Impact of Third-Generation-Cephalosporin Administration in Hatcheries on Fecal *Escherichia coli* Antimicrobial Resistance in Broilers and Layers

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We investigated the impact of the hatchery practice of administering third-generation cephalosporin (3GC) on the selection and persistence of 3GC-resistant *Escherichia coli* in poultry. We studied 15 3GC-treated (TB) and 15 non-3GC-treated (NTB) broiler flocks and 12 3GC-treated (TL) and 10 non-3GC-treated (NTL) future layer flocks. Fecal samples from each flock were sampled before arrival on the farm (day 0), on day 2, on day 7, and then twice more. *E. coli* isolates were isolated on MacConkey agar without antibiotics and screened for 3GC resistance, and any 3GC-resistant *E. coli* isolates were further analyzed. 3GC-resistant *E. coli* isolates were found in all 3GC-treated flocks on at least one sampling date. The percentages of 3GC-resistant *E. coli* isolates were significantly higher in TB (41.5%) than in NTB (19.5%) flocks and in TL (49.5%) than in NTL (24.5%) flocks. In the day 2 samples, more than 80% of the *E. coli* strains isolated were 3GC resistant. 3GC-resistant *E. coli* strains were still detected at the end of the follow-up period in 6 out of 27 3GC-treated and 5 out of 25 non-3GC-treated flocks. Many 3GC-resistant *E. coli* strains were resistant to tetracycline, and there were significant differences in the percentages of resistance to sulfamethoxazole-trimethoprim, streptomycin, or gentamicin between treated and nontreated flocks. The results clearly demonstrated that 3GC-resistant strains are introduced early in flocks and that the use of 3GC in hatcheries promotes the selection of 3GC-resistant *E. coli*. Measures must be implemented to avoid the spread and selection of 3GC-resistant strains.

Third-generation cephalosporins (3GC) have been classified as "critically important antimicrobials" in human medicine by the World Health Organization. In many European countries, there has been a dramatic increase in the prevalence of 3GC-resistant *Escherichia coli* in broilers and broiler meat (1–3), and these isolates are considered of public health concern.

Several studies have suggested a possible link between use of 3GC in hatcheries and the increase in 3GC resistance in *E. coli*. The presence of these resistant isolates in the broiler production pyramid has been recently demonstrated (2, 4). 3GC antimicrobials are sometimes used to control the early mortality rate associated with *E. coli*. It is automatically administered *in ovo* to broilers (3) or by subcutaneous injection to 1-day-old future layers (6), together with Marek’s disease vaccination. It is strongly suspected that use at hatcheries is responsible for the increase in 3GC resistance in poultry production (3, 7, 8). However, to our knowledge, no direct evidence of this potential cause-effect relationship has been published.

The purpose of this study was to investigate the impact of hatchery use of 3GC on the selection and persistence of 3GC-resistant *E. coli* in poultry intestinal flora throughout the lifetime of the birds, by comparing flocks of broilers and future laying hens (layers) that have received cefetium (a 3GC antimicrobial) infections *in ovo* and subcutaneously, respectively, with those that have not. To our knowledge, this is the first longitudinal follow-up of flocks for which there is information on the presence or absence of cefetium treatment in hatcheries.

**MATERIALS AND METHODS**

**Study and sampling design.** The farms were chosen by the poultry breeding companies, which agreed to collect samples throughout the lifetime of the flocks. Two types of poultry production were included in this study: free-range broilers (B) and future laying hens (L). In both cases, 3GC-treated and non-3GC-treated flocks were followed. Broiler chickens had access to an outdoor run during at least the second half of the production period and were slaughtered at more than 78 days of age. Pullets were transferred from rearing houses to laying farms after approximately 120 days.

