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# Dynamics of CMY-2 producing *E. coli* in a broiler parent flock

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ABSTRACT

Extended-spectrum β-lactamase and plasmid mediated AmpC β-lactamase (ESBL/pAmpC) producing bacteria are resistant to Extended Spectrum Cephalosporins (ESC), and are present in all levels of the broiler production chain. We determined the prevalence, concentration, and persistence of ESBL/pAmpC-*Escherichia coli* in a broiler parent flock during the rearing and laying period. One-day old chickens were housed in four separate pens. Until week 33 no antibiotics or coccidiostatics were used. During rearing 57 chickens in each pen ($n$ = 228), and in the laying period two groups of 33 chickens were individually sampled ($n$ = 66). Environmental samples were taken from week 16 onwards. ESBL/pAmpC-*E. coli* presence was determined by selective culturing. In the samples of week 16–19 the concentration of ESBL/pAmpC-*E. coli* was determined. All ESC-resistant isolates found were positive for pAmpC gene $bla_{CMY-2}$ located on IncA/C plasmids, in several *E. coli* MLST types. CMY-2-*E. coli* prevalence decreased from 91% (95%CI 86–94%) at day 7 (week 1) to 0% (95%CI 0–5%) in week 21. However, CMY-2-*E. coli* remained present in the environmental samples during the whole study. CMY-2-*E. coli* concentration varied between detection limit (< 10^3) and 2·10^4 cfu/g faeces. The sharp reduction of CMY-2-*E. coli* in this broiler parent flock in absence of antibiotics suggests a selective disadvantage of $bla_{CMY-2}$ on IncA/C plasmids on animal level. The underlying mechanism should be studied further as this may provide new insights on how to reduce ESBL/pAmpC prevalence and transmission in the broiler production chain.

## 1. Introduction

Extended-spectrum β-lactamase and plasmid mediated AmpC β-lactamase (ESBL/pAmpC) producing bacteria are resistant to Extended Spectrum Cephalosporins (ESC). In the Netherlands, 56.5% of the broilers at slaughter were carriers of ESBL/pAmpC-*Escherichia coli* in 2015 (MARAN, 2016). Although prevalence in poultry varies between farms (Blaak et al., 2015), ESBL/pAmpC producing bacteria are present in all levels of the broiler production chain (Dierikx et al., 2013). The broiler production chain has a pyramidal structure, thus the presence of ESBL/pAmpC in the upper levels of the chain might influence the ESBL/pAmpC status of lower levels in the chain, e.g. through vertical transmission (Nilsson et al., 2014; Zurfluh et al., 2014). To our knowledge reports on the dynamics of ESBL/pAmpC-*E. coli* in parent stock are lacking. The aim of this study is to determine prevalence, faecal concentration, and persistence of ESBL/pAmpC-*E. coli* in a broiler parent flock during the rearing and laying period.

## 2. Material and methods

### 2.1. Chickens

One-day old broiler parent stock chickens ($n$ = 3184) were housed in a rearing house of an experimental poultry farm in the Netherlands. The chickens were divided over four completely separated pens. Each pen housed 693 females and 103 males, separated by a fence. At week 20 all chickens were moved to the laying house. Two groups of 30 females and three males were selected from the four rearing pens, and randomly allocated to two separate pens. During the laying phase two females died and one lame male was replaced in pen 1 and one female died in pen 2.

Chickens received feed without antibiotics or coccidiostats. Feed and water were available *ad libitum* during the first seven days, thereafter feed was supplied based on body weight. Drinking water pipes were cleaned before entry of the chickens and thereafter weekly by acidifying the water using peracetic acid. Chickens received a microflora product (Aviguard®) at day of arrival. A standard vaccination

