Increasing temperature and pH can facilitate reductions of cephapirin and antibiotic resistance genes in dairy manure slurries

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INTERPRETIVE SUMMARY: *Li et al* (2019). "*Short communication:* Raising temperature and pH can facilitate reductions of cephapirin and antibiotic resistance genes in dairy manure slurries". Quantifying the fate of antibiotics and antibiotic resistance genes (ARGs) exposed to various temperatures and pH adjustments in manure slurries will help guide manure management practices to minimize the emergence and subsequent spreading of antimicrobial resistance. In the current study, effects of temperature and initial pH shock on cephapirin and ARGs in dairy manure slurries were investigated using a microcosm setup. Our results suggest a change in temperature or initial pH adjustment during storage of dairy manure slurries could mitigate the spread of resistance.

Short communication: **Raising temperature and pH can facilitate reductions of cephapirin and antibiotic resistance genes in dairy manure slurries**

Meng M. Li[†1], Partha Ray[§], Christy Teets[†], Amy Pruden[‡], Kang Xia*, Katharine F. Knowlton[†]

[†]Department of Dairy Science, [‡]Department of Civil and Environmental Engineering, and *Department of Crop and Soil Environmental Sciences, Virginia Tech, Blacksburg, VA, USA; [§]Department of Animal Sciences, School of Agriculture, Policy and Development, University of Reading, Reading RG6 6AR, UK;

[1] Corresponding author (mengli@vt.edu)

Abstract

Quantifying antibiotics and antibiotic resistance genes (ARGs) in manure exposed to various temperature and pH treatments could guide the development of cost-effective manure handling methods to minimize the spread of antibiotic resistance following land application of manure. This study aimed to investigate the effect of various temperatures and initial pH shocks on the persistence of a cephalosporin antibiotic and ARGs in dairy manure slurries. Feces and urine were collected from five healthy dairy cows administered with cephapirin (cephalosporin antibiotic) at dry-off via intramammary infusion, and mixed with sterile water to generate manure slurries. In a 28-day incubation study, dairy manure slurries were either continuously exposed to one of three temperatures (10, 35, and 55ºC) or received various initial pH (5, 7, 9, and 12) shocks. Cephapirin was detected in the initial samples and on day 1 following all treatments, but it was undetectable thereafter. This indicates that cephapirin can be rapidly degraded irrespective of temperature and pH treatments. However, degradation was greater on day 1 with the mesophilic (35ºC) and thermophilic (55ºC) environments compared to the psychrophilic environment at 10ºC ($P < 0.001$). Increasing pH beyond neutral also accelerated degradation as cephapirin concentrations were lower on day 1 after initial alkaline adjustments (pH 9 and 12) than neutral and acidic adjustments (pH 7 and 5; $P < 0.001$). No significant effect of temperature or initial pH was observed on abundances of a beta-lactam ARG, *cfx*A, and a tetracycline ARG, *tet*(W), implying that bacteria that encoded *cfx*A or *tet*(W) genes were not sensitive to temperature or pH in dairy manure slurries. However, abundances of a macrolide ARG, *mef*A, were decreased in the psychrophilic and thermophilic environments, and also following exposure to a strong alkaline shock (pH 12). Our results suggest that increasing temperature or pH during storage of dairy manure slurries could be used together with other on-

40 farm practices that are tailored to reduce the transfer of ARGs from manure to the environment
41 following land application.

42 **Key words:** antibiotic resistance genes; cephapirin; dairy manure slurry

43 **Short Communication**

44 In 2017, approximately 10.93 million kg of antimicrobial drugs were sold in the United
45 States for use in food-producing animals, and ~50% of that was used in cattle (USFDA, 2018).
46 Previous studies indicated that up to 90% of administrated antibiotics are eliminated from
47 animals' bodies through feces or urine (Kemper, 2008; Ray et al., 2014), implying that manure
48 generated from animal production represents a major route of antibiotics transfer to the
49 environment. The presence of antibiotics, even at a very low concentrations, can contribute to
50 emergence of antibiotic resistance genes (ARGs) and selection of antibiotic resistant bacteria
51 (Knapp et al., 2008; Martínez, 2008; Sandegren, 2014). Many studies have demonstrated that
52 land application of untreated, antibiotic-laden manure can substantially enhance the abundance
53 of ARGs in agricultural soils (Fahrenfeld et al., 2014; Hu et al., 2016; Gou et al., 2018). These
54 ARGs can be transferred to animals and humans through drinking water or the food chain,
55 leading to decreased effectiveness of subsequent antibiotic therapies (Alcaine et al., 2005; Tello
56 et al., 2012). Therefore, it is vital to develop cost-effective methods to degrade antibiotics in
57 manure before land application to mitigate the dissemination of antibiotic resistance.