We studied 30 flocks of broilers originating from two hatcheries (15 from A and 15 from B). Of these, 15 were treated by automatic injection of 0.1 mg cefetium *in ovo* at 18 days of incubation, together with Marek’s disease vaccination, according to hatchery practice. These 3GC-treated broiler (TB) flocks and 15 non-3GC-treated broiler (NTB) flocks from the same hatcheries were monitored for up to 77 days. Each flock was sampled five times, from the first day of life, before arrival on the production farm (day 0 [D0]), and on D2, D7, D41 (just before access to outdoor runs), and D77 (a few days before slaughter). Fresh meconium droppings were collected from transport box papers on D0, and fecal samples were obtained from both swabs collected on the farm thereafter. The number of TB and NTB flocks from A and B hatcheries are given in Table 1.

In addition, we studied 22 flocks of future laying hens originating from two other hatcheries (8 from hatchery C and 14 from hatchery D). For 12 future layer flocks (3GC-treated future layers [TL]), the 1-day-old chicks were treated subcutaneously with 0.1 mg cefetium at the hatchery, and for the 10 other flocks, no antibiotic treatment was administered at the hatchery (nontreated future layers [NTL]). Sampling design included five sampling dates: on the first day of life, before arrival on the farm (D0), on D2,
D7, and D120 (before transfer of pullets to laying barns), and on D200. Fresh meconium droppings were collected from transport box papers on D0, and then pools of feces were collected on the farm thereafter. However, for logistic reasons, some layer flocks (mostly from hatchery C) could not be monitored up to the end of the study as initially planned. The distribution of the 241 collected samples is summarized in Table 1.

For both types of chicken, the flocks and the farms were chosen by the breeding companies that were in charge of collecting the samples. During the breeding period, no antimicrobial treatments were administered to the flocks from hatcheries A, B, and D. Data were not available for flocks from hatchery C.

### Sample analysis
Upon arrival at the laboratory, within 48 h after sampling, samples were diluted in peptone water with 20% glycerol as described below and stored at −70°C until analysis. Bags containing the boot swabs were filled with 200 ml of peptone water and shaken manually for 30 s. Then, 50 ml of the mixture was transferred into a tube and centrifuged at 5,000 × g for 15 min. The pellet was resuspended in 2 ml of peptone water containing 20% glycerol and stored at −70°C.

### Analysis of E. coli isolates
All samples were inoculated on MacConkey agar plates without antibiotic and were incubated at 37°C for 24 h. Insofar as possible, five colonies with typical E. coli morphology were selected for each sample. Additionally, samples from broiler flocks were also seeded on MacConkey agar plates supplemented with 1 mg/liter of ceftoxime (CTX) (9).

Species identification was confirmed using an E. coli PCR amplification protocol (10). Then, a standardized inoculum (0.5 McFarland) of each E. coli isolate (collected on MacConkey agar without antibiotic) was plated on Mueller-Hinton agar (MHA) supplemented with 2 mg/liter of CTX to determine whether the MIC was greater than the EUCAST clinical breakpoints or epidemiological cutoffs.

### Detection of beta-lactamase genes
For 3GC-resistant E. coli strains isolated in the first week of life, the most frequent 3GC resistance genes usually detected in poultry (13) and encoding the TEM, CTX-M, SHV, and CMY-2 enzymes were screened for by PCR amplification as described previously (14–17).

### Statistical analysis
The aim is to test the influence of the 3GC treatment on resistance data. From broiler and layer flocks were analyzed separately. In addition, data were analyzed from two levels, i.e., the isolate and the sample levels. At the isolate level, resistance was expressed in terms of positive versus negative results, whereas at the flock level the proportion of 3GC-resistant E. coli isolates among the five sampled isolates was calculated for each flock and each sampling date. First, the effect of 3GC treatment on resistance to CTX was studied over the whole data set (n = 679 for broilers and n = 416 for layers at the isolate level; n = 137 for broilers and n = 84 for layers at the sample level). Then, the influence of 3GC treatment on resistance to antimicrobials of other classes as well as the proportions of resistance genes on 3GC-resistant E. coli or samples, respectively (n = 182 for broilers and n = 146 for layers at the isolate level; n = 67 for broilers and n = 43 for layers at the sample level), were determined. More specifically, at the isolate level, mixed logistic regression models were applied to test the effects of 3GC treatment, hatchery, sampling time, and their two-level interactions (if possible), the flock being considered a random effect, on the resistance to CTX (glm function in R software). At the sample level, analyses of variance were conducted to test the effects of 3GC treatment, hatchery, sampling time, and their two-level interactions on the proportion of CTX-resistant isolates in samples (lm function in R).