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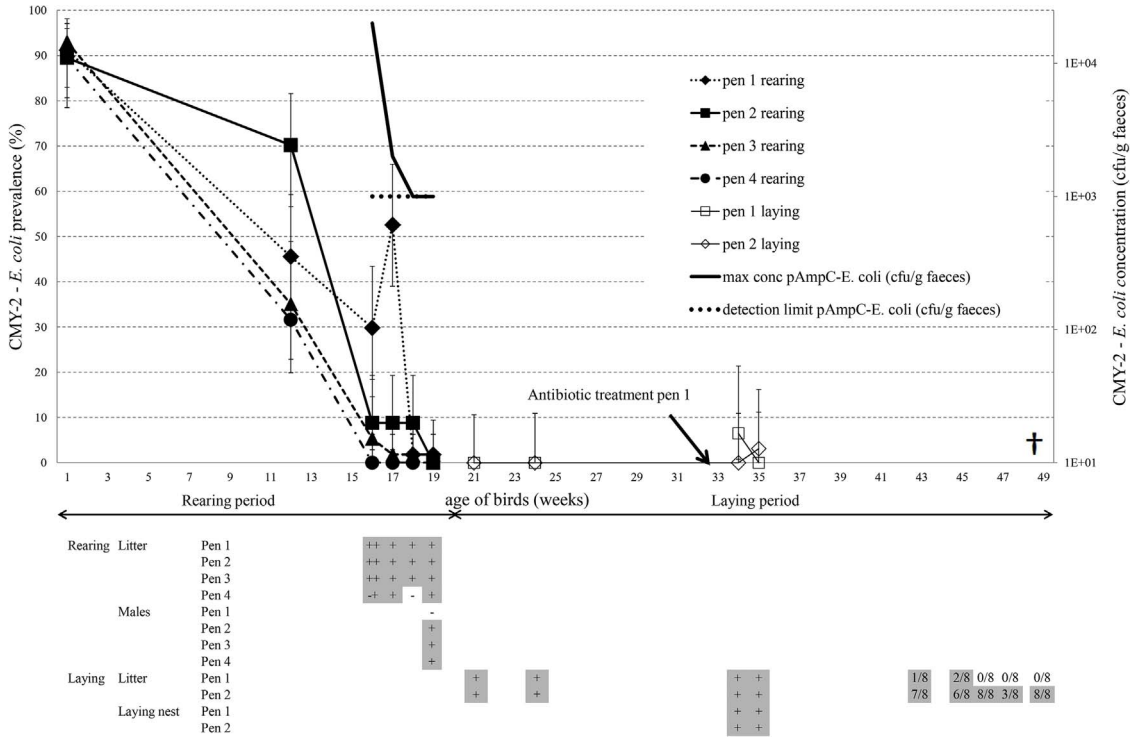



**Fig. 1.** CMY-2-*E. coli* prevalence (%), concentration (cfu/g faeces) and presence in the environment in a broiler parent flock during the rearing and laying period.

and lighting scheme was applied.

### 2.2. Identification and sampling

#### 2.2.1. Rearing period

At day 7 (week 1), in each pen 57 females (minimal sample size to detect 5% prevalence) were selected randomly, and sampled by individual cloacal swabs. At week 12 per pen 57 females were randomly selected, tagged and individual cloacal swabs were taken. In week 16–19 the tagged females were sampled weekly and environmental samples were taken using bootsocks. In week 19 environmental samples were taken in the male pens.

#### 2.2.2. Laying period

At week 20, the females and males were moved to the laying house. All females found ESBL/pAmpC-*E. coli* positive at least once during week 16–19 and a random selection of females being ESBL/pAmpC-*E. coli* positive in week 12 were selected and housed in two groups of 30 females. To each group three randomly selected and tagged males were added. All chickens were sampled individually in week 21, 24, 34 and 35. Environmental samples from the litter were taken at week 21, 24, 34 and 35 (morning) and 43, 45, 46, 47 and 49 (noon). In week 34 and 35 also environmental samples from the laying nests were taken, by hand wiping using bootsocks. In week 49 chickens were euthanized and caecal content was collected.

### 2.3. Antibiotic treatment

In week 33 the chickens in pen 1 were administered amoxicillin via the drinking water for five days (20 g/1000 kg live weight/day).

### 2.4. Follow up offspring

During week 34, 160 eggs were collected from both pens and disinfected with formaldehyde. Forty eggs were crushed, eggshells and egg content was mixed and analysed for ESBL/pAmpC presence, 120 eggs were incubated. After hatching, individual cloacal swabs were taken from the broilers at day of hatch, daily until day 7 and at days 14 and 21. Environmental samples were taken at the same days, starting the day after hatch. At day 21 broilers were euthanized and caecal content was collected.

### 2.5. Ethics

The animal procedures at Utrecht University were approved by the Animal Ethical Committee of Utrecht University (Utrecht, the Netherlands), in full compliance with all relevant legislation.

