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Impact of the administration of a third-generation cephalosporin (3GC) to one-day-old chicks on the persistence of 3GC-resistant *Escherichia coli* in intestinal flora: An *in vivo* experiment

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ABSTRACT

The aim of the experiment was to evaluate under controlled conditions the impact on the excretion of 3GC-resistant *Escherichia coli* of the injection of one-day-old chicks with ceftiofur, a third-generation cephalosporin (3GC). Three isolators containing specific-pathogen-free chicks were used. In the first one, 20 birds were injected with ceftiofur then ten of them were orally inoculated with a weak inoculum of a 3GC-resistant *E. coli* field isolate containing an IncI1/ST3 plasmid encoding a $bla_{CTX\text{-}M\text{-}1}$ beta-lactamase. The other chicks were kept as contact birds. None of the 20 birds in the second isolator were injected with ceftiofur, but ten of them were similarly inoculated with the 3GC-resistant strain and the others kept as contact birds. A third isolator contained ten non-injected, non-inoculated chicks. Fecal samples were collected regularly over one month and the *E. coli* isolated on non-supplemented media were characterized by antimicrobial agar dilution, detection of selected resistance genes and determination of phylogenetic group by PCR. The titers of 3GC-resistant *E. coli* in individual fecal samples were evaluated by culturing on 3GC-supplemented media. Results showed that the inoculated strain rapidly and abundantly colonized the inoculated and contact birds. The ceftiofur injection resulted in significantly higher percentages of 3GC-resistant *E. coli* isolates among the analyzed *E. coli*. No transfer of the 3GC-encoding plasmid to other isolates could be evidenced. In conclusion, these results highlight the dramatic capacity of 3GC-resistant *E. coli* to colonize and persist in chicks, and the selecting pressure imposed by the off-label use of ceftiofur.

1. Introduction

In many European countries, a dramatic increase in the prevalence of third-generation cephalosporin-resistant (3GC-R) *Escherichia coli* in broilers has been observed (EFSA, 2011). The resistance most often results from the production of extended-spectrum beta-lactamases (ESBL) encoded by plasmids. A possible link between the off-label use of 3GC in hatcheries and the increase in 3GC resistance of *E. coli* is suspected (EFSA, 2011). Previously, we monitored the prevalence of 3GC-R *E. coli* in commercial flocks of 3GC-treated or non-treated broilers and future layers, and demonstrated that 3GC-R strains are introduced early in flocks and that the use of 3GC in hatcheries promotes the selection of 3GC-R *E. coli* (Baron et al., 2014). To expand knowledge on the behavior of 3GC-R *E. coli* in young chicks, an *in vivo* experiment was conducted under controlled conditions to evaluate the colonization, persistence and spread of 3GC-R *E. coli* or its ESBL-encoding plasmid in experimentally inoculated one-day-old chicks previously injected or not with a 3GC.

2. Material and methods

2.1. Bacterial strain

The *E. coli* 369 strain had previously been isolated from fecal samples of seven-day-old non-treated broilers (Baron et al., 2014). The strain had been isolated on non-supplemented MacConkey

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media and was characterized by determining its susceptibility using the broth dilution method (CLSI, 2008) with Sensititre plates (Biocentric, Bandol, France). Its phylogenetic group was determined (Clermont et al., 2013) and the presence of the bla_{CTX-M} gene detected by PCR (Woodford et al., 2006). The strain was further characterized by determining the entire sequence of the plasmid encoding the bla_{CTX-M} gene using Mi-seq Illumina technology (paired-end 2×250 nucleotides). Sequences were cleaned with Trimmomatic 0.32 (Bolger et al., 2014) software (ILLUMINACLIP: illumina_oligos_and_reverse_complements:2:30:5:1:true LEADING:3 TRAILING:3 MAXINFO:40:0.2 MINLEN:36 options). Two bowtie2 (Langmead and Salzberg, 2012) alignments were performed (non-deterministic, very-sensitive options) on cleaned sequences. The first was aligned to the $bla_{CTX-M-1}$ gene (DQ915955) so as to evaluate sequencing depth, and the second to phiX174. This second alignment was performed to remove reads matching phiX174 material which is used in Illumina sequencing in the event of very redundant samples. The unaligned reads were downsampled to fit a global coverage estimation of 80 times. Raw reads were deduced from remaining unaligned reads and provided for MIRA *de-novo* assembly (Chevreux et al., 1999). Redundant or poorly covered contigs were filtered out. The resulting assembly was submitted to ResFinder (Zankari et al., 2012) to identify resistance genes and to the RASTA-Bacteria program version 2.12 (http://genoweb1.irisa.fr/duals/RASTA-Bacteria/) (Sevin and Barloy-Hubler, 2007) to identify putative toxin-antitoxin systems. The online Rapid Annotation Subsequencing technology (RAST: http://rast.nmpdr.org/rast.cgi) was used to annotate the plasmid sequence (Aziz et al., 2008). The BLASTn search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to find similar nucleotide sequences. The nucleotide sequence of the p369 plasmid was deposited under GenBank accession number KT779550.

The *E. coli* 369 strain was grown on Mueller Hinton media to prepare the inoculum. The titer of the inoculum was determined by plating serial dilutions on Mueller Hinton agar plates.