58 Manure management practices such as composting and anaerobic digestion could
59 effectively increase degradation of antibiotics and reduce the prevalence of ARGs (Qian et al.,
60 2016; Sun et al., 2016; Ray et al., 2017; Gou et al., 2018). Ray et al. (2017) indicated that
61 thermophilic temperature during the manure composting process could completely degrade
62 cephalosporin, lincosamide, and sulfonamide antibiotics. Sun et al. (2016) reported that the

63 abundances of 8 of 10 detected ARGs declined during thermophilic anaerobic digestion of dairy
64 manure. However, high maintenance and operation costs, as well as high technical requirements,
65 limit implementation of composting or anaerobic digester operations on many farms
66 (Pandyaswargo and Premakumara, 2014; Garfí et al., 2016). Physico-chemical factors, such as
67 temperature, pH, CO_2 and O_2 levels, likely explain the effect of composting and anaerobic
68 digestion on persistence of antibiotics and ARGs (Sun et al., 2016; Youngquist et al., 2016; Li et
69 al. 2019). The objective of the current study was to isolate two of these factors, temperature and
70 pH, and evaluate their independent effects on antibiotic and ARG persistence. Understanding
71 these mechanisms will inform future development of best management practices for preventing
72 the spread of antibiotic resistance from livestock farms.

73 Five dairy cows at the end of lactation with similar body weight (574.9 ± 32.8 kg) were
74 fitted with urinary catheters to allow separate collection of feces and urine and moved to
75 individual metabolic stalls at the Virginia Tech Dairy Center (Blacksburg, VA). After 24 h of
76 acclimation to the barn and catheters, the cows were treated with a single dose of 300 mg
77 cephapirin benzathine (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) into each of the
78 four mammary glands through the teat as in the standard dry-off protocol (dry cow antibiotic
79 treatment 60 days before calving) of the Virginia Tech Dairy Center. All cows were allowed ad
80 libitum access to water and a ration formulated to meet their nutritional needs.

81 Feces and urine were collected from day 1 (the day cephapirin was administered) through
82 day 3 following treatments, and then all feces and urine excreted by a cow over the three days
83 were combined to form one homogenous pool of manure. Approximately 1 kg of the manure
84 mixture was mixed with sterile water to generate a manure slurry with a final solid content of
85 5%. Aliquots (200 mL) of slurry were transferred into glass beakers (400 mL) and incubated at

10, 35, and 55ºC or at 25ºC with initial pH adjustments to 5, 7, 9, or 12 using 1 M (mol/L) HCl (for pH 5 and 7) or NaOH (for pH 9 and 12) solution. Each treatment had 4 replicates. Beakers were covered with aluminum foil with a hole in the middle to maintain aerobic conditions. Beakers were weighed daily, and sterile water was added to each beaker to replace weight loss due to water evaporation. Samples were collected on days 0, 1, 3, 7, and 28 and were immediately stored at -20ºC for future analysis.

Cephapirin concentrations were quantified following the method developed by Ray et al. (2014). To quantify ARGs, frozen manure slurries were freeze-dried, and then DNA was extracted from the freeze-dried manure samples using the QIAamp DNA stool extraction kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Quantitative PCR (qPCR) was performed to quantify a beta-lactam ARG, *cfx*A, a macrolide ARG, *mef*A, and a tetracycline ARG, *tet*(W), using an EvaGreen assay (Biotium, Hayward, CA) with previously reported primers (Aminov et al., 2001; Sóki et al., 2011; Looft et al., 2012). 16S rRNA gene copy numbers were determined using the method by Suzuki et al. (2000). Bacterial gene abundances were represented as 16S rRNA gene copies per g dry manure, and ARG relative abundances were expressed as the proportion to 16S rRNA gene abundance.

