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Antibiotics and antibiotic resistant bacteria and genes in northeastern dairy manure management systems – Project overview and preliminary findings from an 11 farm case study

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ABSTRACT. ***Modern U.S. dairies use antibiotics primarily for disease treatment and prevention. Consequently, antibiotic residues, antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARG) present in dairy manure may potentially enter the environment and contribute to the spread of antibiotic resistance (AR). Working cooperatively with 11 production dairies in three states (NY, PA, MD) our objective is to investigate the efficacy of existing on-farm manure management practices (e.g. long-term storage, composting, anaerobic digestion, etc.) at mitigating these potential contaminants. At each farm manure system performance is being assesses, antibiotic usage is being tracked, and manure samples are being collected at roughly 6-week intervals over 2-years pre- and post- each treatment step of the various manure handling systems. Samples are being analyzed for tetracycline, sulfonamide and macrolide antibiotic residues. Additionally, using culturing techniques and qPCR, ARB in manure solids before and after treatment are being quantified to study the effectiveness of bedding recovery systems at mitigating ARB pathogens. Select samples are also being analyzed for their diversity and abundance of ARGs. Preliminary data shows variable mitigation potentials of the different manure handling systems and is improving our understanding of the fate of these potential contaminants in dairy manures. As the project advances the efficacy of specific treatment systems to mitigate AR will be tested and knowledge on the fate of AR in dairy manure management systems will be extended to the dairy industry.***

Keywords. anaerobic digestion, antimicrobial resistance, compost, long-term manure storage, solid liquid separation

Introduction

Modern dairy production practices use antimicrobials for disease treatment and prevention, as well as for improving animal growth, feed efficiency, and milk production (Pol & Ruegg, 2007; Zwald et al. 2004). Consequently, any antibiotic residues, antibiotic-resistant bacteria (ARB), and antibiotic resistance genes (ARG) present in dairy manure may potentially enter the environment and contribute to the spread of antibiotic resistance (AR). AR is considered a major global risk to the treatability of infections and human health (WHO, 2012). Though the prevalence of antibiotic resistant bovine pathogens may not be increasing (Cummings et al. 2013), dairy farms are one of many sources of antibiotic residues, ARB and ARG to the environment (Pruden et al 2013) and due to their large-scale antibiotic use there is concern that animal agriculture is a major contributor to the development of antibiotic resistant human pathogens (Oliver at al. 2011, Williams-Nguyen et al. 2016). To better understand the exact role dairies play in the spread of AR there is a need to characterize the effectiveness of on-farm practices, manure handling and treatment systems at mitigating antibiotic residues, ARB and ARG in dairy manure. To characterize AR-dairy system dynamics and identify potential on-farm control options for AR, the fate and removal of antimicrobials, ARB, and ARG are being tracked at 11 production dairy farm manure handling and treatment systems. As this effort is still in the data collection stage, here we will outline our approaches, share some preliminary data, and discuss the potential and expected outcomes of the work.

Materials & Methods

Descriptions of Participating Dairies

Eleven dairy farms in three states (NY, PA, MD) were selected to represent the diverse approaches used by modern dairies of various herd sizes to collect and manage their manure. The six NY dairy farms have lactating herds of 650 to 4,200 cows, scrape manure, and have a mix of handling systems that include collection pits, anaerobic digestion, solid-liquid separation, rotary drum and aerated floor composting, bedded packs, covered and open air long-term storages. The two PA dairies have lactating herds with 650 and 4,000 cows, scrape manure, and have a mix of handling systems that include collection pits, anaerobic digestion, solid-liquid separation, windrowed composts, bedded packs, covered and open air long-term storages. The three MD dairies have lactating herds of 65 to 1,000 cows, scrape and flush manure, and have a mix of handling systems that include collection pits, anaerobic digestion, solid-liquid separation, aerated and windrowed composts, stockpiling, bedded packs, open air long-term storages and anaerobic lagoons. At each of these farms antibiotic usage is being collected from interviews with farm herdspersons and farm treatment protocols and treatment and dosage information is being collected from on-farm records (electronic and hand written).

