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frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 10 April 2012
doi: 10.3389/fmicb.2012.00139

Excretion of antibiotic resistance genes by dairy calves fed milk replacers with varying doses of antibiotics

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Elevated levels of antibiotic resistance genes (ARGs) in soil and water have been linked to livestock farms and in some cases feed antibiotics may select for antibiotic resistant gut microbiota. The purpose of this study was to examine the establishment of ARGs in the feces of calves receiving milk replacer containing no antibiotics versus subtherapeutic or therapeutic doses of tetracycline and neomycin. The effect of antibiotics on calf health was also of interest. Twenty-eight male and female dairy calves were assigned to one of the three antibiotic treatment groups at birth and fecal samples were collected at weeks 6, 7 (prior to weaning), and 12 (5 weeks after weaning). ARGs corresponding to the tetracycline (*tet*C, *tet*G, *tet*O, *tet*W, and *tet*X), macrolide (*erm*B, *erm*F), and sulfonamide (*sul*1, *sul*2) classes of antibiotics along with the class I integron gene, *int*I1, were monitored by quantitative polymerase chain reaction as potential indicators of direct selection, co-selection, or horizontal gene transfer of ARGs. Surprisingly, there was no significant effect of antibiotic treatment on the absolute abundance (gene copies per gram wet manure) of any of the ARGs except *erm*F, which was lower in the antibiotic-treated calf manure, presumably because a significant portion of host bacterial cells carrying *erm*F were not resistant to tetracycline or neomycin. However, relative abundance (gene copies normalized to 16S rRNA genes) of *tet*O was higher in calves fed the highest dose of antibiotic than in the other treatments. All genes, except *tet*C and *int*I1, were detectable in feces from 6 weeks onward, and *tet*W and *tet*G significantly increased ($P < 0.10$), even in control calves. Overall, the results provide new insight into the colonization of calf gut flora with ARGs in the early weeks. Although feed antibiotics exerted little effect on the ARGs monitored in this study, the fact that they also provided no health benefit suggests that the greater than conventional nutritional intake applied in this study overrides previously reported health benefits of antibiotics. The results suggest potential benefit of broader management strategies, and that cost and risk may be avoided by minimizing incorporation of antibiotics in milk replacer.

Keywords: antibiotic resistance genes, manure, dairy calves, milk replacer

INTRODUCTION

The extensive use of antibiotics in animal agriculture and the development of antibiotic resistant bacteria have been cause for increasing concern. Livestock operations are often cited as a reservoir for resistant bacteria and antibiotic resistance genes (ARGs; Chee-Sanford et al., 2001; Smith et al., 2004; Sawant et al., 2007; McKinney et al., 2010); and antibiotic use has implications for both animal and human health. Antibiotics are fed to livestock at subtherapeutic levels for both growth promotion and disease prevention, because their use can reduce morbidity and mortality (e.g., Wileman et al., 2009). However, the unintentional selection of bacteria that are resistant to antibiotics could have important human health consequences, with several studies noting identical resistance elements in both humans and food animals (e.g., Boerlin et al., 2001; Lauderdale et al., 2002; Ho et al., 2010).

Based on concerns about human health effects, subtherapeutic use of antibiotics was banned in the European Union in the late 1990s (Boerlin et al., 2001). A major trigger for earlier bans in Denmark and other Nordic countries, was linkage of avoparcin use in broiler chickens and swine to vancomycin resistant *Enterococcal* infections (VRE) in humans (Bager et al., 1997; Bates, 1997). In the United States (U.S.), the Food and Drug Administration (FDA) recently issued new rules limiting antibiotic use in milk replacers for calves (21CFR §520.1484; 21CFR §520.1660d). The precise effects of antibiotics in milk replacer is currently unknown, and the general consequences of antibiotic amendment in livestock feed are complex (Marshall and Levy, 2011). In the 3-years following the European ban, a slight decrease in antibiotic resistance was observed in rectal swabs collected from food animals at slaughter, and a documentable decrease in acquired fecal enterococci resistance in humans was also observed (Casewell et al., 2003). Following the Taiwan avoparcin ban in chickens in 2000, the incidence of VRE decreased significantly (Lauderdale et al., 2007). However, VRE did still persist following the ban and resistance to

other classes of antibiotics, including tetracyclines and macrolides, stayed the same or even increased. In support of this observation, vancomycin and macrolide ARGs have been noted to be present together on the same genetic element from isolates obtained from Danish pig herds (Aarestrup, 2000). Thus, it is critical to consider co-selection of resistance across antibiotic classes in evaluating impacts of feed antibiotics.