2.2. Experiment design

Fifty male and female specific-pathogen-free (SPF) one-day-old Leghorn chicks were obtained from the ANSES experimental flock. They were randomly assigned to five experiment groups. The first isolator (A) contained 20 ceftiofur-treated birds, of which ten were inoculated with *E. coli* 369 (inoculated treated group (IT)) and ten kept as non-inoculated contact birds (contact treated group (CT)). The second isolator (B) contained 20 birds not treated with ceftiofur. In this group, ten birds were inoculated with the *E. coli* 369 strain (inoculated non-treated group (INT)) and ten kept as non-inoculated contact birds (contact, non-treated group (CNT)). The third isolator (C) housed ten chicks neither treated with ceftiofur nor inoculated with *E. coli* 369 (control). On the first day of life (Day 0), all 20 birds in isolator A received a subcutaneous injection of ceftiofur (0.1 mg per bird, CEVAXEL 50 mg/l, CEVA, Libourne, France) whereas the 20 chicks in isolator B were injected with a sterile buffer. Five hours later, ten randomly chosen birds from each isolator were orally inoculated with 0.1 ml peptone buffer containing 10^3 colony forming units (CFU) of *E. coli* 369, whereas the other birds in the isolators were kept as non-inoculated contact birds. As far as possible, individual fecal samples were collected daily for the first five days and then on days 14, 26 and 34, the end of the experiment. During the first 14 days, the chicks were placed in individual boxes inside the isolator for one hour, then fecal samples (0.25–2 g) were collected from the bottom of the box. On Days 26 and 34, cloacal pressure was applied. However, sometimes (particularly on Day 1) it was not possible to obtain fecal material from all the birds. Feed was provided *ad libitum* and consisted of a home-made feed sterilized by vapor injection. No antibiotic additive was present in the feed. The experiment was performed in accordance with the animal welfare experimentation recommendations issued by the *Direction Départementale de la Protection des Populations des Côtes d'Armor* (ANSES registration no. B-22-745-1), and was approved by the ComEth ANSES/ENVA/UPEC ethics committee (authorization no. 12–005).

2.3. Bacteriological analysis

The titers of 3GC-resistant *E. coli* in the individual fecal samples collected on Days 3, 4, 5, 14, 26 and 34 were determined by spreading 100 μl of tenfold dilutions on TBX (Bio-Rad) agar plates containing 2 mg/l cefotaxime. After incubation, the colonies were enumerated and the mean titers calculated for each group and day. The detection limit was 1000 CFU/g of feces. The diluted fecal samples were then frozen after addition of 20% glycerol. Then 100 μl of the 1/100 diluted fecal samples collected on Days 1, 2, 3, 4, 5, 14, 26 and 34 were inoculated on MacConkey agar plates. As far as possible, one colony with typical *E. coli* morphology was stored for each sample and studied further. After identification by PCR (Bej et al., 1991) and determination of the phylogenetic group (Clermont et al., 2013), the susceptibility of the isolates was evaluated by inoculation of a standardized inoculum on cefotaxime (CTX) (2 mg/l), nalidixic acid (NAL) (16 mg/l), streptomycin (STR) (16 mg/l), sulfamethoxazole (SMX) (512 mg/l) and tetracycline (TET) (128 mg/l) supplemented Mueller Hinton agar plates for comparison with the resistance profile of the inoculated *E. coli* 369. The presence of the bla_{CTX-M}, *sul2*, *tet*A and IncI1 genes was detected by PCR (Carattoli et al., 2006; Nolvak et al., 2013; Pei et al., 2006; Woodford et al., 2006). A selection of 20 isolates – including 16 randomly chosen CTX-resistant isolates obtained from the four inoculated and contact groups on four different days and sharing the characteristics of the inoculated strain (one random isolate per group and per day) and four isolates with different resistance profiles – was submitted to pulse field gel electrophoresis after digestion with the restriction enzyme *Xba*I and their profiles were compared to that of the inoculated *E. coli* 369 strain (Ribot et al., 2006).

2.4. Statistical tests

The individual titers of 3GC-resistant *E. coli* were $\log_{10}$-transformed and, for each day, significant differences between groups identified using analysis of variance. Parametric hypotheses (*i.e.*, normality, variance equality and random distribution of the associated residuals) were carefully checked. Groups were then compared using the Student–Newman–Keuls test. Analyses were carried out with R software by means of the lm and SNK test functions (agricolae package).

Distributions of CTX-susceptible and CTX- resistant *E. coli* isolates obtained on non-supplemented media for each group were compared through a Fisher exact test (http://marne.u707.jussieu.fr/biostatgv/?module=tests/fisher). For all tests, *P* values below 0.05 were considered statistically significant differences.

3. Results

3.1. Characterization of the E. coli 369 strain

The minimum inhibitory concentrations were found to be: CTX: >2 mg/l; STR: 128 mg/l; NAL: 128 mg/l; SMX: >512 mg/l; TET: 128 mg/l; cefoxitin (FOX): $<=2$ mg/l and ciprofloxacin (CIP): 0.25 mg/l. It was determined that the strain belongs to phylogenetic group B1 and contains an ESBL gene (bla_{CTX-M}).