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Enterococci are Gram-positive, facultative anaerobic cocci. They are found in many environments (including milk and dairy products, vegetables, plants, cereals, and meats). *Enterococci* are considered commensal organisms, but can also be opportunistic pathogens associated with morbidity and mortality of humans and animals. A longitudinal study of antibiotic resistance of *Enterococcus* to ampicillin, erythromycin, and tetracycline was conducted on an academic teaching farm. Environmental samples were collected by drag swabs at select locations prior to and after the introduction of livestock. All samples were initially processed and screened with specialized media, and then replica plated on tryptic soy agar containing a predetermined amount of antibiotic. There was some variation in the quantity of bacterial and antibiotic-resistant colonies; however, resistance to tetracycline was extremely high. The increases of too numerous to count populations were not time-dependent and appeared consistently after the placement of cows. There is little information on the prevalence and epidemiology of antibiotic resistance of *Enterococci* outside of the hospital setting, including on dairy farms. Longitudinal studies are important in providing insight into the dynamics of establishment and proliferation of bacteria and of antibiotic resistance.

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A Longitudinal Study of the Establishment and Proliferation of *Enterococcus* on a Dairy Farm

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Abstract

Enterococci are Gram-positive, facultative anaerobic cocci. They are found in many environments (including milk and dairy products, vegetables, plants, cereals, and meats). *Enterococci* are considered commensal organisms, but can also be opportunistic pathogens associated with morbidity and mortality of humans and animals. A longitudinal study of antibiotic resistance of *Enterococcus* to ampicillin, erythromycin, and tetracycline was conducted on an academic teaching farm. Environmental samples were collected by drag swabs at select locations prior to and after the introduction of livestock. All samples were initially processed and screened with specialized media, and then replica plated on tryptic soy agar containing a predetermined amount of antibiotic. There was some variation in the quantity of bacterial and antibiotic-resistant colonies; however, resistance to tetracycline was extremely high. The increases of too numerous to count populations were not time-dependent and appeared consistently after the placement of cows. There is little information on the prevalence and epidemiology of antibiotic resistance of *Enterococci* outside of the hospital setting, including on dairy farms. Longitudinal studies are important in providing insight into the dynamics of establishment and proliferation of bacteria and of antibiotic resistance.

Introduction

Enterococci are Gram-positive, facultative anaerobic cocci that occur singly, in pairs or as short chains (Gilmore, 2002). The enterococci as a group were first described by Thiercelin (1899), and the genus *Enterococcus* was identified by Thiercelin and Jouhaud (Franz *et al.*, 1999). The genus was later described in more detail by Shleifer and Kilpper-Bälz (1984). They demonstrated that *Streptococcus faecalis* and *Streptococcus faecium* were distinct enough from other streptococci to warrant their transfer to the genus *Enterococcus* (Franz *et al.*, 1999). Enterococci are members of the Lactic Acid Bacteria (LAB) group of organisms and are found in a large variety of foods including milk and dairy products, vegetables, plants, cereals and meats (Sewell, 2005). Unlike other LAB, enterococci are not "Generally Recognized as Safe" (GRAS) organisms because they are considered emerging pathogens, are sometimes implicated in food spoilage, and can indicate fecal contamination (Ogier *et al.*, 2008).

Enterococci are considered commensal organisms in humans, but can also be opportunistic pathogens associated with significant morbidity and mortality (Marrow *et al.*, 2009). They typically cause infections in patients who have severe underlying disease, are immunocompromised, or are elderly (Garcia-Migura *et al.*, 2007; Ogier *et al.*, 2008). In addition, enterococci can cause many economically important veterinary diseases such as bovine mastitis and diarrhea (Gilmore, 2002; Petersson-Wolfe *et al.*, 2007). The role that non-human sources and reservoirs other than hospitalized patients may play in the spread of *Enterococcus* is controversial and poorly understood (Hershberger *et al.*, 2005). The epidemiology of *Enterococcus* in bovine mastitis has not been totally clarified, but enterococci are generally associated with infections related to poor hygiene (Gilmore, 2002).

There have been numerous studies of the presence of enterococci on cattle farms, such as in manure (Klein *et al.*, 2011), water (Soupir *et al.*, 2010), and feed (Pradhan *et al.*, 2009), as well as in bedding (Godden *et al.*, 2008) and on the animals themselves (e.g., on hides and in mammary glands) (Fluckey *et al.*, 2009; Petersson-Wolfe *et al.*, 2007). However, to our knowledge, prior to 2011 (Shipp and Dickson, 2011), there have been no longitudinal studies published regarding relocating dairy cattle to previously unused farm land. Longitudinal studies are those that collect data from the same sample elements (such as location) on multiple occasions over time (Lynn, 2009). In addition, there are few studies regarding the establishment and proliferation of antibiotic-resistant enterococci in farm environments (Esiobu *et al.*, 2002; Walczak and Xu, 2011).

