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Impact of colistin sulfate treatment of broilers on the presence of resistant bacteria and resistance genes in stored or composted manure

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ABSTRACT

The application of manure may result in contamination of the environment with antimicrobials, antimicrobial-resistant bacteria, resistance genes and plasmids. The aim of this study was to investigate the impact of the administration of colistin and of manure management on (i) the presence of colistin-resistant *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and (ii) the prevalence of various antimicrobial resistance genes in feces and in composted or stored manure. One flock of chickens was treated with colistin at the recommended dosage and a second flock was kept as an untreated control. Samples of feces, litter and stored or composted manure from both flocks were collected for isolation and determination of the colistin-susceptibility of *E. coli*, *K. pneumoniae* and *P. aeruginosa* and quantification of genes coding for resistance to different antimicrobials. The persistence of plasmids in stored or composted manure from colistin-treated broilers was also evaluated by plasmid capturing experiments. Results revealed that colistin administration to chickens had no apparent impact on the antimicrobial resistance of the dominant *Enterobacteriaceae* and *P. aeruginosa* populations in the chicken gut. Composting stimulated an apparently limited decrease in genes coding for resistance to different antimicrobial families. Importantly, it was shown that even after six weeks of composting or storage, plasmids carrying antimicrobial resistance genes could still be transferred to a recipient *E. coli*. In conclusion, composting is insufficient to completely eliminate the risk of spreading antimicrobial resistance through chicken manure.

1. Introduction

Poultry may suffer from various infectious diseases, such as colibacillosis, fowl cholera, mycoplasmosis or necrotic enteritis. In such cases, veterinarians may prescribe antimicrobials which are given to the whole flock, most often orally (Chauvin et al., 2005). The most frequently administered pharmaceuticals are beta-lactams, macrolides, polymyxins, quinolones, sulfonamides and tetracyclines (Chauvin et al., 2007). The impact of these antibiotics on gut microbiota may include modifications to the bacterial populations and their metabolism, and selection of resistant bacteria either through chromosomal mutations or acquisition of resistance genes borne by mobile genetic elements (Modi et al., 2014). The presence of such resistant bacteria in the bird gut is worrisome not only for poultry health, but also for the consumer, because of possible contamination of carcasses at the slaughterhouse (EFSA, 2012). Furthermore, the safety of spreading manure obtained from poultry flocks has been questioned because it is a source of environmental contaminants (Graham et al., 2009; Leal et al., 2012): manure from treated animals may contain antimicrobials and their metabolites, resistant bacteria and resistance genes, the last two items also being commonly present in manure from non-treated herds (Sharma et al., 2009). Thus, the aim of the present study was to evaluate the impact of oral colistin administration on the presence of resistant bacteria or resistance

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genes in feces and stored or composted manure from both treated and non-treated broilers. Colistin was chosen because it is one of the most frequently used antimicrobials in broilers (Anses-ANMV, 2014). Colistin was also used initially in humans for treatment of infections caused by Gram-negative bacteria. Because of its nephrotoxicity, it was withdrawn but with increasing microbial resistance to current antibiotics, colistin has now been reintroduced into human therapy as a drug of last resort to treat multidrug resistant Gram-negative bacteria, such as *Pseudomonas aeruginosa*, the main pathogen implicated in cystic fibrosis. Colistin is a bactericidal antimicrobial that binds to lipopolysaccharide (LPS) and phospholipids in the outer cell membrane of Gram-negative bacteria. It competitively displaces divalent cations from the phosphate groups of membrane lipids, which leads to disruption of the outer cell membrane, leakage of intracellular contents, and bacterial death. Resistance of *Enterobacteriaceae* to colistin is associated with chromosomal mutations, but it is rarely encountered in *Escherichia coli* of avian origin (Kempf et al., 2013). The mechanisms of resistance of Gram-negative bacteria to colistin were recently reviewed by Olaitan et al. (2014). They include LPS modifications, such as modifications of lipid A with phosphoethanolamine and 4-amino-4-deoxy-L-arabinose, efflux pumps, formation of capsules and over-expression of the outer membrane protein OprH. A recent study of *E. coli* strains of animal origin presenting a low level of colistin resistance (MIC, 4 mg/L) characterized mutations in the *pmrAB* gene (Quesada et al., 2015). This gene encodes a two-component system composed of a sensor histidine kinase (PmrB) and its cognate regulator (PmrA), which, once phosphorylated, activates the expression of *pmr* genes (polymyxin resistance) leading to the covalent modification of lipopolysaccharide.

