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Antibiotic resistance genes in the faeces of dairy cows following short-term therapeutic and prophylactic antibiotic administration

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ABSTRACT

The objective of the research was to quantify three antibiotic resistance genes (*tetQ*, *cfxA* and *mefA*) in the faeces of dairy cows following therapeutic and prophylactic antibiotic treatments. Manure collected from dairy cows treated with either no antibiotic, pirlimycin hydrochloride (PIRL), ceftiofur crystalline free acid (CCFA) or cephapirin benzathine (CEPH) were submitted to quantitative PCR analysis. No treatment effects on the abundance of the *tetQ* and *cfxA* were observed. There was a trend for the abundance of the *mefA* to be increased in cows treated with PIRL ($P = 0.07$). Overall, the results showed no difference of measured three ARGs from cows receiving different antibiotics. Considering the limited scope of our investigation, further investigation is needed to provide more information on ARGs excretion from cows that received therapeutic and prophylactic antibiotic treatment.

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Antibiotics, commonly used for therapeutic and prophylactic purposes in livestock, are playing an important role in disease treatment and disease prevention. However, its use has caused a significant increase in antibiotic resistance genes (ARGs) and resistant bacteria selection in the environment (Fahrenfeld et al. 2014). Runoff from farms and land application of manure have been reported to carry over ARGs into the environment and agricultural produce (Marti et al. 2013). Rapid and widespread emergence of both animal and human pathogens resistant to multiple antibiotics, also known as 'superbugs', has caused great concerns of public (Aslam et al. 2018). An example would be methicillin-resistant *Staphylococcus aureus* (MRSA) that is resistant to methicillin, amoxicillin, penicillin, oxacillin and other common antibiotics known as cephalosporins. In Germany, it was found that at least 10% of sporadic infections of healthcare-associated (HA)-MRSA and community-associated (CA)-MRSA is due to livestock-associated MRSA (LA)-MRSA, which is initially associated with livestock (Cuny et al. 2015).

In the dairy industry, common antibiotic uses involve the treatment or prevention of bacterial infections in dairy calves as well as the prevention (dry cow therapy) or treatment of mastitis in dairy cows. When cattle are therapeutically and prophylactically treated with antibiotics, impacts on their intestinal bacteria are likely, such as increased resistance to the antibiotics used (Mirzaagha et al. 2011).

At present, resistance to tetracycline has spread to almost all bacterial genera due to its previous overuse in human and veterinary medicine, such as growth promoters in the animal industry (Aminov et al. 2001). It was reported tetracycline accounted for 64% of the total amount of antibiotics sales and distribution of medically important antimicrobials approved for use in food-producing animals in 2017 (FDA 2018). One of the tetracycline resistance genes is *tetQ*, which encodes a protein that modifies the ribosome, thus abolishing the inhibitory effects of tetracycline on protein synthesis (Chopra and Roberts 2001). The β-lactam antibiotics are a broad class of antibiotics, consisting of all antibiotic agents that contain a β-lactam ring in their molecular structures. The β-lactam resistance genes, such as *cfxA*, encode β-lactamase, which provides antibiotic resistance by breaking the antibiotic's ring structure (Munita and Arias 2016). The lincosamide, macrolide and streptogramin share the 50S subunit binding site, although they all possess a different structure. Since they function similarly, these antimicrobials are often linked together and called the Macrolide-Lincosamide-Streptogramin (MLS) group (Roberts 2008). The *mefA*, an example of MLS resistance genes, encodes the efflux pump, which can pump the antibiotics out of the bacterial cell, thus rendering it ineffective (Daly et al. 2004). Knowledge of the identity and distribution patterns of these resistance genes remains limited. The objective of this research was to use qPCR techniques to determine the effect of pirlimycin hydrochloride (mastitis treatment), ceftiofur crystalline free acid (metritis treatment) and cephapirin benzathine (dry cow therapy) on the temporal pattern of excretion of antibiotic resistance genes (*tetQ*, *cfxA* and *mefA*) to identify key time points for producers to focus their manure management strategies, helping to prevent the spread of antibiotic resistance.

The experiment was conducted under the review and approval of the Animal Care and Use Committee (protocol 12-184-DASC) at Virginia Polytechnic Institute and State University.

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Table 1. Primers for the quantitative PCR.