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Table 1. Iowa State University (ISU) Dairy Farm Sampling Dates/Groups

Prior to introducing dairy cattle	*After introduction of dairy cattle*
October 20, 2007 (A)	January 27, 2008 (C)
November 13, 2007 (B)	February 29, 2008 (D)
	March 12, 2008 (E)
	April 23, 2008 (F)
	May 28, 2008 (G)

In this article, we present the results of a longitudinal study on the Iowa State University's Dairy/Animal Science Education Facility (henceforth referred to as the "ISU Dairy Farm") regarding the establishment and spread of antibiotic resistance. The study aimed to provide insight into the epidemiology of *Enterococcus* (and other bacteria of importance to food safety) in dairy farm environments.

Methods

The ISU Dairy Farm is located on an 887-acre site, 3 miles south of ISU's central campus. The farm houses 450 milking cows, plus a similar number of heifers, dry cows, and calves. A complex of buildings was constructed on 27 acres of land, including a free-stall barn, maternity barn, and a calf research barn for nutrition and husbandry research. The land the ISU Dairy Farm now occupies was donated to the university. The farm had been recreational, and no livestock had ever been introduced. Experiments were conducted to review antibiotic resistance of enterococci specifically resistant to ampicillin, erythromycin, and tetracycline before and after the placement of livestock.

Environmental samples were collected at selected locations on the ISU Dairy Farm on two occasions before the introduction of livestock in order to determine initial antibiotic resistance in the enterococci occurring in such samples (if any) (Table 1). Additionally, sampling took place on five occasions after cattle placement (Table 1). All sampling was conducted in monthly intervals during the morning hours (beginning at 9:00 AM Central Standard Time). A total of 140 drag swabs were processed in this study; one swab was used for each sampling location for a total of 20 drag swabs per sampling date (Fig. 1) and seven sampling dates/groups in total (A–G, Table 1).

During sampling, one sterile 3 cm × 3 cm drag swab moistened with 10 mL of skim milk (Solar Biologics, Ogdensburg, NY) was used per sample. Briefly, a drag swab was pulled through the environment for 60 s at normal walking pace (approximately 6.7 m). Care was taken to obtain samples in the same locations during subsequent sampling periods. Each drag swab was placed in a sterile bag and stored in a container kept at 4°C. After collection, samples were taken to the laboratory and immediately processed. Each drag swab was aseptically added to a sterile Whirl Pack 24 oz/720 mL homogenizer bag (Nasco, Fork Atkinson, WI) containing 10 mL of buffered peptone water (BPW; Difco, Becton Dickinson Company, Sparks, MD) to moisten sample for pipetting. The sample was homogenized for 45 s at 250 rpm (Seward 400 Circulator Stomacher; Seward Laboratory Services, Bohemia, NY). One milliliter of sample was then added to a tube containing 9 mL of BPW creating a diluent concentration of 10^{-1}. The samples were serially diluted until a concentration of 10^{-5} was reached. The 10^{-3} and the 10^{-5} dilutions were plated on Enterococcosel agar (Becton Dickinson Company, Sparks, MD). Plates were incubated for 48 h at 37°C.

Resistance to antibiotics was investigated over time via replica plating (Natarajan *et al.*, 2007; Osterblad *et al.*, 1995).