Manure Sampling, Characterization & Treatment System Monitoring

Approximately every six weeks from June 2016 to May 2017 manure was collected pre- and post- each manure handling process/treatment (e.g. composting, anaerobic digestion) at each dairy farm. Manure was also collected 3 times during both the spring and fall manure storage drawdowns from manure trucks or draglines at each dairy. Manure was collected according to the methods of the Eastern Research Group (2011). In brief, static manure (e.g. compost pile) was sampled by combining 6 separate 3.5 L representative samples into a cleaned and surface sterilized (10% bleach then 70% EtoH) bucket. Continuously flowing manure (e.g. anaerobic digester effluent) was sampled by collecting 6 separate 3.5 L representative samples over a 1 h period into a cleaned, and surface sterilized bucket. Each composite sample was then thoroughly mixed for 2 minutes; if manure solids by hand with clean Nitrile gloves, if liquid using a clean 5-gallon paint mixer (Warner Manufacturing Co., Plymouth, Minnesota, USA) attached to a 20V cordless drill. The mixed samples was then aliquoted into a sterile amber Nalgene™ bottles (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for basic characterization into a nitric acid washed 50 mL Falcon™ tube (Corning Inc., Corning, New York, USA) for antibiotic residue analysis, and a sterile 50 mL Fulcon™ tube for ARB and ARG analysis. Samples were transported on ice the laboratory. Sample collected for basic characterization were analyzed within 24 hours or frozen immediately at -20°C. Samples collected for antibiotic analysis were frozen at -20°C. Samples collected for ARB analysis were analyzed immediately. Samples collected for ARG analysis were frozen at -80°C.

For each of the manure sample, temperature, pH, total solids, volatile solids, volatile fatty acids, nutrients (total Kjeldahl nitrogen, total Kjeldahl phosphorous, orthophosphate, ammonium) are measured according to the Standard Methods for Examination of Water and Wastewater (APHA, 2005) as outlined in Labatut and Gooch (2012). Measures will be used to normalize and correlate to AMR metrics.

For each farm, manure production is being estimated using herd demographic data and ASABE standards (ASAE, 2005). This data, and when possible, other influents into the manure treatment systems (millhouse wastewater, food waste) are being tracked to quantify loading rates, flows and residence times through the various manure management systems. For anaerobic digesters, daily operating temperature, biogas production, and electrical generation are being tracked, and when possible biogas quality (concentrations of CH_4, CO_2, H_2S). Temperature is also being tracked for rotary drum composters.

Antibiotic Residues

Antibiotic residues were extracted and analyzed for select Sulfonamides, Macrolides, and Tetracycles similarly to the methods of Wallace & Aga (2016). Frozen manure samples were freeze dried, homogenized and 100 mg of dried manure solids were added to a polypropylene centrifuge tube. To this, 50 mL of surrogate solution containing d_4 – sulfamethaxazole, phenyl-$_{13}C_6$-sulfamethazine, demeclocycline, and $_{13}$C-erythromycin (500 ng mL^{-1}) was added and allowed to equilibrate for 30 min. Solids were suspended with 5 mL of 20:30:50 acetonitrile:methanol:0.1 M EDTA-McIlvaine buffer (pH = 4; v/v/v/), vortexed for 30 s, ultra-sonicated (40 kHz, 120 W) for 10 min., and centrifuged at 4000 g for 10 min. The supernatant was decanted into a 500 mL HDPE bottle and the solids were extracted twice more. Extracts were diluted with 400 mL Nanapure water to reduce percentage organic content (≤ 4.0%) and the pH was adjusted to 4.0 ± 0.2 with H_3PO_4. Diluted extracts were subjected to solid phase extraction (SPE) with tandem NH_2-HLB cartridges. Cartridges were conditioned with 6 mL of methanol and 10 mL of 2.7 mM EDTA solution. Samples were loaded at a rate of 2 to 4 mL min^{-1}. Loaded cartridges were washed with 10 mL of water/methanol (95:5, v/v), the NH_2 cartridges were removed, and the HLB cartridges were dried under vacuum. The HLB cartridges were eluted with 10 mL of methanol and evaporated to 200 mL under N_2 at 30°C. Extracts were reconstituted to 1 mL with water/methanol (95:5, v/v) plus 0.1% acetic acid solution, and the mixture was vortexed. A 200 mL aliquot of the extract was placed into a vial insert and spiked with 10 mL of 500 ng mL^{-1} spiking solution and ISTD. A second aliquot of 200 mL was spiked only with 10 mL of 500 ng mL^{-1} ISTD, d10–carbamazepine, and minocycline. Both aliquots were centrifuged at 7000 g for 5 min to remove any fine particles from the final extract before analysis by LC-MS/MS (Thermo Scientific™ TSQ Quantum Ultra™ triple quadrupole mass spectrometer). Signals were normalized to the area of the ISTD within each run to minimize variation resulting from instrument drift between samples. Blank injections of extract diluent were made every three to five samples and the injection needle was washed in methanol between each injection to avoid carryover. Compounds were quantified using standard addition method. Methods are also in development for β-lactam analysis.

