acid medium and fermented at 37 °C overnight. The estimated *E. coli* isolates included those that were β-glucuronidase-positive and were presumed to be *E. coli* due to colony morphology.

Evaluation of ofloxacin susceptibility of E. coli

Heart infusion broth containing pure cultures of *E. coli* was spread onto Mueller-Hinton medium using a sterile cotton swab, and the sensitivity of each *E. coli* isolate to ofloxacin was evaluated using the disk method (Kirby-Bauer (KB) method). Antimicrobial susceptibility testing was performed using KB disks (Eiken Chemicals, Tokyo, Japan).

Statistical analysis

Data between different sample categories were subjected to multiple comparisons of variables using Fisher's exact test with the Bonferroni correction. Statistical analyses were performed using R version 4.3.3 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Selection accuracy of E. coli using XMG or OFX-XMG and Simmons citrate medium

Of 103 putative *E. coli* isolates grown on XMG or OFX-XMG, 101 were negative for citric acid utilization. One hundred isolates that tested negative for citric acid utilization were determined to be *E. coli* using the API20E test, and one isolate was suspected to be contaminated with other bacteria. Two isolates were positive for citric acid utilization, and both were determined to be *Klebsiella pneumoniae* subsp. *pneumoniae* using the API20E test. Based on these results, the isolates that grew on XMG or OFX-XMG and did not utilize citric acid were determined to be *E. coli*.

Detection of OFXR-EC

The *E. coli* detection rates in PSN, PS, CSN, and CS were 84.72%, 99.54%, 79.44%, and 98.68%, respectively. Samples in which *E. coli* grew on both XMG and OFX-XMG were considered OFXR-EC-positive, and the ratio of the number of OFXR-EC-positive samples to the number of samples in which *E. coli* grew on XMG was calculated as the OFXR-EC detection rate. The OFXR-EC detection rates were 22.95%, 72.49%, 7.06%, and 24.62% for PSN, PS, CSN and CS, respectively (Table 1). Significant differences in OFXR-EC detection rates were observed among the PSN:PS, PS:CSN, PS:CS, and CSN:CS comparisons (p < 0.05).

Proportion of OFXR-EC-positive samples

Table 2 shows the ratios of ofloxacin susceptibility levels of *E. coli* colonies in each category isolated from XMG in the OFXR-

Table 2. **Ofloxacin susceptibility distribution of *E. coli* in ofloxacin-resistant *Escherichia coli* (OFXR-EC) detection samples in each category.**

Ofloxacin sensitivity	PSN [a]	PS [b]	CSN [a]	CS [b]
R	83 (63.85%)	85 (10.52%)	31 (62.00%)	102 (8.25%)
I	9 (6.92%)	29 (3.59%)	1 (2.00%)	24 (1.94%)
S	18 (29.23%)	694 (85.89%)	18 (36.00%)	1111 (89.81%)
n	110	808	50	1237

PSN: Container mats collected when PS Chicks arrived at the poultry houses. PS: Fecal swabs collected from the ofloxacin treated PS flocks. CSN: Container mats collected when the offspring broiler chicks arrived at the poultry houses. CS: Fecal swabs collected from the offspring broiler flocks. R: Resistant. I: Intermediate. S:Susceptible. n: Number of *E. coli* isolates from the X-MG agar plates of the samples in which OFXR-EC were detected. Significant differences (p <0.05) exist in the causal relationship between R and S samples in categories with different superscript letters. PSN, neonatal parent stock chicks; PS, broiler parent flocks; CSN, neonatal broiler chicks; CS, broilers at approximately 6 weeks of age.

EC-positive samples. Significant differences were observed in the relationship between resistance (R) and susceptibility (S) to ofloxacin in PSN:PS, PSN:CS, PS:CSN, and CSN:CS comparisons ($p < 0.05$).

Discussion

XMG, which contains 4-chloro-5-bromo-1H-indole-3-yl β-D-glucuronide (X-GLUC) and 5-bromo-6-chloro-3-indolyl-β-D-galactopyranoside (MAGENTA-GAL), is a selective medium used to detect *E. coli* and coliform bacteria. In this medium, the presence of X-GLUC results in blue colonies with β-glucuronidase activity, and the presence of MAGENTA-GAL results in red colonies with β-galactosidase activity. β-galactosidase is produced by a large number of microorganisms, including gram-negative bacteria, such as *Enterobacteriaceae*, *Vibrionaceae*, *Pseudomonadaceae*, and *Neisseriaceae*, as well as some gram-positive bacteria, yeasts, protozoa, and fungi[4]. β-glucuronidase activity is present in most *E. coli* strains (94%–97%), and activity has also been confirmed in some *Enterobacteriaceae* other than *E. coli*[5]. Therefore, an *E. coli* colony that produces both of these enzymes exhibits a hue ranging from blue to green to reddish-brown, depending on the expression ratio of each enzyme. In addition, even if some members of the *Enterobacteriaceae* family other than *E. coli* form colonies similar to *E. coli*, additional testing of citric acid utilization allowed for a more accurate selection of *E. coli*. In this study, β-glucuronidase-positive isolates that grew on OFX-XMG or XMG were identified using the confirmation of citrate utilization and the API20E test. Consequently, it was confirmed that the 100 isolates that did not use citric acid were *E. coli*. One isolate that was determined to be mixed with other bacteria was again selectively isolated, a pure culture was established, and then determined using the API20E test to be *E. coli*. Based on these facts, the citrate utilization-negative isolates that grew on OFX-XMG/XMG were considered to be *E. coli*.