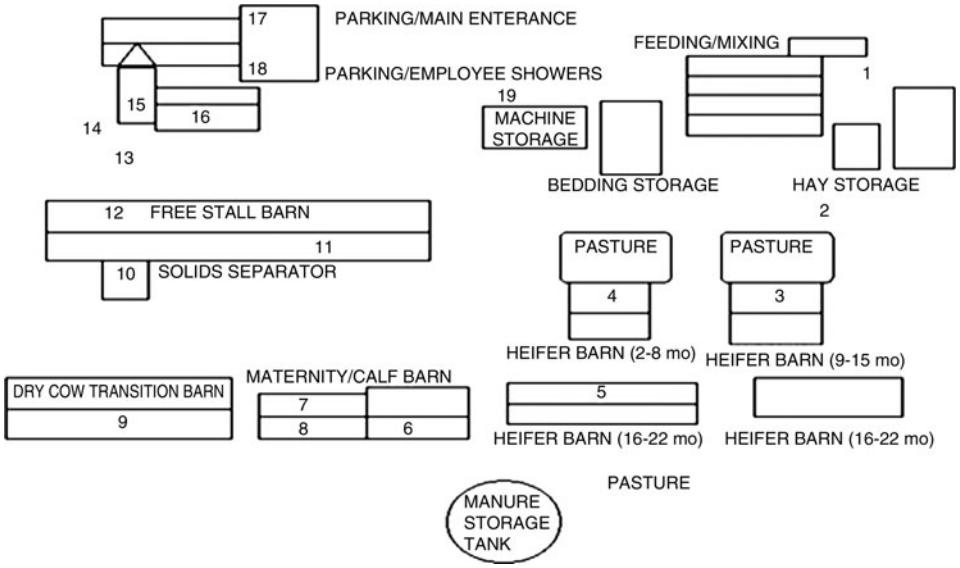

FIG. 1. Diagram of sampling locations at the Iowa State University Dairy/Animal Science Education and Discovery Facility. (1) Feed Storage Area (FSA); (2) Open Field NE (OF); (3) Heifer Barn 9–15 months (H9); (4) Heifer Barn 2–8 months (H2); (5) Heifer Barn 16–22 months (H16); (6) Maternity/Calf Barn (MC 6); (7) Maternity/Calf Barn (MC 7); (8) Maternity/Calf Barn (MC 8); (9) Dry Cow/Transition Barn (DC); (10) Solids Separator (SS); (11) Free Stall Barn (FS11); (12) Free Stall Barn (FS12); (13) Free Stall Barn Walkway (FW); (14) Holding Area Walkway (HW); (15) Holding Area/Milking (HM); (16) Special Needs/Hospital Barn (SH); (17) Parking Main Entrance (PM); (18) Parking/Employee Showers (PS); (19) Equipment Storage (ES); (20) Machine Storage Area (MS).

Colonies from the 10^{-3} and 10^{-5} Enterococcosel plates were replica plated (using a replica plating cylinder covered with sterile velvet) onto tryptic soy agar (TSA) (Becton Dickinson Company, Sparks, MD) containing ampicillin (48 μg/mL; Sigma, St. Louis, MO), erythromycin (48 μg/mL; Sigma), and tetracycline (24 μg/mL; MP Biomedicals, Solon, OH); these quantities were at least 1.5 times the concentrations recommended by the National Antimicrobial Resistance Monitoring System (NARMS) (Gilmore, 2002). All TSA plates were prepared using the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Wilker, 2007) and refrigerated 1–2 weeks before sampling was carried out. Data were analyzed by EpiInfo (version 3.5), a public domain program created by the Centers for Disease Control and Prevention's PulseNet Program (Atlanta, GA).

Medications used to treat conditions such as mastitis, diarrhea, and upper respiratory infections were prescribed by an ISU faculty veterinarian who disclosed uses and doses given. Sick cattle were treated individually. Additionally, a detailed protocol was strictly followed by the ISU Dairy Farm staff. BAC-STOP udder predip dip (Esteam Manufacturing Ltd., Calgary, Alberta, Canada) and Transcend udder post dip (IBA, Millbury, MA) were used prior to and after milking. FC-98 Udder Wash (IBA) was also used throughout the farm as a boot sanitizer.

Results

When assessing sampling dates and antibiotic resistance (Table 1, A–G), the number of colony-forming units (CFU)

Table 2. Total Tryptic Soy Agar (TSA) Colony-Forming Units (CFU) and Resistant Colonies (Ampicillin, Erythromycin, and Tetracycline)