Effects of subtherapeutic antibiotic use on animal health have been difficult to assess. Based on medicated feed prescription rates, decreased rates of respiratory infections were observed in Swiss piglets and fattening pigs in the 3-years following the antibiotic ban relative to the 3-years prior, but gastrointestinal tract infections appeared to go up (Arnold et al., 2004). A marked increase in necrotic enteritis was noted in broiler chickens in Norway following the avoparcin ban, but this seemed to be ameliorated by the subsequent approval of narisin as a feed additive (Grave et al., 2004). Also, while therapeutic antibiotic use rates did indeed decrease in Switzerland (Arnold et al., 2004), Norway, and Sweden (Grave et al., 2006) following the E.U. ban, therapeutic use increased in weaning piglets in Denmark (Grave et al., 2006). Generally, a comprehensive examination of banning subtherapeutic antibiotic use in piglets, beef cattle, and poultry in Sweden, indicated that although temporary health problems and increases in antibiotic uses were noted, strategic management practices make possible competitive animal production and reduced antibiotic use (Wierup, 2001). Few controlled studies have examined the effects of antibiotic use on health (Berge et al., 2009a) or antibiotic resistance (Langford et al., 2003; Berge et al., 2006a; Kaneene et al., 2008; Pereira et al., 2011) in pre-weaned calves, and none have incorporated culture-independent techniques to quantify the establishment of ARGs in calf manure.

The objective of this study was to compare the establishment of a cross-section of ARGs in the feces of calves receiving milk replacer dosed with subtherapeutic and therapeutic antibiotics and their effect on overall health. In particular, the effect of oxytetracycline and neomycin in the milk replacer and weaning to starter grain containing monensin were of interest as typical practices in the U.S. The inclusion of oxytetracycline and neomycin in milk replacer for young calves is one of the most common uses of subtherapeutic antibiotics in the U.S. dairy industry. Neomycin is absorbed only very slightly by the animal (Aschbacher and Feil, 1994) and thus remains in the digestive tract where it controls growth of pathogenic organisms. Oxytetracycline, in contrast, is highly absorbed, so is considered useful for prevention and treatment of respiratory illnesses. One intentional contrast to conventional dairy practice incorporated in this study was feeding calves >2 times more protein than in traditional feeding programs, and >1.5 times more fat. This is because while conventional U.S. milk replacer feeding rates generally provide sufficient nutrients only for maintenance and modest growth [National Research Council (NRC), 2001], which could bias the evaluation of the health benefits of antibiotics.

In this study, 28 male and female dairy calves were assigned to control, subtherapeutic, or therapeutic antibiotic treatment groups at birth and fecal samples were collected at weeks 6, 7 (prior to weaning), and 12 (5 weeks after weaning). The abundance of a representative range of tetracycline (*tet*O, *tet*W, *tet*C, *tet*G, and *tet*X), sulfonamide (*sul*1 and *sul*2), and macrolide (*erm*B and *erm*F) ARGs as well as a class 1 transferable genetic element (*int*I1) was quantified in the calf feces as a culture-independent assessment of resistance. The specific ARGs examined were selected as indicators of direct antibiotic selection (i.e., *tet* ARGs) as well as potential co-selection of resistance to clinically relevant antibiotics (i.e., *sul* and *erm* ARGs) and overall horizontal gene transfer potential (i.e., *int*I1). Quantitative polymerase chain reaction (qPCR) was incorporated to provide a culture-independent assessment of the overall resistance potential incurred by these ARGs. Identifying factors that affect fecal excretion of ARGs by dairy calves will help assess the contribution of dairy farms to the overall loading of anthropogenic sources of antibiotic resistance to the environment and provide insight into effective management strategies for limiting the spread of antibiotic resistance.

MATERIALS AND METHODS

EXPERIMENTAL TREATMENTS

Forty-one newborn calves were blocked according to breed (Holstein or crossbred), birth order (two blocks), and gender. Crossbred calves were either Holstein- or Jersey-sired and out of Jersey × Holstein, Jersey × Holstein × Brown Swiss, or Jersey × Holstein × Swedish Red dams. Within each block, calves were randomly assigned to treatments at birth, resulting in the assignments summarized in **Table A1** in Appendix. Treatments were control (containing no antibiotics in the milk replacer), subtherapeutic (neomycin sulfate and oxytetracycline hydrochloride each fed at 10 mg/calf/day provided from day 1 until weaning), and therapeutic (no antibiotics in the milk replacer until day 36, then neomycin sulfate and oxytetracycline hydrochloride each fed at 1000 mg/calf/day for 14 day). All protocols and procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