The OFXR-EC detection method using OFX-XMG allowed selective detection of OFXR-EC, regardless of the composition ratio of OFXR-EC to the total number of *E. coli* contained in the sample. However, if the OFXR-EC composition ratio was very low or high, the culture result might be the same (positive). Therefore, this detection method only clarifies the proportion of flocks in which OFXR-EC is distributed among the flocks selected for investigation; the proportion of OFXR-ECs among the total *E. coli* in each flock sample could not be determined.

Herein, samples were cultured at 42 °C using mEC broth supplemented with novobiocin to increase the detection sensitivity of *E. coli*. This culture method indicated that the *E. coli* contained in the sample grew dominantly in competition with other bacteria. However, the exponential growth behavior of individual *E. coli* cells might not necessarily be similar. The data obtained in this study (Table 2) showed little difference in the composition ratio of OFXR-EC between PSN and CSN, and between PS and CS. The difference in the distribution ratio of OFXR-EC between the chick and rearing stages was significant, even when considering the number of *E. coli* and OFXR-EC originally present in each sample, as well as the diversity of bacteria. Further investigation into the factors related to these differences is required.

Although this study was conducted using a limited number of field samples, the data obtained confirmed the approximate distribution of antimicrobial-resistant *E. coli* in parent chickens with a history of fluoroquinolone use and their broiler offspring. In the present study, OFXR-EC was introduced into parent flocks at a relatively high rate by neonatal chicks. In addition, the detection rate of OFXR-EC was significantly higher in PS treated with ofloxacin for a continuous short period from the time neonatal chicks arrived at the poultry house, compared to that in PSN. This is consistent with the general veterinary assumption that when antibacterial agents are used, the number of bacteria that are resistant to the antimicrobial agents selectively increases. In contrast, the OFXR-EC detection rate was significantly lower in CSN derived from these parent flocks than that in the parent flocks. Furthermore, in CS, the detection rate was significantly higher at approximately 6 weeks of age than that in CSN, even though ofloxacin was not used throughout the rearing period. These findings cannot infer the factors associated with an increase in the OFXR-EC detection rate of CS at 6 weeks compared to the OFXR-EC detection rate of CSN. Further investigation is required to consider other factors, such as the geographical location of the poultry farm, feed, water, and other materials used during rearing, as well as the unintentional invasion of wild animals and insects.

Smith et al. (2007) have suggested that patterns of antimicrobial agent use do not correlate with the prevalence of resistant bacteria. Genetic analysis of *E. coli* isolated from poultry farms reveals that some resistant strains persist in the farm environment and colonize new flocks[6]. Schulz et al. (2019) have found that fluoroquinolone preparations in poultry and livestock houses significantly increase the probability of fluoroquinolone detection in the house dust and *E. coli* with reduced susceptibility to these agents has been detected[7]. This suggests that the agents diffused into the environment via the exhaust air. Schulz et al. (2012) have revealed that bacteria originating from swine pens are detected within a range of at least 300 m from the pens[8]. Thus, when using antibacterial agents on poultry farms, there is a possibility that the level of antimicrobial-resistant bacteria that selectively proliferate and spread to surrounding poultry and livestock farms through air currents will increase. Houseflies may also act as vectors of *E. coli*, including FQR-EC, between livestock and poultry farms and human living environments[9]. Therefore, there is a possibility that houseflies from nearby livestock farms and waste disposal sites may bring FQR-EC into the broiler-rearing environment. It is difficult to prevent housefly invasion in most commercial poultry housing systems. To reduce the risk of accidental transmission of FQR-EC in poultry farms, it is necessary to improve the farm environment and reduce the factors that increase the number of houseflies throughout the region. If antibacterial agents are used at poultry and livestock sites, the amount used is often high. Therefore, it is assumed that the use of antibacterial agents influences the bacterial flora in the rearing and surrounding environments. When administered orally, fluoroquinolones are highly effective in treating infections caused by gram-negative aerobic bacteria. Nevertheless, fluoroquinolone activity against gram-positive aerobic bacteria is relatively low; therefore, there is concern regarding the emergence of resistant bacteria[3]. To eliminate concerns associated with the use of antibacterial agents and to enable these agents to be used effectively in situations where they are needed, knowledge about antimicrobial agent use and their effects on *E. coli*, as well as on several indicator bacteria, including gram-positive bacteria is required.

In conclusion, this study revealed that in parent flocks where ofloxacin was used at the neonatal chick stage, the distribution of OFXR-EC was higher than that in neonatal chicks before ofloxacin use. However, further research is required to determine the dynamics of OFXR-EC distribution ratios over time during rearing. In addition, the proportion of OFXR-ECs in the offspring decreased to the same level as that in the parental pre-ofloxacin administration group. However, the distribution ratio of OFXR-ECs increased, even without the use of antibacterial agents containing ofloxacin, during the offspring-rearing period.

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Author Contributions

The author was solely responsible for the study conception and design; data collection; analysis and interpretation of results; and manuscript preparation.

Conflicts of Interest

The author declares no conflict of interest.

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