### 2.6. Analysis

#### 2.6.1. ESBL/pAmpC-E. coli detection

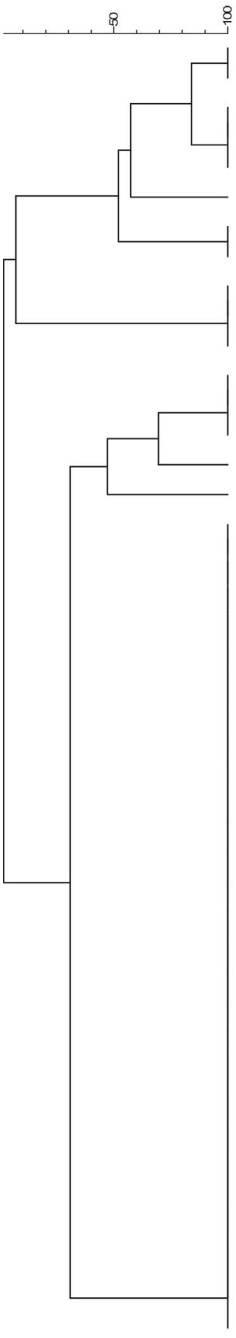
Cloacal and caecal samples, eggs and bootsocks were selectively cultured (3 mL LB broth versus 400 mL LB, supplemented with 1 mg/L cefotaxime). After overnight incubation at 37 °C, 10 µL broth was inoculated on MacConkey plates supplemented with 1 mg/L cefotaxime and incubated overnight at 37 °C. Cloacal samples were analysed individually. Eggs and caecal samples were pooled per five, bootsocks were pooled per pen, except for bootsocks taken in week 16 and 43–49.

#### 2.6.2. ESBL/pAmpC-E. coli and E. coli concentration

Swabs used in week 16–19 in pen 1 and 2 were weighed before and after sampling to determine the amount of faeces collected. Swabs were suspended in 1 mL saline solution and tenfold dilution series were made to quantify the colony-forming units (cfu) of ESBL/pAmpC-*E. coli* and total *E. coli* per mL, using MacConkey plates with and without 1 mg/L cefotaxime. Based on the amount of faeces on the swabs cfu/gram faeces was calculated.

#### 2.6.3. Typing

From week 12 onwards, in at least one isolate of every sampling moment, ESBL/pAmpC genes were typed by PCR and sequencing (Dierikx et al., 2010). Plasmids were characterized by transformation (Dierikx et al., 2010) and PCR-based Replicon Typing (PBRT) (Diatheva, Italy). Selection of transformants was performed on LB agar