Consequently, we investigated how treatment with colistin might affect the prevalence of colistin-resistant bacteria (*E. coli*, *Klebsiella pneumoniae* and *P. aeruginosa*) and further assessed how manure treatment affects recovery of colistin-resistant bacteria. Because colistin resistance so far seems limited to chromosomal mutations and no specific colistin resistance gene has been described, it was not possible to monitor the variations of colistin resistance genes. However, because colistin administration and manure treatments may modify the bacterial composition of gut and manure microbiota, we decided to evaluate their impact on the prevalence of indicator genes coding for resistance to other antimicrobial families, and the possibility of such genes being captured via conjugative transfer by viable *Enterobacteriaceae*.

2. Material and methods

Seventeen thousand chickens (Ross PM3) were purchased from a local hatchery. The 170 boxes of 100 chicks were randomly allocated to twelve pens. The chicks were housed in the ANSES Ploufragan animal facilities in two separate but identical rooms each containing six pens of 70 m^2 at an initial density of 19.7 birds/m^2. The concrete floor was covered with untreated wood shavings as litter (about 4 kg/m^2). The chickens were maintained at an age-appropriate temperature, and supplied with food and water *ad libitum* throughout the trial period. Room 1 contained three pens on side A for producing composted manure from 4250 non-treated chickens (NT-Comp manure) and three pens on side B for producing stored manure from 4250 non-treated chickens (NT-Sto manure). Similarly, Room 2 contained three pens on side A for producing composted manure from 4250 colistin-treated birds (T-Comp manure) and three pens on side B for producing stored manure from 4250 colistin-treated birds (T-Sto manure). At 25 days of age (D25), the broilers in Room 2 were administered colistin sulfate (Colivet solution, CEVA, Libourne, France) at the recommended dose (37.5 ml Colivet per 1000 kg body weight, equivalent to 2.25 mg colistin base activity or 75,000 I U/kg per day for five consecutive days). Colivet was given daily in the drinking water, by pouring the colistin quantity in the tank containing the volume of water to be drunk during the next 24 h. The consumption of the total volume of medicated water was checked every day. The administration period was chosen so as to minimize the delay between administration and manure recovery after slaughter of broilers and to maximize the quantity of colistin administered, as it is proportional to the broiler weight. During the treatment period, 240 randomly sampled chickens were individually weighed daily to evaluate the mean chicken weight, and the water consumption of the group was recorded daily during the treatment period to calculate actual consumption. The birds in Room 1 were not treated with any antibiotics. The two rooms were separated by a technical room with prefabricated panels, the ventilation, water and feed circuits for the two rooms were independent and biosecurity measures were implemented to avoid diffusion of resistant bacteria between groups. The chickens were slaughtered at 33 days of age.

Immediately after departure of the broilers, the litter from each group of animals was collected separately, homogenized and transported to a meadow. Each type of manure (NT-Comp, NT-Sto, T-Comp or T-Sto) was gathered into a heap of approximately $6 \times 3 \times 1.5$ m on plastic sheets in order to be able to collect the manure leachates. All four heaps were covered with a polypropylene geotextile fabric for protection against bad weather. No amendment was added. Two heaps (T-Sto, from treated and NT-Sto, from non-treated broilers) were stored with no disturbance or change for six weeks, while the other heaps were turned over after three weeks to encourage composting (the T-Comp and NT-Comp heaps from treated and non-treated broilers, respectively)(AFNOR, 2010). During the first ten days, the temperature was recorded every 15 s by two sensors (Kistock KTT310 (KIMO)) placed in the middle of each heap. Thereafter, up to the sixth week, six sensors (Campbell Scientific) per heap placed near the core (approximately 1.5 m below the surface) and nearer the surface (20–50 cm deep) recorded temperatures every 30 s. The mean was recorded every 30 min.

2.1. Sampling

On arrival of the chicks, fresh meconium droppings were collected from eight transport box papers; pooled samples of fecal material from ten birds from the different parts of the pens were then obtained at different times before colistin treatment (one pooled sample per room on D4 and D25), during treatment (one pooled sample per each side of each room on D26, five per each side of each room on D27) or after treatment (five pooled samples per each side of each room on D30). Drinking-water samples were collected during the treatment days and blood samples were collected from ten treated and ten non-treated birds before and during treatment. Colistin was assayed in blood and drinking water samples by liquid chromatography with tandem mass spectrometry detection (LC–MS/MS) with a limit of quantification in water at 0.02 μg/ml (Gobin et al., 2010).

Samples of litter (a mixture of feces and wood shavings) were collected in the breeding rooms on D33 immediately after the broilers' departure. For each side of each room, litter samples were obtained from the different areas (resting and feeding areas), then pooled per type of area to obtain a total of 12 pooled samples per room. Manure samples were collected from the heaps after three or six weeks. For each heap, twelve samples were obtained 10–20 cm below the surface and sixteen samples 1.5 m below the surface. They were then pooled to produce three (Week 3) or four (Week 6) samples per type of manure (T-Sto, T-Comp, NT-Sto or NT-Comp). Leachates flowing from the different manure heaps were collected whenever possible.

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