Farm Group	*Antibiotic/concentration*	*Total CFU*	*Resistant colonies*	*Percentages*
A	Ampicillin (10^{-3})	24	1	4%
A	Erythromycin (10^{-3})	24	0	0%
A	Tetracycline (10^{-3})	24	0	0%
A	Ampicillin (10^{-5})	Data not shown	Data not shown	Data not shown
A	Erythromycin (10^{-5})	0	0	0%
A	Tetracycline (10^{-5})	0	0	0%
B	Ampicillin (10^{-3})	10	5	50%
B	Erythromycin (10^{-3})	10	35	350%
B	Tetracycline (10^{-3})	10	0	0%
B	Ampicillin (10^{-5})	Data not shown	Data not shown	Data not shown
B	Erythromycin (10^{-5})	0	0	0%
B	Tetracycline (10^{-5})	0	0	0%
C	Ampicillin (10^{-3})	142	24	17%
C	Erythromycin (10^{-3})	142	16	11%
C	Tetracycline (10^{-3})	142	126	89%
C	Ampicillin (10^{-5})	Data not shown	Data not shown	Data not shown
C	Erythromycin (10^{-5})	77	18	23%
C	Tetracycline (10^{-5})	77	133	172%
D	Ampicillin (10^{-3})	227	4	2%
D	Erythromycin (10^{-3})	227	70	31%
D	Tetracycline (10^{-3})	257	249	97%
D	Ampicillin (10^{-5})	Data not shown	Data not shown	Data not shown
D	Erythromycin (10^{-5})	42	18	43%
D	Tetracycline (10^{-5})	42	42	100%
E	Ampicillin (10^{-3})	407	142	35%
E	Erythromycin (10^{-3})	407	196	48%
E	Tetracycline (10^{-3})	407	338	83%
E	Ampicillin (10^{-5})	Data not shown	Data not shown	Data not shown
E	Erythromycin (10^{-5})	29	4	14%
E	Tetracycline (10^{-5})	29	48	166%
F	Ampicillin (10^{-3})	127	16	13%
F	Erythromycin (10^{-3})	127	54	42%
F	Tetracycline (10^{-3})	127	232	183%
F	Ampicillin (10^{-5})	Data not shown	Data not shown	Data not shown
F	Erythromycin (10^{-5})	58	38	66%
F	Tetracycline (10^{-5})	58	223	384%
G	Ampicillin (10^{-3})	82	0	0%
G	Erythromycin (10^{-3})	82	3	4%
G	Tetracycline (10^{-3})	82	61	74%
G	Ampicillin (10^{-5})	Data not shown	Data not shown	Data not shown
G	Erythromycin (10^{-5})	19	60	316%
G	Tetracycline (10^{-5})	19	132	695%

Data not shown, minimal/no CFU detected; total CFU, CFU counted on Enterococcosel plates; CFU resistance, grown on TSA with specified antibiotic (10^{-3} and 10^{-5} concentrations); percentages, percent of resistant colonies.

Table 3. Too Numerous to Count (TNTC) Locations and Sampling Groups A–G

Location	*Date A*	*Date B*	*Date C*	*Date D*	*Date E*	*Date F*	*Date G*	*N = number*
1 (FSA)							×	1
2 (OF)			×					1
3 (H9)								0
4 (H2)								0
5 (H16)								0
6 (MC 6)				×				1
7 (MC 7)								0
8 (MC 8)			×	×			×	3
9 (DC)								0
10 (SS)			×	×	×	×	×	5
11 (FS 11)			×		×	×	×	4
12 (FS 12)			×	×	×	×	×	5
13 (FW)			×	×	×	×	×	5
14 HW			×	×		×	×	4
15 (HM)			×	×	×	×		4
16 (SH)					×			1
17 (PM)								0
18 (PS)								0
19 (ES)								0
20 (MS)								0
N (group)	0	0	8	7	6	6	7	34

Example: Date C, Location 2, Sampled on January 27, 2008; open field NE.
Also see Table 1 and Figure 1 for reference.

growing on 10^{-3} and 10^{-5} TSA plates containing varying amounts of ampicillin, erythromycin, and tetracycline were as shown in Table 2. The number and percentages of bacterial CFU detected on Enterococcosel plates varied (e.g., in the 10^{-3} plates, there were increases in farm groups A–E and a noticeable decrease in groups F and G). In TSA antibiotic-resistance plates, there was also variation; however, resistance to tetracycline in both the 10^{-3} and 10^{-5} plates was extremely high.

We also reviewed trends regarding too numerous to count (TNTC) locations on the ISU Dairy Farm. Table 3 depicts where TNTC Enterococcosel plates were found (farm location and farm number). Prior to the introduction of dairy cattle, no TNTC locations were detected. However, after dairy cattle were introduced, TNTC locations were consistent throughout the study (from six to eight TNTC plates/locations) detected.