Data analyses were conducted in R software (version 3.3.0; R development Core Team, 2015). All qPCR results were normalized using log base 10 transformation. The temperature or initial pH effect on cephapirin concentrations and ARGs was analyzed in a mixed statistical model with temperature or initial pH as a fixed effect and incubation day as a repeated measurement using the gls function in the nlme package (Pinheiro et al., 2018). The first-order autoregressive covariance with heterogeneous variance of time were considered in the repeated

108 measures ANOVA model. The Tukey's honestly significant difference post hoc test was used to
109 test differences among treatments, and $P < 0.05$ was considered significant.

110 Cephapirin was present in manure slurries on day 0 (initial samples) and day 1 following
111 either temperature or initial pH adjustment, but it was undetectable thereafter. On day 0, the
112 mean concentration of cephapirin was 12.5 ± 1.3 ng per g dry manure; this rapidly decreased to
113 less than 1 ng per g dry manure on day 1 regardless of temperature or pH treatments (Figure 1).
114 Ray et al. (2017) reported that cephapirin was only present in day 0 compost samples. Gilbertson
115 et al. (1990) indicated that another cephalosporin (ceftiofur) was quickly degraded within 8 h in
116 cattle feces. Thus, our result was consistent with the previous studies, suggesting that cephapirin
117 has a poor stability in dairy manure slurries. Compared to the psychrophilic environment (10ºC),
118 the mesophilic (35ºC) and thermophilic (55ºC) environments decreased cephapirin
119 concentrations on day 1, with no difference between the mesophilic and thermophilic conditions
120 (Figure 1A).

121 Cephapirin belongs to the β-lactam class of antibiotics which have a basic β-lactam ring
122 structure for the antibacterial activity. Ray et al. (2017) indicated that cephapirin was unstable at
123 high temperature and Cha et al. (2006) demonstrated that the β-lactam ring has poor stability in
124 animal manure and lagoon effluent. Wagner et al. (2011) observed that multiple β-lactamases
125 were detected in bacteria isolated from the digestive tract of cows. In the current study, the
126 number of bacterial 16S rRNA gene copies increased in the mesophilic and thermophilic
127 environments compared to the psychrophilic environment (Table 1), suggesting that elevated
128 temperature might facilitate the biotic degradation of cephapirin via the secretion of β-lactamases
129 by an increased population of manure microbes.

On day 1, cephapirin concentration was lower in manure exposed to initial alkaline pH shock (pH 9 and 12) compared to neutral or acid pH shock (pH 7 or 5; Figure 1B). Gilbertson et al. (1990) demonstrated that the hydrolysis of ceftiofur was dramatically increased when pH was changed from pH 5 to 9. Ivaska and Nordström (1983) found that cephapirin exhibited a stable peak on differential pulse polarography at pH 7 to 8.5, implying that the chemical structure of cephapirin is more stable in a slightly alkaline condition.

In the current study, none of the animals had received any antibiotic treatment for at least 280 days before the cephapirin administration, but all three of the ARGs quantified (*cfx*A, *mef*A, and *tet*(W)) were detected in all of the manure slurry samples. Muurinen et al. (2017) detected 8 common ARGs and mobile genetic elements in stored manure as well as in soil (including fertilized and unfertilized) and tile drainage water collected from 2 dairy farms, which supports the co-occurrence of ARGs in various farm environments. It is likely that the detected ARGs could be transferred to the cows from the dairy farm environment or from other cows in the dairy herd, and therefore, various modes of ARG transmission deserve further attention and research.

Tetracycline resistance genes are the common ARGs observed in dairy manure (Sun et al., 2016; Zhou et al., 2016). In the current study, the average log base 10 transformed relative abundances for *tet*(W), *mef*A, and *cfx*A cross all the temperature and pH treatments were -1.8, -2.2, and -4.3, respectively. A greater abundance of the *tet*(W) gene was detected than of the *cfx*A or *mef*A gene. The *cfx*A gene encodes class A β-lactamase, a gene that codes for resistance specific to cephalosporin antibiotics (García et al., 2008). In the current study, the *cfx*A gene had a low abundance compared to the abundance of *tet*(W) and *mef*A genes. This could be due to the rapid dissipation of cephapirin during manure incubation leading to low or no selective pressure among manure microbes.