Antibiotic Resistant Bacteria (ARB)

Separated manure solids (SMS) before and after advanced treatments from two collaborating farms were tested for ARB. At one farm SMS were treated with high calcium quicklime (Graymont, Richmond, British Columbia) at approximately a 6% mass ratio for 12 hours prior to their use as bedding. At the other farm, SMS were partially composted in a rotary drum bedding recovery unit (FAN SEPARATOR, GmbH, Marktschorgast, Germany) operated at 58°C prior to their use as bedding. Serial dilutions of samples were aseptically plated onto AccuMast™ mastitis diagnostics plates (FERA Animal Health, LLC, Dryden, New York, USA) to select for coliforms, *Staphylococcus* and *Streptococcus* spp. Colony forming units were counted, and pathogen communities were collected into 5 mL microfuge tubes with sterile water and frozen at -80°C for ARG analysis.

Antibiotic Resistance Genes (ARG)

To assess the robustness of a quantitative metagenomic approach to measure antimicrobial resistance genes (ARGs) in dairy manure, the QIAamp Power Fecal DNA kit (MO BIO Laboratories, QIAGEN Co., Carlsbad, California, USA) was used to extract DNA from manure pre- and post- select treatments (composting, plug flow and mixed anaerobic digestion). DNA from a well-studied marine bacterium was spiked into each sample as an internal control (denoted IS DNA) and samples were sequenced using Illumina HiSeq4000 (Illumina Inc., San Diego, California, USA) at the University of Michigan sequencing core. A calibration curve with different ratios of IS DNA to total community DNA (0.1%, 1% and 10%, by mass) was also sequenced to establish the relationship between IS reads and total sample DNA reads. Reads were then assembled to increase the length of the alignment, improving annotation of resistance genes (Thomas et al., 2012), prior to quantification at the read-level. The Resfam (Gibson et al., 2014) and Comprehensive Antimicrobial Resistance Databases (Jia et al., 2017) in combination with manual curation is now being used to annotate ARGs from the assembled reads. Selected ARGs found in the samples will then be quantified using qPCR to validate the approach.

Initial Results

Preliminary Findings – Manure System Performance

Manure in collections pits typically have a pH between 7 and 8, total solids content between 5 and 11%, and may be as cool as 5°C in the winter and as warm as 25°C in the summer. These and the other measured characteristics all change as manure moved through the various treatment systems. On-farm technologies that are being more intensively monitored include the rotary drum composters and anaerobic digesters. Rotary drum systems operate at different temperatures depending on the farm and specifications of the units and the manure entering them. While some units operate steadily at 56°C, others fluctuate in temperature but may operate between 70°C and 80°C. Anaerobic digester systems being monitored also vary in their operating conditions and performance from farm to farm and over time (Figure 1).