MANAGEMENT OF NEWBORN CALVES

Within 2 h of birth, calves were moved from the calving pen to individual stalls in an enclosed barn. Calf navels were dipped in a 7% tincture of iodine, and calves were vaccinated intranasally with Nasalgen (Merck Animal Health, Millsboro, DE). Birth date, weight, and identity of the dam were recorded. Calves received 1.89 l of high quality thawed colostrum as soon as possible after birth, usually within 2 h. A second feeding of 1.89 l of colostrum was administered 6 h after the first feeding and calf navels were dipped a second time.

FEEDING AND HOUSING

Calves were enrolled and treatments were imposed at 1 day of age. Calves were fed milk replacer (Cow's Match, Land O'Lakes Animal Products Co., Arden Hills, MN, USA) twice daily, at 6:00 AM and 6:00 PM. Milk replacer contained 28% crude protein and 20% fat, with whey protein as the protein source and human grade edible lard as the fat source. Milk replacer powder was reconstituted with water to contain 17.6% solids and fed individually to calves via nipple buckets. Amount of liquid fed provided 1.1–0.68 kg of dry matter per calf, depending upon birth weight, resulting in feeding 2.1–3.4 times more protein and 1.5–2.4 times more fat than traditional feeding programs. This feeding rate has been

termed "intensive feeding" is higher than conventional calf feeding programs, which deliver only 0.454 kg of milk replacer dry matter containing 20% fat and 20% protein. The intensive feeding program thus more closely approaches amounts calves would consume if allowed free choice to liquid diets. Antibiotic treatments were added directly to the nipple buckets, which were used exclusively for a specific treatment to avoid cross-contamination. Calves were supervised during feeding, and 15 min was allowed for consumption. After feeding, all equipment was thoroughly cleaned. Calves were fed starter grain (Intensity −22% crude protein, 10.13 mg/kg of monensin, Southern States Cooperative, Richmond, VA, USA) beginning at 1 day of age.

Calves were housed at the Virginia Tech Dairy Cattle Center in individual fiberglass or plastic hutches (1.83 m × 1.37 m) with metal hog panels to create a fenced area (∼2.5 m^2). Bull calves were housed in hutches until 39 day, when they were moved into metabolism crates to facilitate total feces and urine collection (described below). After the total collection period was complete, bull calves were moved back to their original hutches until weaned.

Weaning was initiated at day 50 by reducing milk replacer offered by 50% and feeding only once daily at 6:00 AM. Calves were initially fed 0.22 kg of starter grain, and the amount offered increased in 0.22 kg increments as consumption increased. When starter grain consumption reached 1.81 kg daily, calves were fully weaned (59 ± 2 day of age). Post-weaning, calves were housed in the calf hutches for 7 days then were housed in small groups in a 3-sided barn with access to a drylot. Weaned calves were group-fed first cutting alfalfa hay and 22% crude protein starter grain ad lib. Starter grain contained monensin at a concentration of 10.13 mg/kg.

SAMPLE AND DATA COLLECTION

Following the 6:00 AM feeding, body temperatures, fecal scores, and respiratory scores (Larson et al., 1977) were recorded daily. Calves were weighed at birth and at weaning to calculate average daily gain.

Bull calves were housed in metabolism crates (1.2 m × 0.6 m) to facilitate total collection of feces beginning at 39 day of age, for 3 day of adaptation to the stall followed by 7 day of total collection. Trays were placed under the metabolism crates to collect excreted feces. Feces were collected daily, weighed, and subsampled. Because heifer calves are anatomically ill-suited for total collection of feces uncontaminated by urine, grab samples of feces were collected from heifer calves at 6:00 AM, 2:00 PM, 8:00 PM, and 12:00 AM for 7 day beginning at 6 weeks of age. Fecal samples were collected from the gravel surface if a fresh sample was available at collection time. If no fresh sample was available, calves were rectally stimulated to produce a fresh sample. After weaning, samples were collected weekly from calves (now group-housed) via rectal palpation.

REMOVED CALVES

A total of 13 calves were removed from the study (3, 7, and 3 were on control, subtherapeutic, and therapeutic treatments, respectively), because of health problems requiring clinical antibiotic treatment.