Gene	Primer	Annealing temperature	Reference
16S rRNA	F: 5′-CGGTGAATACGTTCYCGG-3′ R: 5′- GGWTACCTTGTTACGACTT-3′	56°C	Suzuki et al.(2000)
tetQ	F: 5′-AGAATCTGCTGTTTGCCAGTG-3′ R: 5′CGGAGTGTCAATGATATTGCA-3′	63°C	Aminov et al.(2001)
mefA	F: 5′-CGGTTACACCACTTTTAGTACCAGAAG-3′ R: 5′-TGCAACTGCCGGACTAACAA-3′	53°C	Looft et al.(2012)
cfxA	F: 5′-TGACTGGCCCTGAATAATCT-3′ R: 5′-ACAAAAGATAGCGCAAATCC-3′	55°C	Sóki et al.(2011)

Twelve Holstein cows in their first lactation (days in milk 110–200) from the Virginia Tech Dairy Center (Blacksburg, VA) were assigned to one of the four antibiotic treatments. Farm records showed that these cows had not received any antibiotic treatment for at least 9 months prior to parturition. During the study, cows were housed individually in tie-stalls with rubber mats and were fed a total mixed ration twice daily with *ad libitum* access to water. Except for the three dry cows that were assigned cephapirin benzathine for dry cow therapy, all of the other nine cows were randomly assigned to the rest of the three treatments. Cows for cephapirin benzathine treatment were selected based on the previously mentioned criteria as well as a scheduled date for dry-off that overlapped with the time of the experiment. Cows ($n = 3$) receiving the cephapirin benzathine (CEPH) treatment were dried-off on day 0. Before treated, the cows were milked, and each teat was cleaned with a gauze pad soaked in 70% isopropyl alcohol. Each teat was injected with 10 mL cephapirin benzathine (300 mg cephapirin, (ToMorrow®, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO),) and then sealed with Orbeseal® (bismuth subnitrate, colloidal silicon dioxide and liquid paraffin; Zoetis, Madison, NJ). Cows ($n = 3$) receiving the pirlimycin hydrochloride (PIRL) treatment were infused with 10 mL of pirlimycin hydrochloride (50 mg pirlimycin, Pirsue®, Zoetis, Madison, NJ) in their front left-quarter on day 0 and day 1. Again, similar to for the cows receiving dry-off therapy, the teat was cleaned before treatment. Cows ($n = 3$) receiving the ceftiofur crystalline free acid (CCFA) treatment were injected subcutaneously with 1.5 mL ceftiofur crystalline free acid sterile suspension (150 mg ceftiofur, Excede®, Zoetis, Madison, NJ) per 45.4 kg live weight at the base of the right ear on day 0 and again at the base of the left ear on day 3. The control group cows ($n = 3$) received no antibiotic. Faecal samples were collected from all cows 30 min prior to antibiotic administration on day 0 and once daily on day 1, 3 and 14 following treatment. Faecal samples were collected rectally using a clean palpation sleeve and sterile lubricant for each collection and were immediately stored in a −20°C freezer until analysis. DNA from samples was extracted using the QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Germantown, MD) and analysed with qPCR for 16S rRNA genes: *tetQ*, *cfxA* and *mefA* (Table 1). Statistical analysis was conducted using PROC GLIMMIX in SAS 9.2 (SAS Institute Inc., Cary, NC) with treatment, day and treatment by day interaction as fixed effects in the model. Data were logarithmically transformed to achieve normality before statistical analysis. Samples from d 0 were used as a covariate. Significance was declared at $P < 0.05$. Trends were declared at $P < 0.10$.

The PIRL-treated groups had a numerically lower abundance of 16S rRNA gene but not statistically different. The *tetQ* and *cfxA* gene abundance was not affected by treatment, day and treatment by day interaction (Table 2). There was a trend that the PIRL-treated cows had a higher absolute abundance of the *mefA* gene ($P = 0.07$).

Quantifying 16S rRNA gene, which exists in all bacteria cells, is one of the most popularly used techniques for quantifying bacteria community population in environmental samples (Suzuki et al. 2000). Hornish et al. (1992) reported that approximately 24% of the intra-mammary administered prilimycin was excreted through faeces. In the current study, the numerically lower bacteria counts (16S rRNA gene) of PIRL-treated cows could be due to a large percentage of pirlimycin entering the gut thus reducing bacteria population. In contrast, cephapirin does not easily cross from the udder into the blood (Gehring and Smith 2006); thus, the bacterial population in CEPH-treated cows was close to the control cows.

In most dairies, treatment for mastitis, metritis and dry cow therapy are mostly performed by antibiotic use. Pirlimycin hydrochloride for mastitis treatment belongs to the lincosamide class of antimicrobials. There was a trend that *mefA*, which encodes for resistance to the Macrolide-Lincosamide-Streptogramin group, was higher than other groups. Ceftiofur crystalline free acid for metritis treatment and cephapirin benzathine for dry cow therapy all target bacteria-producing β-lactamase. The resistance gene *cfxA* coding for β-lactamase was not different in the CCFA and CEPH groups when compared with the control (Figure 1, Table 2). This could be partly due to the short term of the antibiotic administration. Compared with the CCFA groups, the CEPH group had a numerically lower

Table 2. Effect of antibiotic treatment on the absolute abundance of antibiotic resistance genes in dairy cow faeces.