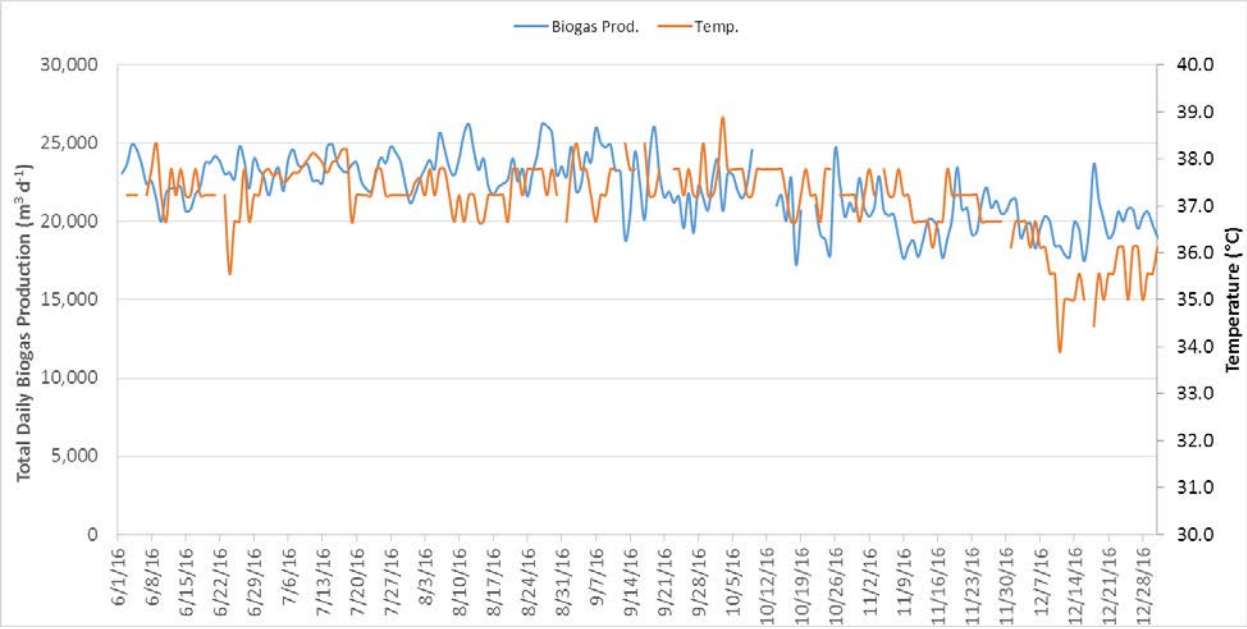
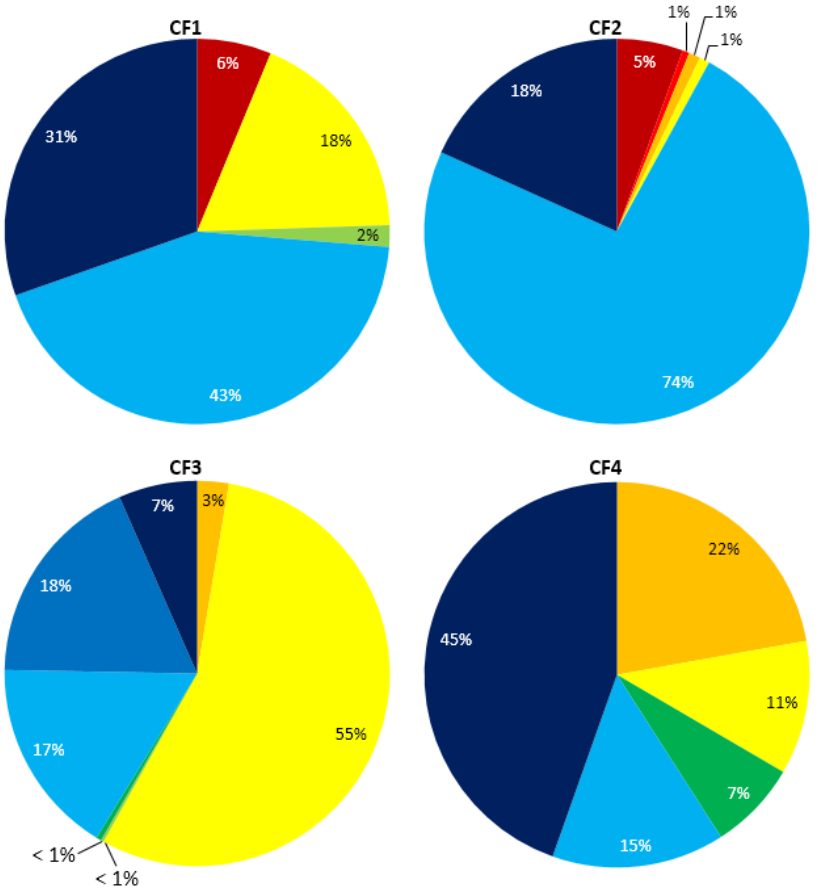


Figure 1. Total daily biogas production and internal temperature of an anaerobic digester systems operating on one of the collaborating dairy farms.