SAMPLE ANALYSIS

Feces samples were frozen immediately at −20°C after collection. Samples collected from heifer calves were thawed and pooled by date collected on an equal wet weight basis. Pooling was not necessary for bull calf feces samples because one sample was collected of each day's total excretion. DNA was extracted from 500 mg of wet feces per calf from week 6, 7, and 12. The FastDNA® Spin Kit for soil was applied across all samples. The FastPrep® instrument (MP Biomedicals, Santa Ana, CA, USA) was employed with homogenization for 40 s at a speed of 6.0. The DNA was suspended in purified water and aliquots were stored in 0.5 ml cryovials at −80°C.

Previously reported qPCR protocols were implemented to quantify the following ARGs: *erm*B, *erm*F (Chen et al., 2007), *sul*1, *sul*2 (Pei et al., 2006), *tet*C, *tet*G (Aminov et al., 2002), *tet*O, *tet*W (Aminov et al., 2001), and *tet*X (Ng et al., 2001) as well as *int*I1 (Hardwick et al., 2008) and 16S rRNA (Suzuki et al., 2000) genes. Primer sequences are provided in **Table A2** in Appendix. The 9-μl per reaction master mix consisted of 2.8 μl of sterile water, 0.6 μl (5 M) of forward primer, 0.6 μl (5 M) of reverse primer, and 5 μl of SsoFastEvaGreen (Bio-Rad, Hercules, CA, USA) with 1 μl of diluted DNA extract added. A dilution series qPCR was conducted for each gene and each sample matrix, through which it was determined that a 1:70 dilution provided the most consistent results across all samples. All qPCR were conducted in triplicate on a CFX-96 (Bio-Rad). Calibration curves were constructed from serial dilutions of positive controls over seven orders of magnitude. Positive controls were obtained from cloned PCR products of target genes verified by DNA sequencing. Gene copies for any samples with numbers of gene copies below the limit of quantification were recorded as 0 for the purposes of statistical analysis.

STATISTICAL ANALYSIS

Fecal scores, respiratory scores, and body temperatures were analyzed using the Mixed procedure of SAS (9.2, 2008) with the model:

$$Y = \mu + D_i + T_j + B_k + G_l + BG_{kl} + TB_{jk} + TG_{jl} + e_{ijkl}$$

where:

μ = overall mean;

D = effect of day (i = 1–65);

T = effect of treatment (j = control, subtherapeutic, therapeutic);

B = effect of breed (k = crossbred, Holstein);

G = effect of gender (l = bull, heifer); and

e = error (interaction of day, treatment, breed, and gender).

Average daily gains were analyzed using the Mixed procedure of SAS (9.2, 2008) with the model:

$$Y = \mu + T_i + B_j + G_k + BG_{jk} + TB_{ij} + TG_{ik} + e_{ijk}$$

where:

μ = overall mean;

T = effect of treatment (i = control, subtherapeutic, therapeutic);

B = effect of breed (j = crossbred, Holstein);

G = effect of gender (k = bull, heifer); and
e = error (interaction of treatment, breed, and gender).

Because they were not normally distributed, ARG data were log-transformed prior to statistical analysis to determine significance of main effects and interactions. Log-transformed values were used to calculate LSM. All gene data were analyzed using the GLIMMIX procedure of SAS (9.2, 2008) with the model:

$$Y = \mu + T_i + B_j + G_k + W_l + TB_{ij} + TG_{ik} + BG_{jk} + WT_{il} + WB_{jl} + WG_{kl} + e_{ijkl}$$

where:
μ = overall mean;
T = effect of treatment (i = control, subtherapeutic, therapeutic);
B = effect of breed (j = crossbred, Holstein);
G = effect of gender (k = bull, heifer);
W = effect of week (l = 6, 7, 12); and
e = error (interaction of treatment, breed, gender, and week).

After the log-transformed data set was used to determine significance, contrasts were performed to separate means for significant effects and interactions. All data are reported as LSM ± SE. Significance was determined at $P < 0.10$.

RESULTS

EFFECT OF ANTIBIOTICS ON GROWTH AND HEALTH

No effects of antibiotic treatment, breed or gender were observed on average daily gain (1.30 ± 0.13 kg/day), fecal scores (2.5 ± 0.17), respiratory scores (1.2 ± 0.04), or body temperature (102.2° ± 0.95).

EFFECT OF ANTIBIOTICS ON ARGs IN FECES

Two of the analyzed genes (*tet*C and *int*I1) were not detected in fecal samples of any calves. Four genes encoding resistance to tetracyclines were detected in feces (*tet*G, *tet*O, *tet*W, *tet*X; **Figure 1**) as were two genes encoding resistance to sulfonamides (*sul*1, *sul*2) and two genes encoding resistance to erythromycin (*erm*B, *erm*F). All calves harbored at least one of the *tet* genes in the feces by week 12, and multiple ARGs related to tetracyclines were present in nearly all of the fecal samples analyzed. ARGs corresponding to antibiotics not fed to calves were less abundant across all three treatments (**Figure 2**). About one third of the calves did not have *sul*1 present in their feces at any individual sampling point.