### RESULTS
Detection of 3GC-resistant E. coli
From the 241 samples, 1,095 E. coli isolates were collected (679 isolates from 137 samples out of 150 broiler samples and 416 isolates from 84 samples out of the 91 future layer samples) (Table 2). No E. coli strains were isolated from 19 samples out of 52 collected on D0 (9 TB, 4 NTB, 3 TL, and 3 NTL). All other samples were positive for E. coli culture, and five distinct colonies were obtained from most of them. Out of these 1,095 isolates, 31.05% (340 isolates: 137 from TB, 45 from NTB, 111 from TL, and 47 from NTL) grew on CTX-supplemented MHA and were thus categorized as 3GC resistant (Fig. 1A and B).

At the isolate level, the presence of 3GC-resistant E. coli was significantly higher in TB (137 out of 330 isolates) than in NTB (45...
out of 349 isolates) \( (P < 0.001) \). Moreover, a significant increase in the presence of 3GC-resistant *E. coli* was observed for D2 and D7 compared to that for D0 \( (P < 0.001) \).

In both production types, 3GC-resistant *E. coli* strains were still detected at the end of the follow-up period: 10 3GC-resistant *E. coli* isolates out of 149 in broiler flocks on D77 and 6 out of 60 in layer flocks on D200.

At the flock level, 3GC-resistant *E. coli* isolates were detected on nonsupplemented medium in all 27 3GC-treated flocks on at least one sampling date. Only two nontreated flocks (one of each production type) never showed 3GC-resistant *E. coli* on nonsupplemented medium. The percentage of 3GC-resistant *E. coli* isolates in treated flocks was significantly higher than in the respective nontreated flocks \( (P < 0.001) \) for both production types (Fig. 2). The increase in the percentage of 3GC-resistant *E. coli* isolates in treated flocks was significantly higher than in nontreated flocks for D2 compared to D0 \( (P < 0.001) \).

On the last sampling day (D200 for layer flocks and D77 for broiler flocks), resistant isolates were obtained from 6 of the 27 3GC-treated and 5 of the 25 nontreated flocks.

However, when CTX-supplemented media were used for broiler samples, 3GC-resistant *E. coli* strains were detected even more often. Thus, on D41, 3GC-resistant *E. coli* was detected in all TB flocks and in 14 out of 15 NTB flocks. On D77, the last sampling date before slaughter, only one TB and one NTB flock did not show 3GC-resistant *E. coli*.

Characterization of the 3GC-resistant *E. coli* isolates collected during the first week of life (D0 to D7). Because it was not possible to obtain samples for all flocks included in the study for their whole lifetime (i.e., in layer flocks), characterization of 3GC-resistant *E. coli* was limited to the isolates obtained from the samples collected during the first week of life to ensure statistical comparisons. We thus screened for genes encoding the TEM, CTX-M,

### Table 2

<table>
<thead>
<tr>
<th>Flock</th>
<th>No. of samples</th>
<th>No. of <em>E. coli</em> isolates</th>
<th>No. (%) of 3GC-resistant <em>E. coli</em> isolates</th>
<th>No. of bla&lt;sub&gt;CTX-M&lt;/sub&gt;-positive isolates</th>
<th>No. of bla&lt;sub&gt;CMY-2&lt;/sub&gt;-positive isolates</th>
<th>No. of bla&lt;sub&gt;TEM&lt;/sub&gt;-positive isolates</th>
</tr>
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<tr>
<td>TB</td>
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<td>330</td>
<td>116 (35.1)</td>
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<td>26</td>
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<tr>
<td>NTB</td>
<td>71</td>
<td>349</td>
<td>39 (11.2)</td>
<td>22</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>TL</td>
<td>45</td>
<td>224</td>
<td>104 (46.4)</td>
<td>81</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>NTL</td>
<td>39</td>
<td>192</td>
<td>42 (21.9)</td>
<td>20</td>
<td>16</td>
<td>1</td>
</tr>
</tbody>
</table>

\( ^{a} \) TB, treated broilers; NTB, nontreated broilers; TL, treated layers; NTL, nontreated layers.