Preliminary Findings - Antibiotic Usage

One year of antibiotic usage has been completely tabulated for four of the 11 farms with treatment records from the remaining NY, MD, and PA farms still being processed. Based on data from the four farms, antibiotic usage varies significantly from farm-to-farm. While particular antibiotics were commonly used for preventing and treating mastitis and for treating metritis and pneumonia, treatments of other infections were less consistent across farms (Appendix 1). As a result, each dairy farms daily antibiotic use was unique (Figure 2). Of the farm data that has been tabulated, average daily usage of β-lactam (Penicillin and Cephalosporin), tetracycline, sulfonamide and macrolide ranged from 0.5 to 45.1, 3.0 to 91.3, 0.0 to 8.2, and 0.0 to 15.4 g d^{-1}, respectively. On average across all four farms, 49.2 mg cow^{-1} day^{-1} Tetracyclines, 25.2 mg cow^{-1} day^{-1} Penicillins, 13.2 mg cow^{-1} day^{-1} Ceftiofurs, and 3.15 mg cow^{-1} day^{-1} Sulfonamides are used.



	Ave. daily antibiotic usage (g d-1)*			
Drug Class	CF1	CF2	CF3	CF4
Aminocoumarin	4.2	3.4	-	-
Aminoglycosidases	-	0.4	-	-
Amphenicol	-	0.6	1.2	45.4
Cephalosporins	12.3	0.5	25.2	23.0
Lincosamides	1.2	-	0.1	-
Macrolides	-	-	0.2	15.4
Penicillins	29.1	45.1	7.5	29.7
Sulfonamides	-	-	8.2	-
Tetracyclines	20.4	11.2	3.0	91.3
Quinolones	-	-	-	-

* Average based on number of days usage was tracked.

Figure 2. Average daily antibiotic usage at four collaborative farms (CF) in 2016. Pie charts depict percentage (average daily mass of active ingredient) of 10 common antibiotic classes used at each farm. The table provides the average mass values.

Antibiotic usage fluctuated somewhat over time with occasional short-term spikes in usage in response to the treatment of illness/infection outbreaks. For example, for the four farms where data is completely tabulated, usage of Penicillin-type antibiotics averaged 51-316 g wk^{-1}, but exceeded 1,600 g wk^{-1} in week 32 at CF4 in response to a respiratory infection outbreak and its treatment with Penicillin G Procaine (Figure 3).