No significant temperature or initial pH effects on the relative abundance of *cfx*A and *tet*(W) were observed in dairy manure slurry (Table 1). Sun et al. (2016) reported that there was no significant temperature effect on *tet*(W) abundance during anaerobic digestion of dairy manure. Huang et al. (2019) demonstrated that high temperature cannot always remove ARGs in manure anaerobic digestion unless antibiotic resistant bacteria or gene transfer elements are more efficiently decreased by raising temperature. Therefore, we can conclude that manure bacteria that encoded *cfx*A or *tet*(W) genes were not sensitive to incubation temperature or initial pH shock in dairy manure slurries.

Compared to the mesophilic condition, the *mef*A gene abundance decreased in psychrophilic and thermophilic environments ($P < 0.0001$; Table 1) probably due to changes in bacteria community (Qian et al., 2016; Sun et al., 2016). Miller et al. (2016) indicated that high temperature could remove some ARGs carried by bacterial hosts that were not thermotolerant. The optimum growth temperature for most bacterial species is around 37ºC (Zhu, 2000). Therefore, the psychrophilic and thermophilic environments might inhibit the growth rate of bacteria that encoded *mef*A gene, leading to the decrease of *mef*A abundance.

The strong alkaline treatment (pH 12) resulted in 0.9 log base 10 fold reduction of *mef*A abundance as compared to the other pH treatments ($P < 0.0001$; Table 1). Although there was no pH effect on total bacterial 16S rRNA gene copies (Table 1), it is possible that the microbial community structure shifted due to pH stress. Lin et al. (2013) reported that bacteria *Clostridium alkalicellum* and *Corynebacterium humireducens* were enriched at pH 10 in swine manure, while the abundance of *Butyricimonas sp.* was decreased. In the current study, the strong alkaline condition might have changed the bacterial community to have less bacteria carrying *mef*A gene, which led to the reduction in *mef*A abundance.

In conclusion, the thermophilic temperatures and strong alkaline treatments can facilitate rapid dissipation of cephapirin. Both psychrophilic and thermophilic temperatures as well as strong alkaline treatments can decrease the abundance of *mef*A ARG. However, cephapirin was rapidly and completely removed from manure slurries regardless of heat and pH treatment and thus, likely, no additional treatments are needed for its degradation. On the other hand, heat and pH influenced the abundance of *mef*A gene. This indicates that there could be treatment strategies (such as change in temperature or initial pH adjustment) developed to aid the removal of *mef*A. For instance, adding hydrated lime into manure slurries could be an effective method to reduce antibiotic resistance, since it will generate heat and increase pH. Heinonen-Tanski et al. (2004) reported that a dose of 10 g/L of hydrated lime with good stirring could destroy coliform bacteria to concentrations below 10 CFU/g in diluted cattle slurries. Jamal et al. (2011) indicated that adjusting pH to 12 or higher can halt or retard the microbial reactions leading to decreased odor production and vector attraction. Meanwhile, high pH can also reduce the availability of heavy metals, enhancing agricultural benefits and lowering environmental risks (Wong and Selvam, 2006). However, the role of elevated manure temperature and pH to nitrogen losses via volatilization of ammonia should be mentioned. The significant volatilization of ammonia during manure liming is a safety concern for animals and workers, and the loss of N also represents a significant loss of value for the manure as a fertilizer. Therefore, large stores of manure slurry should never be treated with lime due to a safety concern and an economic loss. Rather, liming of manure might be conducted within small loads of manure being hauled to a land application site in a tank wagon. One potential limitation of this study is that manure samples that had been treated to adjust pH were not neutralized before freezing. Without neutralizing samples at the completion of the incubation time, pH might impact the samples during storage and later

analysis. Although immediate storage at -20ºC would stop almost all microbial growth and chemical properties of fresh manure (Pan et al., 2019), it may be worth highlighting that -80ºC instead of -20ºC is the best storage temperature for microbial community analyses, and pH may have impacted the degradation of cephapirin. Overall, our results suggest increasing temperature or pH during storage of dairy manure slurries could be used together with other on-farm practices that are tailored to reduce the transfer of ARGs from manure to the environment following land application.