Feeding milk replacer with subtherapeutic or therapeutic doses of neomycin and oxytetracycline had no effect on the absolute abundance (log copy per gram wet weight) of *tet*G, *tet*O, *tet*W, *tet*X, *sul*1, *sul*2, or *erm*B in manure, but calves fed medicated milk replacers yielded reduced abundance of *erm*F as compared to control calves (**Table 1**). The relative abundance (ARG per 16S rRNA; normalized) of *erm*F was not different between antibiotic fed and non-antibiotic control groups (**Table 2**). Calves fed the higher (therapeutic) dose of antibiotics resulted in increased relative abundance of *tet*O as compared to subtherapeutic and control calves (**Table 2**).

EFFECT OF OTHER FACTORS ON ARG ABUNDANCE

Abundance of the ARGs analyzed was not different between genders or between breeds, but abundance of all *tet* ARGs except *tet*O increased with time in all treatments (**Figure 1**), including the no antibiotic control. For *tet*G and *tet*X, gene abundance increased between weeks 6 and 12. For *tet*W, the increase in abundance occurred during the period following weaning, and was detected at the week 12 sampling point. Effects of time on *tet* ARGs were no longer observed when the data were

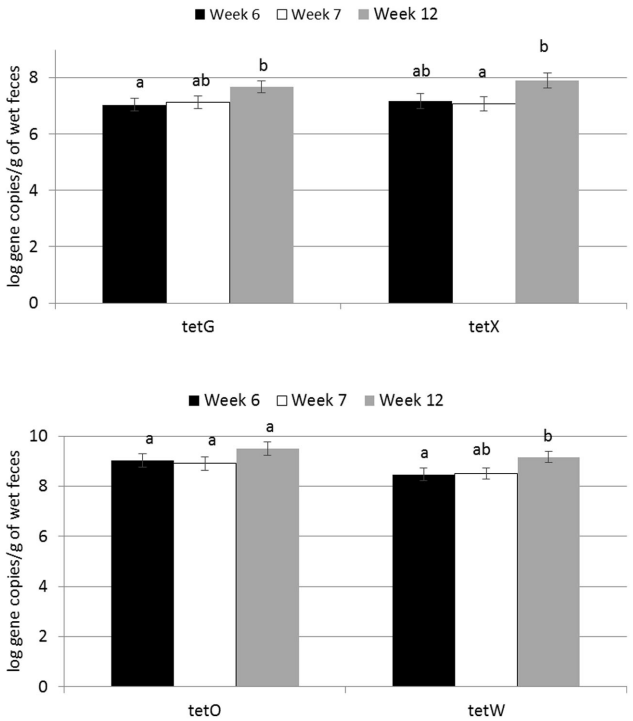

FIGURE 1 | Effect of time on abundance of tetracycline ARGs in calf feces. Bars represent the pooled averages across calves fed medicated or non-medicated milk replacers. Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

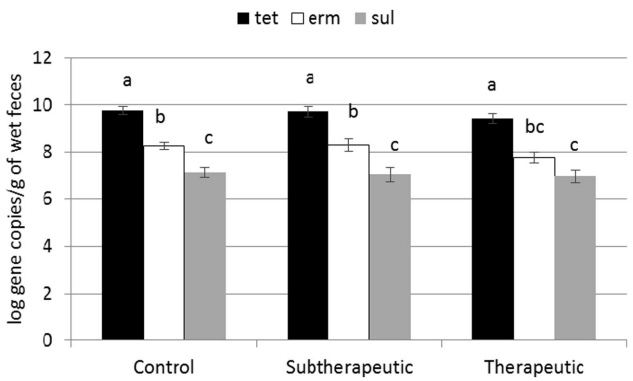

FIGURE 2 | Effect of antibiotics in milk replacer on abundance of tetracycline (*tet*), macrolide (*erm*), and sulfonamide (*sul*) classes of ARGs. Bars represent the pooled averages of the total ARGs quantified within each class. Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

Table 1 | Effect of milk replacer medication, breed, gender, week, and the interaction of milk replacer with these on abundance of selected antibiotic resistance genes in the feces of dairy calves.