![FIG 1](http://aac.asm.org) Numbers of 3GC-resistant *E. coli* (black) or 3GC-susceptible *E. coli* (white) strains in samples from broiler flocks without *in ovo* ceftiofur treatment (A), with *in ovo* ceftiofur treatment (B), from future laying hen flocks without (C) and with (D) subcutaneous injection of ceftiofur in 1-day-old chicks.
FIG 2. Boxplot of the distribution of the percentage of 3GC-resistant *E. coli* strains (*E. coli* R-C3G) in each flock. Data are grouped by flock and give the sample statistics of the rate of 3GC-resistant *E. coli* strains for each flock and sampling date. The black band in the box is the median, the filled square is the mean, the bottom and the top of the box are the 25th and 75th quartiles, and the open circles are the highest and lowest observed values; the number above the sampling date is the number of flocks. (A) Nontreated broiler flocks; (B) treated broiler flocks; (C) nontreated layer flocks; and (D) treated layer flocks.
The bla<sub>CTX-M</sub> gene was the most frequently detected gene, being present in 208 out of 301 (69.1%) 3GC-resistant <i>E. coli</i> isolates. For broiler and layer production types, the percentage of isolates harboring this gene was, respectively, higher and significantly higher in 3GC-treated flocks (73.3% for TB and 77.9% for TL) than in non-3GC-treated flocks (56.4% for NTB and 47.6% for NTL) (P < 0.01 for layers). The bla<sub>CMY-2</sub> gene was the second most frequently detected gene. However, the difference of frequencies in NTL flocks (38.1%) and in TL flocks (16.3%) was not significant. In broiler flocks, the proportion of 3GC-resistant <i>E. coli</i> isolates harboring the bla<sub>CMY-2</sub> gene was 23.3% in treated flocks and 28.2% in nontreated flocks. For both production types, the presence of this gene was significantly associated with the hatchery of origin (P < 0.05). No significant difference was observed between 3GC-treated and non-3GC-treated flocks for the detection of bla<sub>TEM</sub>. Nevertheless, the bla<sub>TEM</sub> gene was more frequent in NTB flocks than in NTL flocks (21.3% of 3GC-resistant <i>E. coli</i> isolates compared to 6.2%). The bla<sub>SHV</sub> gene was never detected.

The majority of the 3GC-resistant <i>E. coli</i> isolates (255 out of 301) harbored only one of the screened beta-lactamase genes, with no significant difference between 3GC-treated and non-3GC-treated flocks. Only two isolates, both obtained from treated flocks, harbored three genes (bla<sub>CTX-M</sub>, bla<sub>CMY-2</sub>, and bla<sub>TEM</sub>), and six strains harbored bla<sub>CMY-2</sub> and bla<sub>CTX-M</sub>. Out of the 301 isolates tested, 24 harbored none of the four screened genes.

For each poultry production type, there were no differences in the frequencies of resistance to tetracycline, kanamycin, and ciprofloxacin of 3GC-resistant <i>E. coli</i> from 3GC-treated and non-3GC-treated flocks. More than 75% of 3GC-resistant isolates harbored tetracycline, and less than 5% were resistant to kanamycin. Isolates from TB flocks were more often resistant to streptomycin (P < 0.05) and to gentamicin (P < 0.05) than those from NTB flocks (Table 3).

Multiresistance (defined as resistance to at least three different antimicrobial classes, including beta-lactams [18]) was observed in 46.2% of 3GC-resistant <i>E. coli</i> isolates. Multiresistant isolates were more frequent in 3GC-resistant <i>E. coli</i> isolates from TB flocks (66.1%) than from NTB flocks (35.9%) (P < 0.01). Among the 50 isolates, which were resistant to four or five antimicrobial classes, 29 were resistant to all five tested classes. These pentaresistant isolates originated mainly from treated flocks (24 out of 29 flocks), especially from broiler flocks (21 out of 24). Most were isolated on D2 (18 out of 29). Moreover, 25 multiresistant isolates came from flocks originating from hatchery A; the four others were from hatchery D.