| MLST ST | pAmpC gene | Other gene | Plasmid | Week | Pen | Sample type |
|---|---|---|---|---|---|---|
| 224 | CMY-2 | TEM-1 | IncA/C | 21 | L2 | environment |
| 224 | CMY-2 | TEM-1 | IncA/C | 34 | L1 | cloacal |
| 2485 | CMY-2 | | IncA/C | 17 | R3 | environment |
| 2485 | CMY-2 | | IncA/C | 18 | R3 | environment |
| 2485 | CMY-2 | | IncA/C | 19 | R3 M | environment |
| 648 | CMY-2 | TEM-1 | IncA/C | 16 | R2 | environment |
| 155 | CMY-2 | | IncA/C | 12 | R4 | cloacal |
| 155 | CMY-2 | | IncA/C | 19 | R1 | environment |
| 156 | CMY-2 | TEM-1 | IncA/C | 17 | R2 | environment |
| 156 | CMY-2 | TEM-1 | IncA/C | 19 | R2 M | environment |
| 156 | CMY-2 | TEM-1 | IncA/C | 19 | R4 M | environment |
| 355 | CMY-2 | | IncA/C | 16 | R3 | environment |
| 355 | CMY-2 | | IncA/C | 18 | R3 | cloacal |
| 355 | CMY-2 | | IncA/C | 19 | R3 | environment |
| new | CMY-2 | | IncA/C | 19 | R2 | environment |
| new | CMY-2 | | IncA/C | 44 | L1 | environment |
| 10 | CMY-2 | TEM-1 | IncA/C | 12 | R1 | cloacal |
| 10 | CMY-2 | | IncA/C | 12 | R2 | cloacal |
| 10 | CMY-2 | TEM-1 | IncA/C | 12 | R3 | cloacal |
| 10 | CMY-2 | TEM-1 | IncA/C | 16 | R1 | cloacal |
| 10 | CMY-2 | TEM-1 | IncA/C | 16 | R1 | environment |
| 10 | CMY-2 | | IncA/C | 16 | R4 | environment |
| 10 | CMY-2 | TEM-1 | IncA/C | 17 | R1 | environment |
| 10 | CMY-2 | | IncA/C | 17 | R4 | environment |
| 10 | CMY-2 | TEM-1 | IncA/C | 18 | R1 | environment |
| 10 | CMY-2 | | IncA/C | 18 | R2 | environment |
| 10 | CMY-2 | TEM-1 | IncA/C | 19 | R4 | environment |
| 10 | CMY-2 | TEM-1 | IncA/C | 21 | L1 | environment |
| 10 | CMY-2 | TEM-1 | IncA/C | 24 | L1 | environment |
| 10 | CMY-2 | | IncA/C | 24 | L2 | environment |
| 10 | CMY-2 | | IncA/C | 34 | L1 | laying nest |
| 10 | CMY-2 | | IncA/C | 34 | L1 | environment |
| 10 | CMY-2 | TEM-1 | IncA/C | 34 | L2 | laying nest |
| 10 | CMY-2 | TEM-1 | IncA/C | 34 | L2 | environment |
| 10 | CMY-2 | TEM-1 | IncA/C | 35 | L2 | cloacal |
| 10 | CMY-2 | | IncA/C | 35 | L1 | laying nest |
| 10 | CMY-2 | | IncA/C | 35 | L1 | environment |
| 10 | CMY-2 | TEM-1 | IncA/C | 35 | L2 | laying nest |
| 10 | CMY-2 | | IncA/C | 35 | L2 | environment |
| 10 | CMY-2 | | IncA/C | 44 | L2 | environment |
| 10 | CMY-2 | TEM-1 | IncA/C | 45 | L2 | environment |
| 10 | CMY-2 | TEM-1 | IncA/C | 46 | L2 | environment |
| 10 | CMY-2 | | IncA/C | 47 | L2 | environment |
| 10 | CMY-2 | TEM-1 | IncA/C | 49 | L2 | environment |

**Fig. 2.** Multilocus sequence typing (MLST), gene- and plasmid characteristics of cloacal and environmental samples from females and males (M) in different pens during rearing (R1-R4) and laying (L1, L2) period.

containing 1 mg/L cefotaxime. *E. coli* genotyping was performed by MLST (Wirth et al., 2006). MLST patterns were analysed using Bionumerics version 6.1.

## 3. Results and discussion

At day 7 (week 1) prevalence of ESBL/pAmpC-*E. coli* ranged between pens from 89 to 93% (Fig. 1). All isolates carried the pAmpC gene $bla_{CMY-2}$ on IncA/C plasmids (Fig. 2). Overall CMY-2-*E. coli* prevalence showed a remarkable decrease, from 91% (range 89–93%) at day 7 to 46% (32–70%) in week 12, 11% (0–30%) in week 16, 16% (0–53%) in week 17, 3% (0–9%) in week 18 and 1% (0–2%) in week 19, without intervention. During the laying period (week 21, 24) no positive cloacal swabs were found. All 44 typed isolates carried $bla_{CMY-2}$ and 22 samples were also carrying $bla_{TEM-1}$. The predominant *E. coli* sequence type (ST10, 28 samples) was found in all pens, suggesting clonal spread. The $bla_{CMY-2}$-IncA/C combination was found in different *E. coli* STs suggesting plasmid spread.

The high prevalence in week 1 might be the result of vertical transmission from the grandparent flock or other sources of contamination at the hatchery or during transport. The grandparent flock had been treated with antibiotics in the weeks prior to production of the parent stock. Unfortunately, no data about the ESBL/pAmpC prevalence in this flock is available. Despite the high prevalence at day 7, CMY-2-*E. coli* was not able to persist in the chickens. Other studies in poultry have also shown a decreasing prevalence of antibiotic resistant bacteria, with and without the use of antibiotics (Diarra et al., 2007; Baron et al., 2014; Huijbers et al., 2015). However, most of these studies report limited reduction. Factors as ageing (Lu et al., 2003), diet (Amerah et al., 2011), litter (Torok et al., 2009), probiotics (Nakphaichit et al.,

2011), disease (Stanley et al., 2012) and stress (Burkholder et al., 2008) might influence the microbiota composition and thus the potential of CMY-2-*E. coli* to persist in the gut. Until week 33 no antibiotics were used, resulting in no selective advantage to CMY-2-*E. coli*. After applying amoxicillin in week 33 in one of the pens, 2/31 chickens became positive in week 34, in the non-treated pen prevalence was 0%. However, one week later, 1/32 chickens was positive in the non-treated pen, whereas no positive samples were found in the treated pen (Fig. 1).