	Log gene copies per gram wet feces			SE[4]	*P* <				
	Control[1]	Subtherapeutic[2]	Therapeutic[3]		Trt	Breed	Gender	Day	Interactions[5]
n	12	7	9						
*tet*G	7.40	7.29	7.17	0.18	NS	NS	NS	0.06	Trt*gender; breed*gender
*tet*O	9.18	9.10	9.13	0.34	NS	NS	NS	NS	NS
*tet*W	8.89	8.80	8.43	0.28	NS	NS	NS	0.07	NS
*tet*X	7.47	7.70	6.99	0.34	NS	NS	NS	NS	NS
*sul*1	6.29	5.88	6.34	0.28	NS	0.10	NS	NS	Trt*gender; gender*week
*sul*2	6.78	6.64	6.61	0.23	NS	NS	NS	NS	NS
*erm*B	7.20	7.31	6.82	0.37	NS	NS	NS	NS	NS
*erm*F	7.91	7.36	6.95	0.32	0.04	NS	NS	0.003	NS

[1] *Control milk replacer containing no antibiotics fed from day 2 to weaning (59± 2 day).*

[2] *Medicated milk replacer containing 10 mg/calf/day of tetracycline and neomycin fed from day 2 to weaning.*

[3] *Medicated milk replacer containing 1000 mg/calf/day of tetracycline and neomycin fed from day 36 to weaning.*

[4] *SE for treatment LS means.*

[5] *Significant (P < 0.10) two and three way interactions of treatment, breed, gender, and week.*

Table 2 | Effect of milk replacer medication, breed, gender, week, and the interaction of milk replacer with these on selected antibiotic resistance genes in the feces of dairy calves.

	Gene copies/16S rRNA			SE[4]	*P* <				
	Control[1]	Subtherapeutic[2]	Therapeutic[3]		Trt	Breed	Gender	Day	Interactions[5]
*tet*G	0.65	0.90	13.5	14.3	NS	NS	NS	NS	NS
*tet*O	2.27	2.13	5.35	1.53	0.02	NS	NS	NS	NS
*tet*W	10.3	4.00	1.84	9.13	NS	NS	0.07	NS	NS
*tet*X	0.50	0.69	0.88	0.68	NS	0.03	NS	NS	Trt*gender; breed*gender; breed*week
*sul*1	0.13	0.12	1.11	0.58	NS	NS	NS	NS	Gender*week
*sul*2	0.06	0.06	0.13	0.12	NS	NS	NS	NS	Breed*week
*erm*B	0.89	0.78	0.67	0.99	NS	NS	NS	NS	NS
*erm*F	0.66	0.09	0.16	0.75	NS	NS	NS	0.004	NS

[1] *Control milk replacer containing no antibiotics fed from day 2 to weaning (59± 2 day).*

[2] *Medicated milk replacer containing 10 mg/calf/day of tetracycline and neomycin fed from day 2 to weaning.*

[3] *Medicated milk replacer containing 1000 mg/calf/day of tetracycline and neomycin fed from day 36 to weaning.*

[4] *SE for treatment LS means.*

[5] *Other significant (P < 0.10) two and three way interactions of treatment, breed, gender, and week.*

expressed per unit of 16S rRNA gene (**Table 2**). However, relative abundance of *erm*F was observed to increase with time across treatments, only when expressed per unit of 16S rRNA gene (**Figure 3**).

For the majority of the ARGs, breed, gender, and week did not have any effect except that crossbred and bull calves harbored reduced relative abundance of *tet*X and *tet*W, respectively (**Table 2**). Interaction of treatment with breed, gender, and week of treatment had no significant effects on gene abundance for the majority of the analyzed genes. The interactions of treatment and gender were significant for *tet*G and *sul*1 (**Table 1**). Among control calves, heifers harbored higher abundance of *tet*G more than did bulls; that was reversed in the subtherapeutic group but the difference was not significant (**Figure 4**). There was no effect of gender on abundance of *sul*1 among calves fed the subtherapeutic milk replacer, but among control calves, heifers had far more copies of *sul*1 in their feces than did bulls (**Figure 5**). The interaction of week and gender was significant for *sul*1 (**Table 1**). Heifers had higher abundance of *sul*1 at week 6 than did bulls, (**Figure 6**). The interaction of breed with gender was significant for *tet*G (**Table 1**). Crossbred bulls had the higher abundance of *tet*G than crossbred heifers; there was no effect of gender among Holstein calves (**Figure 7**).