Discussion

In this longitudinal study, the numbers and percentage of bacterial CFU varied. However, antibiotic-resistant CFU increased as cattle were placed at the facility; this is to be expected since many bacterial organisms (including pathogens) originate in the ruminant intestinal tract (Lefebvre *et al.,* 2006; Oliver *et al.,* 2005; Pradhan *et al.,* 2009). It is important to note that, in this study, antibiotic-resistant *Enterococcus* grew on TSA plates containing extremely high levels of ampicillin (48 μg/mL), erythromycin (48 μg/mL), and tetracycline (24 μg/mL)—much higher than the amount deemed resistant by CLSI. In addition, there were enterococci present that were multi-drug resistant. In the 10^{-3} dilutions plated on antibiotic-containing TSA, 25 out of 38 (66%) of plates were multi-drug resistant; of those 25 antibiotic-resistant plates, 18 (72%) exhibited resistance to erythromycin and tetracycline. In the 10^{-5} dilutions plated on antibiotic-containing TSA, 19 of 25 plates (76%) were multi-drug resistant. Nine of those plates (26%) demonstrated ampicillin and tetracycline resistance. This is significant because multi-drug resistance is becoming more prevalent (French, 2008) and is compromising the treatment of disease in both humans and animals (Leclercq, 2009; Sawant *et al.,* 2007).

In Table 3, TNTC Enterococcosel plates by farm locations are diagrammed. In locations A and B (before dairy cattle placement), there were no TNTC plates (farm locations) detected. However, immediately after the placement of cattle, TNTC plates (farm locations) were detected. The numbers of TNTC plates were consistent throughout the sampling periods: C ($n = 8$); D and G ($n = 7$); and E and F ($n = 6$). Since the TNTC Enterococcosel plates could not be replicated on antibiotic-containing TSA, the actual numbers of bacterial CFU and the antibiotic resistant profiles (derived from antibiotic-containing plates) are likely much higher and more extensive.

One limitation of this study is that slight differences in making specialized media (e.g., concentration of powdered agar mix, length of time autoclaving) could have influenced microbial counts. Additionally, this study took place over a 9-month period, but it would have been better to continue sampling over a longer period of time. Measures based on long-term data collection would be more useful in devising strategies to limit the spread of resistant infectious bacterial organisms in farming environments (Burgos *et al.,* 2005).

Conclusion

There is little information on the prevalence and epidemiology of antimicrobial resistance in *Enterococci* outside the hospital setting, including on dairy farms (Hershberger *et al.,* 2005). However, there have been many studies of antibiotic resistance of enterococci in food items such as cheeses, meats, and fermented foods (Teuber *et al.,* 1999). Increased resistance of enterococci in foods is of interest because the ability of these organisms to infect immunocompromised hosts and cause

serious medical conditions in humans (Teuber *et al.,* 1999). In dairy cows, animals that develop diseases such as mastitis and post-parturient disease may become chronically infected and be sent to slaughter prematurely (Makovec and Ruegg, 2003; Sischo, 2006). The lack of surveillance data is especially evident in important agricultural environments such as dairy farms (Burgos *et al.,* 2005). When completing a literature review concerning *Enterococcus* resistance in a new dairy farm environment (analysis of bacteria prior to, and after placement of dairy cattle), no information was found at the time of this writing. To our knowledge, this is the second study of the establishment and proliferation of bacteria (and antibiotic resistance) before and after the introduction of production livestock on previously unused farm land. As noted, the first was conducted in our laboratory (Shipp and Dickson, 2011).

While there may be an abundance of information regarding treatment of conditions in dairy cattle such as mastitis and post-parturient disease (DeGaris and Lean, 2008; Petersson-Wolfe *et al.,* 2008; Sischo, 2006; Zhao and Lacasse, 2008), the role that non-human sources and reservoirs (other than hospitalized patients) may play in the spread of *Enterococcus* is controversial and poorly understood (Hershberger *et al.,* 2005). Most antimicrobial studies have focused on bacteria such as *Salmonella*, enterotoxigenic *Escherichia coli*, and bacteria isolated from clinical cases (Sawant *et al.,* 2007). While there are studies that question the risks of using antibiotics in animal production (Cox *et al.,* 2009; Wilhelm *et al.,* 2009), the general consensus is that increased surveillance of bacteria (such as *Enterococcus)* and compliance with appropriate use of antibiotics would be beneficial to human and animal health (Allerberger and Mittermayer, 2008; Cox *et al.,* 2009; Prescott, 2008). Minimizing antibiotic resistance requires a multidisciplinary approach (French, 2008). Efforts such as creating new antibiotics (Leclercq, 2009), conducting research to minimize bacterial infections in livestock and in humans (Anderson *et al.,* 2008; Chingwaru *et al.,* 2003; Makovec and Ruegg, 2003), improving diagnostic skills of laboratory workers (Emori and Gaynes, 1993; Hageman *et al.,* 2003), using antibiotics in a responsible (or "prudent") fashion (Prescott, 2008), and better farm management (Sischo, 2006) would be helpful in addressing the issue of antibiotic resistance among humans and animals.

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