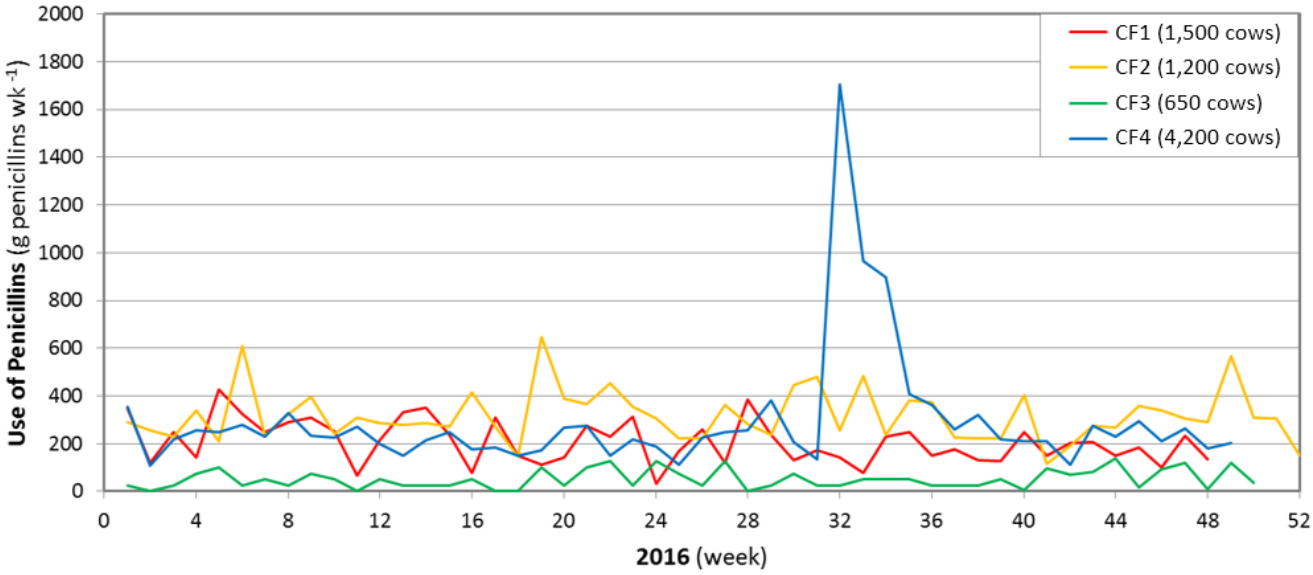


Figure 3. Weekly usage of Penicillin-type antibiotics at four collaborative farms (CF) in 2016.

Preliminary Findings - Antibiotic Residues

Antibiotics were detectable at low levels (Sulfonamides, 0-50 μg g^{-1} TS; Tetracyclines, 0-500 μg g^{-1} TS; Macrolides 0-0.3 μg g^{-1} TS) in many of the raw and treated manure samples. Based on the results to date, regardless of the complexity of the farm handling/manure treatment system, sulfonamides and macrolides were not detected in the long-term manure storages. However, Tetracycline antibiotics (both parent compounds and transformation products) were detected in long-term manure storage samples, though typically at levels an order of magnitude less than in freshly collected manure. Robust analysis of the effectiveness of particular manure treatment systems cannot completed until additional data has been completely analyzed. One primary lesson learned from our sampling to date is that significant sampling and technical variability can result from the heterogeneity of manure (Table 1). Researchers should thus anticipate that large sampling numbers may be required to adequately sample these heterogeneous manure matrices, and that many technical replicates may be required to accurately, and precisely, test these samples for antibiotic residues.

Table 1. Uncertainty analysis for a raw dairy manure sampled and extracted in triplicate for Anhydrotetracycline (ATC), 4-Epitetracycline (ETC), 4-Epichlorotetracyclines (ETCT), Tetracycline (TC).

Variability Measure	Triplicate	ATC	ETC	ECTC	TC
Mean ± SD (ng antibiotic g^{-1} total manure solids)	Sample	2608 ± 466	2649 ± 519	5497 ± 705	2761 ± 196
	Extraction	2514 ± 418	1884 ± 220	4837 ± 588	2470 ± 382
Sum Square Error (% of Total)	Sample	55%	65%	56%	28%
	Extraction	45%	35%	44%	72%
$Variability_{sample}$:$Variability_{extraction}$		0.62	0.82	0.72	0.38

Preliminary Findings - ARB

AccuMast plates were successfully used to isolate mastitis pathogens from separated manure solids pre- and post-treatment. DNA has been extracted from communities of *Staphylococcus* and *Streptococcus* spp. and tetracycline resistance genes, such as tet(O) have been detected in extracted DNA from these communities. Quantitative PCR efforts are underway to test if the proportion of tet(O) changes from mastitis communities pre- and post-advanced manure solids treatment.

Preliminary Findings - ARG

Sequencing of the initial six samples (pre- and post- rotary drum composting, plug-flow and continuously mixed anaerobic digestion) has been completed with > 1 billion reads generated. Read- and gene-level annotation are currently being conducted where, 1) individual reads are being mapped back to the reference genome and the ratio of genes mapped to the reference and total reads are being determined, 2) and at the gene-level, reads are first being assembled, binned and then annotated.