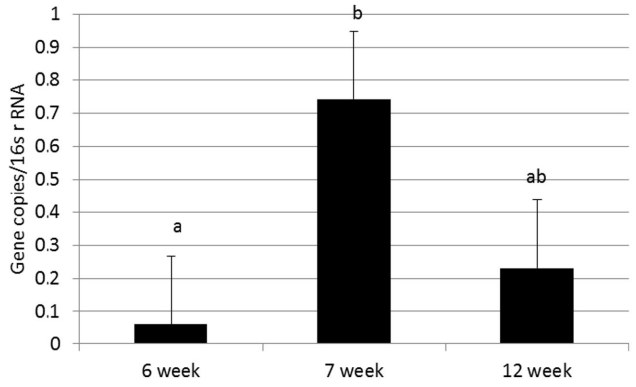

FIGURE 3 | Effect of time on relative abundance of *ermF* ARGs in calf feces. Bars represent the pooled averages across calves fed medicated or non-medicated milk replacers. Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

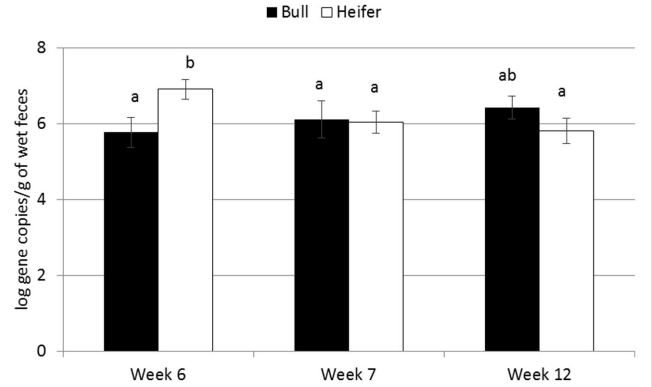

FIGURE 6 | Effect of the gender by time interaction on abundance of *sul1* ARGs in calf feces. Bars represent the pooled averages across calves fed medicated or non-medicated milk replacers. Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

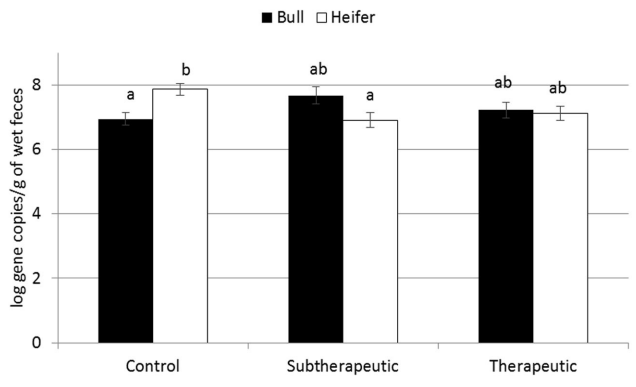

FIGURE 4 | Effect of the gender and antibiotic treatment interaction on abundance of *tetG* ARGs in calf feces. Bars represent the pooled averages across the three sampling events. Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

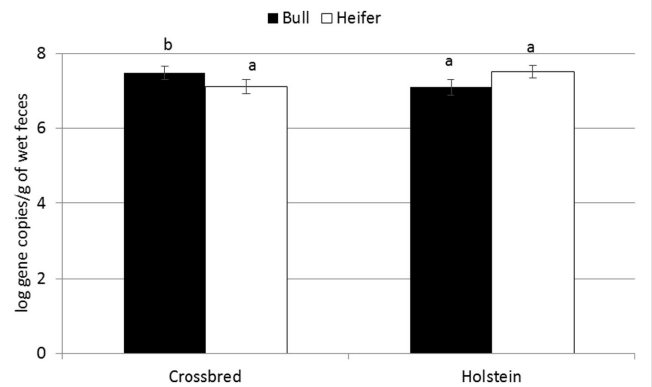

FIGURE 7 | Effect of gender by breed interaction on abundance of *tetG* ARGs in calf feces. Bars represent the pooled averages across calves fed medicated or non-medicated milk replacers and over the three sampling events. Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

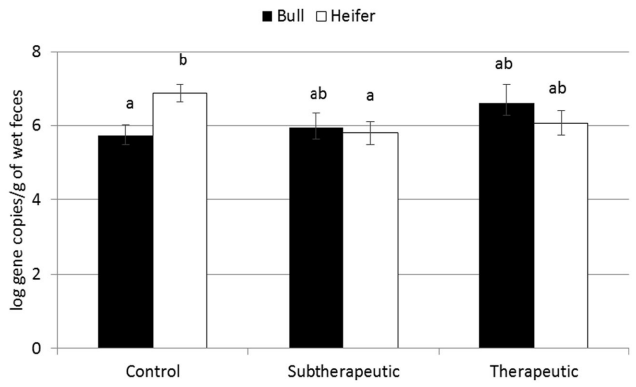

FIGURE 5 | Effect of the gender and antibiotic treatment interaction on abundance of *sul1* ARGs in calf feces. Bars represent the pooled averages across the three sampling events. Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