The inability to persist on animal level might be due to an unsuccessful combination of CMY-2-*E. coli* on plasmid IncA/C. In European broiler meat, CMY-2-*E. coli* is often found in combination with plasmids IncI1 or IncK (Borjesson et al., 2013; Egervarn et al., 2014). The low occurrence and the observed decrease in this study may suggest that plasmid IncA/C is less able to conjugate and spread in bacterial populations as was previously described for *Salmonella* (Poole and Crippen, 2009).

The decreasing prevalence was also represented by decreasing concentrations of CMY-2-*E. coli* in faeces. The maximum concentration of CMY-2-*E. coli* observed decreased from 2·10^4 cfu/g faeces in week 16, to 1·10^3 (detection limit) in weeks 18 and 19 (Fig. 1). During week 16–19 the total *E. coli* counts remained between 10^4 and > 10^8 cfu/g faeces.

Contrary to the decreasing prevalence and CMY-2-*E. coli* concentration in the faeces, almost all environmental samples (89/116) were positive (Fig. 1). Before placement of the chickens the laying house tested negative for ESBL/pAmpC-*E. coli*. Based on the positive environmental samples, negative cloacal swabs during the laying period and negative caecal samples at the end of the experiment, the chickens most likely introduced CMY-2-*E. coli* into the laying pens and after that ceased shedding CMY-2-*E. coli*. Environmental contamination might have persisted after the birds ceased excretion. Others report survival of *E. coli* and ESBL-*E. coli* in faeces and soil for months (Merchant et al., 2012). Although CMY-2-*E. coli* was still present in the environment during egg collection, none of the samples taken from the eggs and offspring were found positive for ESBL/pAmpC-*E. coli*.

## 4. Conclusion

This study showed that in a parent flock at an experimental farm, in absence of antibiotics, prevalence of pAmpC gene $bla_{CMY-2}$ on IncA/C plasmid decreased and is not detected in the offspring. This may not be true for other farms, with different ESBL/pAmpC-plasmids in *E. coli*, and under field conditions. The mechanism behind this should be studied further as this might lead to possible interventions to reduce ESBL/pAmpC prevalence and transmission in the broiler production chain.

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## Conflicts of interest

None.

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## References

Amerah, A.M., Peron, A., Zaefarian, F., Ravindran, V., 2011. Influence of whole wheat inclusion and a blend of essential oils on the performance nutrient utilisation, digestive tract development and ileal microbiota profile of broiler chickens. Br. Poult. Sci. 52, 124–132.

Baron, S., Jouy, E., Larvor, E., Eono, F., Bougeard, S., Kempf, I., 2014. Impact of third-generation-cephalosporin administration in hatcheries on fecal Escherichia coli antimicrobial resistance in broilers and layers. Antimicrob. Agents Chemother. 58, 5428–5434.

Blaak, H., van, H.A., Hamidjaja, R.A., van der, P.R., Kerkhof-de, H.L., de Roda, H.A., Schets, F.M., 2015. Distribution, numbers, and diversity of ESBL-producing *E. coli* in the poultry farm environment. PLoS One 10, e0135402.

Borjesson, S., Egervarn, M., Lindblad, M., Englund, S., 2013. Frequent occurrence of extended-spectrum beta-lactamase- and transferable ampc beta-lactamase-producing *Escherichia coli* on domestic chicken meat in Sweden. Appl. Environ. Microbiol. 79, 2463–2466.

Burkholder, K.M., Thompson, K.L., Einstein, M.E., Applegate, T.J., Patterson, J.A., 2008. Influence of stressors on normal intestinal microbiota, intestinal morphology, and susceptibility to Salmonella enteritidis colonization in broilers. Poult. Sci. 87, 1734–1741.