Discussion/Expected Outcomes

Work is still ongoing to tabulate antibiotic usage for the remainder of the farms, to analyze antibiotic residues, antibiotic resistant bacteria and resistance genes; data which will be used to assess the efficacy of distinct manure treatment system at mitigating antimicrobial resistance.

Standard characterization of manure samples and monitoring the performances of the on-farm manure systems will enable comparison of AR variables to qualities of manure, such as pH and total solids, and to treatment conditions, such as temperature and residence time. Comparing system performance to AR mitigation efficacy is a primary goal of this research effort. As some farms are using systems of very similar specifications – for example three very rotary drum composters from the same manufacturer are in the study, two are on the same farm but are operated at different temperatures – this study has unique opportunities to compare the effects of different farms influents operating conditions on AR mitigation efficacy. Monitoring rotary drums and the other manure treatment technologies is also providing value to on-farm manure system operators interested in understanding in greater detail the performance of their systems.

Based on antibiotic use data to date we have been able to tailor future sampling efforts for in-depth antibiotic residue and ARG testing. We anticipate that antibiotic use tracking can also be used to estimate antibiotic residue loading in the manure by applying generalized metabolism/excretion rates to the mass of antibiotics used. Where antibiotic use data is adequately detailed to be associated with particular cows and groups, there is also the opportunity to look at the effects of manure handling systems in greater detail. For example, some farms house treated cows in barns that utilize different handling

systems (scraped, flushed, and bedded-packs) providing the opportunity to assess the impact these systems have on initial antibiotic concentrations.

Antibiotic residue testing is a main objective of this project and preliminary data is showing some antibiotic mitigation potential for select manure treatment systems. The antibiotic levels detected to date are around or below those previously published for livestock and dairy manures (Ince et al., 2013; Spielmeyer, 2014). This project's data is beginning to characterize the variability observed in on-farm systems and will inform needed improvements in sampling and monitoring to accurately assess antibiotic mitigation potentials. Antibiotic residue testing has also enabled our collection of antibiotic usage data. For example, on one farm we had detected tetracycline in the manure, but had no record of that antibiotic being used as a treatment. This was mentioned to the herdsperson at the farm, who then identified that tetracycline-powder is sometimes used topically to treat foot problems with select cows. Once this was identified we were able to better account for usage of the antibiotic and gain the confidence of the herdsperson, improving our efforts and ability to robustly characterize dairy antibiotic usage and antibiotic fates in the manure system. Improved resolution of how drug usage impacts antibiotic residues and relates to ARB and ARG levels in manure in on-farm systems is an anticipated outcome of the overall project.

ARB testing is focused on advanced treatments of manure solids for bedding. While antibiotic resistant *Salmonella* isolates have not increased in prevalence based on analysis milk samples (Cummings et al, 2013), recycled manure solids are an environment where ARB development is possible and may be problematic. By exploring the prevalence of ARB in this potentially high-risk environment and testing the efficacy of increasingly popular on-farm advanced treatment of manure solids, this ARB effort is focused on practical questions relevant now to on-farm decision making. Field studies are also being complimented by in-lab testing to more carefully assess the treatment effects of lime on ARB abundance.

The sequencing approach is designed to take a deep and broad look at the resistome, or "the collection of all the antibiotic resistance genes and their precursors in both pathogenic and non-pathogenic bacteria" (Wright, 2007). Specifically, the employed approach will identify bacteria harboring resistance genes within their chromosomes and enable quantification of ARGs in the metagenome. The use of IS DNA will ensure data analysis is robust and minimal in bias and facilitate more rigorous quantitative analyses so the effects of treatments and correlations to antibiotic levels can be carefully assessed. Lastly, separation and extraction of viral DNA, extracellular DNA, and intracellular DNA followed by qPCR on targeted ARGs informed by results from the sequencing effort will suggest the stability of ARGs in these DNA fractions through manure management.