### DISCUSSION

This study monitored 3GC-treated and nontreated flocks, from D0 before arrival on the production farm until a few days before slaughter or until egg production, and was designed to analyze the impact of 3GC use in hatcheries on the selection of 3GC-resistant <i>E. coli</i>. Limits to our study include the absence of knowledge on the 3GC status of the parental flocks and the interrupted follow-up of some of the layer flocks. However, all planned samples were obtained for broiler flocks and at least for the first week of future layers, ensuring nonbiased comparisons of isolates for this period.

Many studies reporting the presence of 3GC-resistant <i>Enterobacteriaceae</i> are based on isolation on 3GC-containing media, which can detect resistant isolates even when present in very low densities, therefore greatly increasing the sensitivity of the method (3). However, when selective media were used for broiler samples, most samples were positive, thereby hindering any comparison between treated and nontreated flocks. We therefore used non-3GC-supplemented media and then screened all the isolates for 3GC susceptibility. Although nonselective media were used, some samples collected on D0 did not yield any <i>E. coli</i> isolates, particularly those collected from TB, i.e., birds having received a 3GC injection a few days prior. This absence of <i>E. coli</i> may be related to the initial sterility of the chick digestive tract which acquires its microbiota during the first hours or days of life. It can also be hypothesized that, for treated groups, the concentrations of ceftiofur and its metabolites present in the flocks prevented colonization by susceptible isolates, because in ovo or day-of-hatch subcutaneous antibiotic administration can interfere with the establishment of a competitive exclusion culture (5). No data on the concentrations of ceftiofur in the flocks after in ovo administration are available, but Heinrich et al. (6) analyzed a single fecal sample collected 2 days after subcutaneous injection of day-old chicks and reported an indicative concentration of around 100 μg/kg, suggesting that ceftiofur is rapidly excreted. This relatively high concentration of 3GC may inhibit some resistant <i>E. coli</i> strains (11), explaining negative culture on D0, the first sampling after administration. Thereafter, 3GC concentrations in the digestive tract probably decrease under the MIC of resistant strains, so that 3GC concentrations reach values that promote selection, strongly favoring resistant isolates, as observed on D2. Moreover, the higher proportion of 3GC-resistant <i>E. coli</i> strains in TL flocks on D0 than in BT flocks may be linked to the period of injection of the ceftiofur: 3 days prior in TL and only a few hours prior in TB. This difference in timing may result in differences in concentrations of ceftiofur or its metabolites in the feces of the chicks and favor a stronger
selection of resistant strains. However, other factors, such as vertical transmission, hatchery practices, poultry genetics, etc., may also play a role in the observed difference.

The impact of 3GC injection was particularly important during the first week of life, when resistant strains were frequently isolated from the dominant flora: more than 80% of the E. coli population in treated flocks were 3GC resistant on D2, whereas the percentages in nontreated flocks were significantly lower. Our results also showed an overall higher frequency of 3GC-resistant E. coli in nontreated flocks and a higher proportion of 3GC-resistant E. coli harboring the gene blaCTX-M in treated flocks than in nontreated ones, independently of the production type (broilers or layers) or of the mode of injection of ceftiofur (in ovo at 18 days of incubation or subcutaneously for 1-day-old chicks). To our knowledge, this is the first study confirming the suspected impact of 3GC use in hatcheries in field conditions.

As previously observed in other studies on French broilers and layers (19, 20), blaCTX-M was the most frequently detected gene, whereas blacMY-2 is more frequent in poultry or poultry meat in other countries, such as Denmark or Norway (21, 22), and is common in the United States (23). However, in the present study, as in a previous study on French layers (19), blacMY-2 was the second most frequently detected gene. It is noteworthy that it was sometimes present in conjunction with blaCTX-M as observed in Belgium (24).

