NOTE *Public Health*

Factors for Occurrence of Extended-Spectrum β-Lactamase-Producing *Escherichia coli* in Broilers

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ABSTRACT. To clarify the factors for occurrence of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* in broilers, two flocks (1 day of age) fed a diet with or without antibiotics were kept in a broiler house sanitized with disinfectants. ESBL-producing *E. coli*, however, was detected at a concentration of over 10^6 CFU/g of feces at 9 days of age to 49 days of age in both broiler flocks. Therefore, this indicated that the antibiotics other than cephalosporins used in this study had no effect due to co-selection on the numbers of ESBL-producing *E. coli* in broiler feces during this period. When a flock was kept with diet containing antibiotics for 49 days in a laboratory animal room, no ESBL-producing *E. coli* was detected in the flock. These results suggest that the occurrence of ESBL-producing *E. coli* may not be related to feeding with antibiotics and that the contamination of broiler houses with ESBL-producing *E. coli* might be an important factor.

KEY WORDS: antibiotics, broiler, *Escherichia coli*, extended-spectrum β-lactamase-producing, occurrence.

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Extended-spectrum β-lactamase (ESBL)-producing bacteria have antibiotic resistance to third generation cephalosporins such as cefotaxime (CTX). Reports on *Escherichia coli* carrying broad-spectrum β-lactamases isolated from food-producing animals and humans have been published worldwide [8]. ESBL-producing *Klebsiella pneumoniae*, *E. coli* and *Proteus mirabilis* have been implicated in numerous outbreaks of nosocomial infections over the last 2 decades [3, 10, 13]. CTX-M-2-producing *E. coli* from cattle in Japan has been reported [11], and the Japanese Veterinary Antimicrobial Resistance Monitoring Program (JVARM) reported isolation of ESBL-producing *E. coli* from poultry in Japan [6]. In addition, a potential increase in the ESBL-producing *E. coli* isolated from broilers was also reported [4]. Our previous study [5] clarified the high prevalence of ESBL-producing commensal enteric bacteria in broilers in Japan. However, use of third-generation cephalosporins on broilers is not permitted in Japan. The factors of occurrence of ESBL-producing *E. coli* in broiler are still unclear. The aim of this study was to clarify these factors.

Two flocks with 16 broilers each (Chunky, 1 day of age) were newly introduced to a hygienic broiler house sanitized with disinfectants. Although this windowless broiler house had been maintained in all-in-all-out system and sanitized with disinfectants immediately after all the broilers were marketed, ESBL-producing *E. coli* strains had been isolated from broiler feces collected in this broiler house in the last two examinations. A flock fed a diet with antibiotics (salinomycin 50 ppm and enramycin 7 ppm from 1 to 21 days of age; salinomycin sodium salt 50 ppm, gentamycin 5 ppm and colistin sulfate 5 ppm from 22 to 42 days of age; no antibiotics from 43 to 49 days of age), and another flock without antibiotics was raised until 49 days of age with hygienic water. The broiler house operating procedures were conventional. Harada *et al.* [2] reported that the use of dihydrostreptomycin and trimethoprim in cattle and pigs apparently contributes to the selection of chloramphenicol (CP)-resistant strains of *E. coli*, despite a ban on the use of CP in Japan. In this study, antibiotics other than third generation cephalosporins, such as salinomycin, were used to investigate the occurrence of ESBL-producing *E. coli* due to co-selection. To monitor ESBL-producing *E. coli* and ESBL-producing bacteria other than *E. coli*, feces from each flock were collected 6 times until 49 days of age. A portion of feces was diluted with 9 times volume of phosphate-buffered saline (PBS) and then 10-fold diluted to 10^{-6} with PBS. The dilutions (0.1 m*l*) were plated onto CHROMagar ESBL (CHROMagar, Paris, France) to isolate ESBL-producing *E. coli* and onto ChromoCult Coliform Agar ES (Merck, Darmstadt, Germany) with 1 μg/m*l* CTX to isolate ESBL-producing *Enterobacteriaceae* except for *E. coli* and without CTX to isolate CTX-sensitive *Enterobacteriaceae* in duplicate. After incubation at 37°C for 24 hr, colonies suspected to be *E. coli* on CHROMagar ESBL were counted, and four of the colonies were isolated. On

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Fig. 1. The occurrence of extended-spectrum β-lactamase-producing *Escherichia coli* in two flocks fed a diet with or without antibiotics.