Diarra, M.S., Silversides, F.G., Diarrassouba, F., Pritchard, J., Masson, L., Brousseau, R., Bonnet, C., Delaquis, P., Bach, S., Skura, B.J., Topp, E., 2007. Impact of feed supplementation with antimicrobial agents on growth performance of broiler chickens Clostridium perfringens and enterococcus counts, and antibiotic resistance phenotypes and distribution of antimicrobial resistance determinants in *Escherichia coli* isolates. Appl. Environ. Microbiol. 73, 6566–6576.

Dierikx, C., van Essen-Zandbergen, A., Veldman, K., Smith, H., Mevius, D., 2010. Increased detection of extended spectrum beta-lactamase producing Salmonella enterica and Escherichia coli isolates from poultry. Vet. Microbiol. 145, 273–278.

Dierikx, C.M., Van, D.G., Smith, H.E., Kant, A., Mevius, D.J., 2013. Presence of ESBL/AmpC −producing *Escherichia coli* in the broiler production pyramid: a descriptive study. PLoS One 8.

Egervarn, M., Borjesson, S., Byfors, S., Finn, M., Kaipe, C., Englund, S., Lindblad, M., 2014. *Escherichia coli* with extended-spectrum beta-lactamases or transferable AmpC beta-lactamases and Salmonella on meat imported into Sweden. Int. J. Food Microbiol. 171, 8–14.

Huijbers, P.M., van Hoek, A.H., Graat, E.A., Haenen, A.P., Florijn, A., Hengeveld, P.D., van Duijkeren, E., 2015. Methicillin-resistant Staphylococcus aureus and extended-spectrum and AmpC beta-lactamase-producing Escherichia coli in broilers and in people living and/or working on organic broiler farms. Vet. Microbiol. 176, 120–125.

Lu, J., Idris, U., Harmon, B., Hofacre, C., Maurer, J.J., Lee, M.D., 2003. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. Appl. Environ. Microbiol. 69, 6816–6824.

MARAN, 2016. Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2015 .

Merchant, L.E., Rempel, H., Forge, T., Kannangara, T., Bittman, S., Delaquis, P., Topp, E., Ziebell, K.A., Diarra, M.S., 2012. Characterization of antibiotic-resistant and potentially pathogenic Escherichia coli from soil fertilized with litter of broiler chickens fed antimicrobial-supplemented diets. Can. J. Microbiol. 58, 1084–1098.

Nakphaichit, M., Thanomwongwattana, S., Phraephaisarn, C., Sakamoto, N., Keawsompong, S., Nakayama, J., Nitisinprasert, S., 2011. The effect of including Lactobacillus reuteri KUB-AC5 during post-hatch feeding on the growth and ileum microbiota of broiler chickens. Poult. Sci. 90, 2753–2765.

Nilsson, O., Borjesson, S., Landen, A., Bengtsson, B., 2014. Vertical transmission of *Escherichia coli* carrying plasmid-mediated AmpC (pAmpC) through the broiler production pyramid. J. Antimicrob. Chemother. 69, 1497–1500.

Poole, T., Crippen, T., 2009. Conjugative plasmid transfer between Salmonella enterica Newport and *Escherichia coli* within the gastrointestinal tract of the lesser mealworm beetle, Alphitobius diaperinus (Coleoptera: Tenebrionidae). Poult. Sci. 88, 1553–1558.

Stanley, D., Keyburn, A.L., Denman, S.E., Moore, R.J., 2012. Changes in the caecal microflora of chickens following Clostridium perfringens challenge to induce necrotic enteritis. Vet. Microbiol. 159, 155–162.

Torok, V.A., Hughes, R.J., Ophel-Keller, K., Ali, M., Macalpine, R., 2009. Influence of different litter materials on cecal microbiota colonization in broiler chickens. Poult. Sci. 88, 2474–2481.

Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L.H., Karch, H., Reeves, P.R., Maiden, M.C., Ochman, H., Achtman, M., 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol. Microbiol. 60, 1136–1151.

Zurfluh, K., Wang, J., Klumpp, J., Nuesch-Inderbinen, M., Fanning, S., Stephan, R., 2014. Vertical transmission of highly similar bla CTX-M-1-harboring IncI1 plasmids in *Escherichia coli* with different MLST types in the poultry production pyramid. Front. Microbiol. 5, 519.