					P value		
Treatment[1]	Control	CCFA	PIRL	CEPH	Treatment	Day	Interaction
16S rRNA	9.48 ± 0.17	8.97 ± 0.18	8.77 ± 0.20	9.46 ± 0.16	0.07	0.44	0.60
tetQ	9.12 ± 0.28	9.52 ± 0.30	9.23 ± 0.26	9.13 ± 0.26	0.75	0.68	0.63
cfxA	7.29 ± 0.18	7.65 ± 0.19	7.20 ± 0.16	7.05 ± 0.16	0.20	0.68	0.69
mefA	8.74 ± 0.15	8.82 ± 0.17	8.91 ± 0.16	8.20 ± 0.15	0.07	0.68	0.68

[1]CCFA: ceftiofur crystalline free acid sterile suspension; PIRL: pirlimycin hydrochloride; CEPH: cephapirin benzathine.
[2]absolute abundance: copies of gene (log10) per gram of faeces.
[3]the data were presented as group mean ± standard deviation of the group.

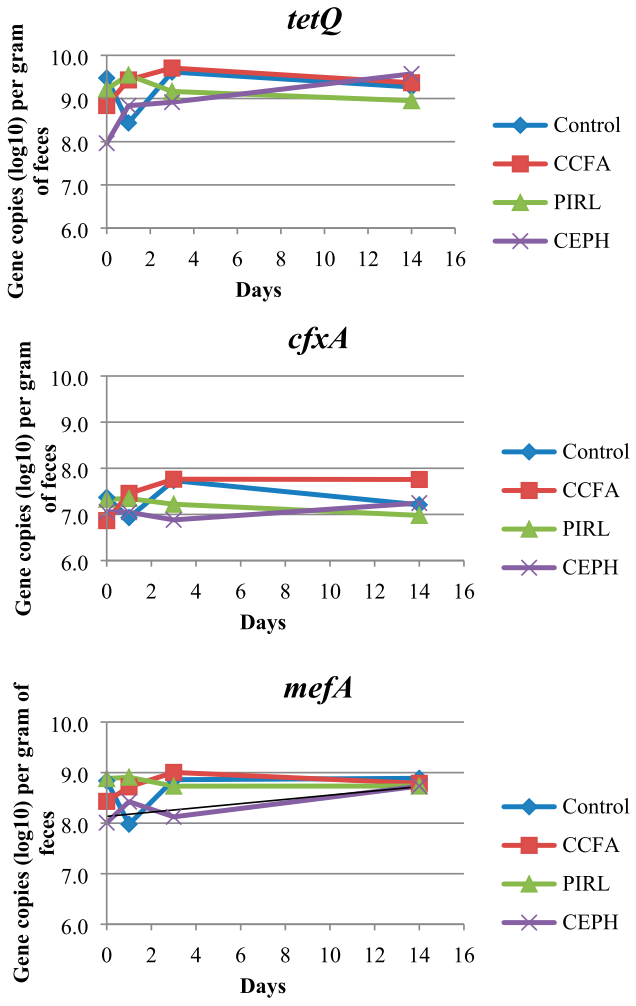

Figure 1. Gene copies (log10) of *tetQ*, *cfxA* and *mefA* per gram of faeces from CCFA (ceftiofur crystalline free acid sterile suspension), PIRL (pirlimycin hydrochloride) and CEPH (cephapirin benzathine) treatment.

abundance of *cfxA*. CCFA was administered on day 0 and day 3, while CEPH was administered only on day 0 could be the possible reason.

It has been reported that resistance genes coding resistance to fluoroquinolones, tetracyclines, β-lactams and other classes of antibiotics have been observed in cattle that were never exposed to antibiotics (Durso et al. 2011). Consistent with the previous results, all the three ARGs were detected in the control cows in this study. Antibiotic residue in the environment can promote the development and spread of ARGs in the environmental bacterial populations which might be the reason ARGs were detected on the animals never exposed to antibiotics. Thames et al. (2012) detected resistance genes for tetracycline, sulphonamide and macrolide-lincosamide-streptogramin in newborn calves. In a study comparing the occurrence of antibiotic resistance, it was found that the frequency of antibiotic resistance in organic farms was not different from conventional farms (Roesch et al. 2006). However, antibiotic use did increase abundance and diversity of ARGs in swine microbiomes whereas the animals from the control group already had a high background of resistance genes (Looft et al. 2012). In the current study, there is no difference between the control group and other treatment group animals for all of the ARGs measured. Except the short term of the antibiotic administration, the dose could be another reason. It should be noted that for the pirlimycin hydrochloride group (mimic mastitis treatment), ceftiofur crystalline free acid group (mimic metritis treatment), the actual results from reality might differ from what was observed in the current study as the cows used here were healthy cows. The antibiotics activity can be affected by the disease status as serum proteins binding action could surrender the antimicrobial ineffective (Li et al. 2017). However, information of disease status on ARGs development was not found.

Overall, the results showed no difference of measured three ARGs from cows receiving different antibiotics. Considering the limited scope of our investigation, the next-generation sequencing techniques should be further used to provide more information on ARGs excretion from cows that received therapeutic and prophylactic antibiotic treatment.

Statement

This experiment was conducted under the review and approval of the Animal Care and Use Committee (protocol 12-184-DASC) at Virginia Polytechnic Institute and State University.

Disclosure statement

No potential conflict of interest was reported by the authors.

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