In nontreated flocks (no 3GC at the hatchery, no other antimicrobials administered on the production farm), 3GC-resistant E. coli strains were detected and represented up to 30% of the E. coli population. This was observed for both production types during the first week of life, but some chickens were still contaminated several months thereafter. Detection of 3GC-resistant E. coli in organic chicken meat (1, 25) has already been reported, and extended-spectrum beta-lactamase (ESBL) contamination may result from ESBL-colonized 1-day-old chicks. In our study, 3GC-resistant E. coli strains were also detected in nontreated flocks (layers and broilers), as early as D0, before arrival on the production farm. This result corroborates previous reports of vertical transmission of resistant strains from parental flocks (26–28) of contamination in the hatchery environment (2). We also detected 3GC-resistant E. coli strains from environmental samples collected in the chick screening room of one of the participating hatcheries (data not shown). Thus, 3GC-resistant E. coli may be introduced in the hatchery facilities, either from true vertical transmission when parental poultry stocks are contaminated or from very early contamination in the hatchery itself or during transport, a period when the immature digestive flora is probably very receptive to early colonization, although other possible contamination events occurring thereafter on the production farm cannot be excluded. 3GC treatment results in the selection of resistant strains in the immature intestinal flora of chicks in the very first days of their life. As noted above, the selective advantage is fully expressed on D2, with a very high proportion of resistant strains in treated flocks.

After D7, the 3GC-resistant E. coli strains were no longer dominant among the E. coli strains in the poultry intestinal flora; nevertheless, they were still present at low levels and could be detected with nonsupplemented media. The decrease in the proportion of 3GC-resistant E. coli strains is probably linked to the decreasing selection pressure in the poultry gut, because the antibiotic is metabolized and excreted. It is also frequently reported that resistant strains are less prevalent in older animals (29). Very few data are available on the metabolism of ceftiofur in chicks (6), and it was unfortunately not possible in this study to determine the concentrations of ceftiofur or its metabolites in feces. Other chicks can be contaminated by other (nonresistant) E. coli strains that are present on the farm and that have a better competitive advantage. However, large numbers of chicks bearing resistant E. coli strains shed huge numbers of resistant isolates, resulting in rapid contamination of the other individuals in the same flock and production barn environment. This high level of contamination is probably difficult to eliminate even with strict disinfection procedures, particularly on farms with outdoor runs. This likely contributes to the persistence of 3GC-resistant E. coli.

On D77 or D200 (last sampling dates according to production type), resistant E. coli could still be detected on the treated and nontreated flocks, although the differences of resistance rates were no longer significant. Future studies include the comparison of isolates and the plasmids obtained at D0 with those isolated before slaughter to evaluate their persistence during the lifetime of the flock. However, later horizontal contamination may be involved and includes the production farm environment, particularly in free-range chickens, because wild birds may harbor ESBL-producing Enterobacteriaceae (30).

With regard to public health, the prolonged excretion of 3GC-resistant E. coli strains in broilers or layers results in contamination of the environment by the dissemination of resistant bacteria via the litter and the manure and, for free-range broilers, contamination of outdoor runs. The relative high density of 3GC-resistant strains at farms and their persistence during the breeding period probably also increase occupational exposure of farm workers to ESBL/AmpC-producing E. coli strains (31). Furthermore, the presence of 3GC-resistant E. coli strains on D77 and D200 in broilers and layers, respectively, is a potential hazard for the consumer (32) and should lead to more investigations of meat and eggs.

The disinfection methods should be applied at all the levels of the poultry pyramid to prevent horizontal as well as vertical contamination. Stopping the use of 3GC and coselecting antibiotics at hatcheries will reduce the selection pressure exerted on these resistant bacteria.

Conclusion. The design of our study clearly demonstrated the impact of use of 3GC in hatcheries, either in ovo or in day-old chicks, on the selection of 3GC-resistant E. coli. Stopping the use of 3GC at hatcheries is an important measure to implement, but this may not be sufficient to control 3GC-resistant E. coli strains, because these organisms, either vertically or horizontally transmitted, can persist in the absence of antibiotic selection pressure during the whole lifetime of the flock. Very strict biosecurity measures must be implemented, and research is needed to find effective ways to reduce the prevalence of 3GC resistance in poultry production.

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