ChromoCult Coliform Agar ES with CTX, the typical *Enterobacteriaceae* (e.g., *Enterobacter cloacae*, *Klebsiella oxytoca, Klebsiella pneumoniae*) morphology formed salmon pink to red colonies due to β-D-glucuronidase-negative and β-D-galactosidase-positive characteristics. *Proteus mirabilis and Salmonella* spp. formed colorless colonies due to β-D-glucuronidase- and β-D-galactosidase-negative characteristics. Some colonies were also isolated, and the species were identified by biochemical characteristics with a commercial kit (API, BioMérieux, Marcy-l'Etoile, France). These isolates from CHROMagar ESBL and ChromoCult Coliform Agar ES with CTX were tested for β-lactamase gene ($bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}9}$, bla_{SHV}, bla_{TEM}, $bla_{CMY\text{-}2}$) by the methods of Lal *et al.* [7], Fang *et al.* [1], Woodford *et al.* [14] and Kojima *et al.* [6]. These ESBL gene-positive isolates were tested for the production of ESBL by a method of the Clinical Laboratory Standards Institute (CLSI). The rate of colonies positive for ESBL gene was multiplied by the number of colonies suspected of being *E. coli* on CHROMagar ESBL to determine the number of ESBL-producing *E. coli*.

ESBL-producing *E. coli* appeared in both flocks with and without antibiotics at a population of 10^6–10^8 CFU/g of feces from 9 days of age to 49 days of age, while the bacterium was not detected at 1 day of age (Fig. 1). All *E. coli* isolates on CHROMagar ESBL were positive for $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}2}$, and they produced ESBL. Most isolates of *Enterobacter cloacae*, *Proteus mirabilis*, *Salmonella* spp., *Klebsiella oxytoca* and *Klebsiella pneumoniae* on ChromoCult Coliform Agar ES without CTX were susceptible to 1 μg/ml CTX and not ESBL-producing bacteria. The isolates such as *E. cloacae* and *Salmonella* spp. on ChromoCult Coliform Agar ES with CTX were resistant to 1 μg/ml CTX and did not produce ESBL. In this context, it was revealed that there were ESBL-producing *E. coli* and enteric bacteria susceptible to cephalosporins and enteric bacteria resistant to cephalosporins, with the exception of ESBL producers, in the same broiler feces. In this study, no ESBL producers except *E. coli* were isolated from the flocks with or without antibiotics. Therefore, it was not clarified whether antibiotic intake of broilers promotes the growth of ESBL producers. In addition, it was suggested that ESBL genes are not essential to enteric bacteria of broilers. These results indicated that feeding with antibiotics had no effects on co-selection of ESBL-producing *E. coli* in the broiler flock. The source of the ESBL producers in the broiler feces was not clarified. However, we found that a small number of ESBL-producing *E. coli* in broiler feces grew to large numbers by 49 day of age. Contamination of the broiler house with the bacterium might be a more important factor for the occurrence than intake of antibiotics.

In a laboratory animal room that had never been used to keep broilers, a flock with 12 broilers (1 day of age) fed the diet with antibiotics was kept for 49 days, and their feces were monitored for ESBL-producing *E. coli* by the same method described above. CTX-susceptive *E. coli* was detected until 49 days of age, and there was no *E. coli* on ChromoCult Coliform Agar ES with CTX and CHROMagar ESBL.

Therefore, this suggests that there are differences between the environments of the laboratory animal room and the broiler house. Raising flocks in hygienic environments might be important for inhibition of infection with ESBL-producing *E. coli*.

Smet *et al.* described that ESBL-producing bacteria grew in an environment without CTX [12]. The present study also indicated the same results. In addition, Murase *et al.* reported that genetically related strains of *E. coli* were isolated from broilers being raised at a broiler farm without antibiotics [9]. However, strains having various patterns of drug resistance appeared at a broiler farm without antibiotics. It seemed that mobile genetic elements such as R-plasmid affected the drug resistance patterns. Analysis by pulsed-field gel electrophoresis of ESBL-producing *E. coli* and CTX-sensitive *E. coli* would produce additional information. Further studies on a large scale and in newly opened farms are required to prevent contamination of ESBL-producing *E. coli* in broilers.

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