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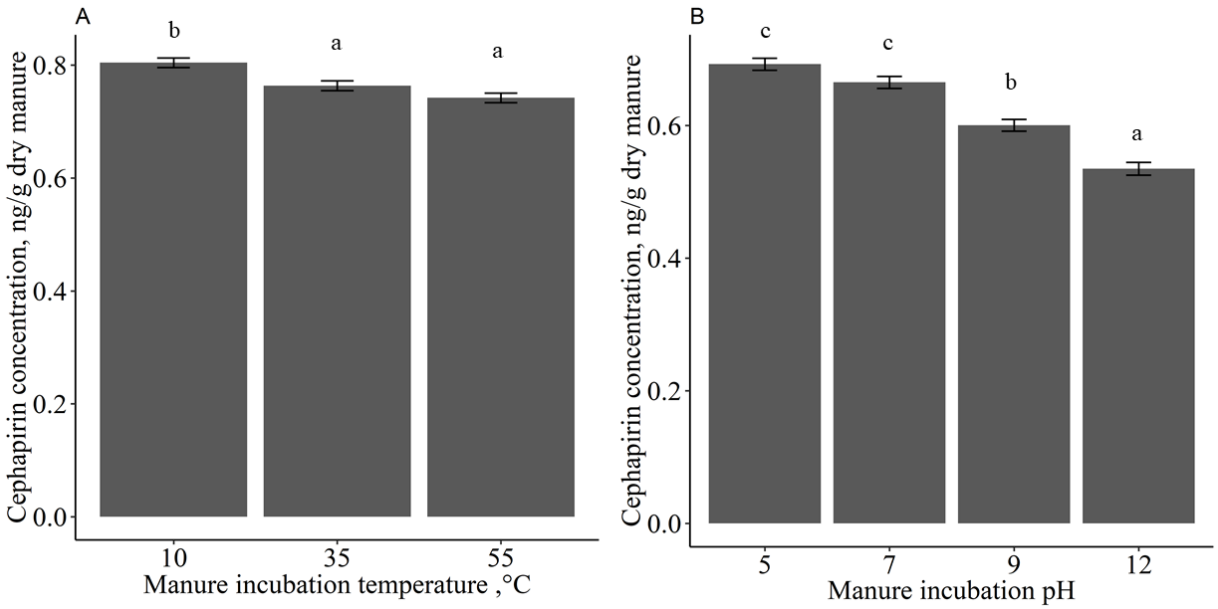

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316 Figure 1. Cephapirin concentrations in dairy manure slurries exposed to various temperatures (A)
317 or initial pH shocks (B) on day 1 following treatments (n = 4). Bars denoted by a different letter
318 indicate significant differences between treatments ($P < 0.05$).

Table 1. The effect of temperature or initial pH on the abundance of 16S rRNA gene and relative abundance of antibiotic resistance genes in dairy manure slurries[1]

Treatment	Bacterial 16S rRNA	*cfx*A	*mef*A	*tet*(W)
Temperature, ºC				
10	8.28[a]	-4.03	-2.40[a]	-1.68
35	8.77[b]	-4.19	-1.70[b]	-1.80
55	8.64[b]	-4.22	-2.69[a]	-1.65
SEM[2]	0.07	0.08	0.22	0.06
P value for temperature	< 0.0001	0.12	< 0.0001	0.19
Initial pH				
5	8.72	-4.30	-1.97[b]	-1.94
7	8.75	-4.51	-1.92[b]	-1.89
9	8.51	-4.32	-1.84[b]	-1.58
12	8.69	-4.82	-2.81[a]	-1.99
SEM	0.16	0.22	0.10	0.17
P value for initial pH	0.17	0.3	< 0.0001	0.25

[1]Bacterial 16S rRNA gene was expressed as gene copies per g dry manure slurry. *cfx*A is a beta-lactam antibiotic resistance gene; *mef*A is a macrolide antibiotic resistance gene; and *tet*(W) is a tetracycline antibiotic resistance gene. Antibiotic resistance genes were expressed as a proportion to bacterial 16S rRNA gene. Data was normalized using log base 10 transformation. The Tukey's post hoc test was used to test differences among treatments. Different superscripts in the same column within temperature or pH group indicate significantly different means ($P < 0.05$). n = 4.

[2]Standard error of mean