Conclusion

While early in the project, preliminary data is offering exciting insights into the complexity of antimicrobial resistance patterns in real-world dairy agroecosystems. These research efforts will provided an important overview of how modern northeastern dairy farms are using antibiotics, insight into what antibiotic residues these farms might being generated, begin resolving how on-farm manure management may impact and potential mitigate antibiotic residues, ARB and ARG. An ultimate goal of these characterization efforts are to inform our regional dairy producers, through concerted outreach efforts, about the AR issue and equip them with the knowledge to move their farms in a positive and more environmentally, economically, and socially sustainable direction.

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Appendices

Appendix 1. Antibiotic usage by six of the collaborative dairy farms. Values represent number of dairies (of the 6 studied) that use a specific antibiotic for a specific treatment. Critically important antibiotics, as defined by the World Health Organization (WHO, 2017), are listed in red. Highly important antibiotics, as defined by the World Health Organization (WHO, 2017), are listed in orange. * indicates the antibiotic is only approved for veterinary usage and is not used as a human medicine. Dry = dry off therapy; Mast. = mastitis; Syst. mast. = systematic mastitis; Toxic = Toxic mastitis (E. coli endotoxemia); Foot rot = foot rot; Feet = other hoof and feet issues including ulcers and warts; Lame = lameness; Injury/ Trauma = various injuries and trauma; RP = retained placenta; Met. = metritis; DA = displaced abomasum; Indig. = indigestion; Diarhea = diarhea; Resp./Pneu. = respiratory infection, pneumonia, cough; Fever = fever, high temperature; Pink eye = pink eye; Metaphyl = metaphylaxis, pneumonia preventive treatment for new stock and calves; Other = Other infections

Drug Class	Antibiotic	Trade Name	Usage																		
			Mastitis				Reproductive			Foot/Lame/Injury				Enteric			Other				
			Dry	Mast.	Syst. mast.	Toxic	RP	Met.	Birth/ C-sect.	Foot rot	Feet	Lame	Injury/ Trauma	DA	Indig.	Diarhea	Resp./ Pneu.	Fever	Pink eye	Metaphyl	Other
Aminocoumarin	Novobiocin	AlbaDry Plus	2																		
Aminoglycosidases	Dihydrostreptomycin Sulfate	Quartermaster	3																		
	Neomycin	Neomycin																	1		
Amphenicol	Florfenicol *	Nuflor										1	1				3				1
	Florfenicol *	Resflor															3				1
Cephalosphorins	Ceftiofur Sodium *	Naxcel		1			2	1						1	1		3				
	Ceftiofur Hydrochloride *	Spectramast LC		5																	
	Ceftiofur Hydrochloride *	Spectramast DC	4																		
	Ceftiofur Hydrochloride *	Excenel		1	1	1	3	5	1	3				4	1		4	3			2
	Ceftiofur Crystalline Free Acid *	Excede		1			3	5	1	2		1		1	1	1	4	2		1	1
	Cephapirin Sodium	ToDay		2																	
Lincosamides	Pirlimycin Hydrochloride	Pirsue		5																	
Macrolides	Erythromycin	Erythromycin																			
	Tildipirosin *	Zuprevo																		1	
	Tulathromycin *	Draxxin															4				1
	Tylosin *	Tylosin								1											
Penicillins	Amoxicillin Trihydrate	AmoxiMast	1	4	1						1										
	Ampicillin	Polyflex		2	1		4	4	2	2	2		1	4	1		5	1	1		1
	Ampicillin	Hetacin		1																	
	Cloxacillin Benzathine	Dryclox	2																		
	Cloxacillin Benzathine	Orbenin	2																		
	Hetacillin Potassium	Polymast		1																	
	Penicillin G	Penicillin		1			2	2	1	1	1		3				1	2			2
	Penicillin G	AlbaDry Plus	2																		
	Penicillin G	Quartermaster	3																		
Sulfonamides	Sulfadimethoxine	Albon								1										1	
Tetracyclines	Oxytetracycline	LA 200		1	1		2	2	1	1	2	1	1		1		2	1	1		1
	Oxytetracycline	TETROXY® 343									1	1									
	Oxytetracycline	Biomycin 200					2	3	2	1	2	1	1				2	1	1	1	2
	Oxytetracycline	Oxytet 100									1										
	Tetracycline	SP 324 POWDER								1	1										
Quinolones	Enrofloxacillin	Baytril															1				