DISCUSSION

EFFECT OF ANTIBIOTICS ON GROWTH AND HEALTH

The lack of effect of milk replacer medication on growth and health is in contrast to some observations (Quigley and Drew, 2000; Berge et al., 2005; Stanton et al., 2010), probably because calves in the current study were fed a more nutrient-dense diet with higher intake than in most early research. Consequently, calves completing the course of the study were relatively healthy and grew quickly. Notably, of the 13 calves that were removed due to health problems, 7 were in the subtherapeutic treatment group. Health problems that were observed in calves in the current study were largely due to protozoan and viral microorganisms, which are not susceptible to antibiotics. Langford et al. (2003) observed no effect of penicillin content of milk in calves fed *ad libitum*; they also attributed the lack of effect of antibiotic to the nutritional benefits to the calf of offering an unlimited supply of milk. Interestingly, Berge et al. (2009a) actually observed antibiotic addition to pasteurized waste

milk to be associated with a higher percentage of diarrhea days in calves compared to no antibiotic addition, while respiratory scores were equivalent between the two treatments. They partially attributed this effect to adequate passage of immunity via colostrum, as advocated in their parallel studies (Berge et al., 2009b). Their study also highlights potentially negative effects of antibiotics on gastrointestinal flora. Kaneene et al. (2008) similarly observed no health benefits of adding oxytetracycline and neomycin to milk replacer and also noted substantial colostrum provisions to the calves. Thus, nutritional status and overall health may override benefits provided by subtherapeutic antibiotic treatment.

EFFECT OF ANTIBIOTICS ON ABUNDANCE OF ARGs IN FECES

The present study represents the first controlled, culture-independent evaluation of the effect of antibiotic addition in milk replacer on antibiotic resistance in calf feces through weaning. The results provide an indication of the effects of subtherapeutic and therapeutic tetracycline and neomycin treatments typical in the U.S. Among the eight ARGs examined across three classes, *tet*O was the only one to exhibit an increase in response to antibiotic treatment, but only when normalized to 16S rRNA genes. The results were somewhat unexpected, given reports of feed antibiotics increasing resistance in manure of mature cattle (Alexander et al., 2009) and other livestock (Marshall and Levy, 2011). In a prior study of calves fed "waste milk" containing penicillin residues, resistance of fecal bacteria to penicillin increased with increasing dose fed (Langford et al., 2003). One possible cause for discrepancy is that of the 25 known tetracycline ARGs (Aminov et al., 2001, 2002; Yang et al., 2004), only five were quantified in this study. Although these represented the three main classes of resistance (efflux, ribosomal protection, and degradation) and have previously been reported to be found in cattle waste (Storteboom et al., 2007; Alexander et al., 2009; Dahshan et al., 2010; McKinney et al., 2010), it is quite possible that key tetracycline ARGs responding to antibiotic treatment in this study were overlooked. Interestingly, though *tet*X was previously reported to be confined within the genus *Bacteroides* (Yang et al., 2004), its abundance was comparable with that of *tet*G, with recent reports suggesting it is more widespread than previously thought (Ghosh et al., 2009). Regardless, the results of this study suggest that removal of chlortetracycline and neomycin from milk replacer will not eliminate shedding of ARGs into the environment. McKinney et al. (2010) similarly observed only a very modest reduction of *tet* and *sul* ARG abundance in waste lagoons on organic dairy farms relative to those of conventional dairies.

The reduced abundance of *erm*F observed in feces of calves fed antibiotics as compared to control calves was intriguing. Macrolide and sulfonamide ARGs were examined in this study as potential indicators of co-selection of resistance to antibiotics other than the oxytetracycline and neomycin administered in the milk replacer. In particular, *sul*1 is carried within the 3′ conserved region of class 1 integrons (Mazel, 2006), while tetracycline resistance has been noted to be prevalent in macrolide-resistant *Streptococcus pyogenes* (Nielsen et al., 2004). In a study by Berge et al. (2006a) it was found that milk replacer containing neomycin sulfate and tetracycline HCl selected for *E. coli* resistant to classes of antimicrobials not used, including: aminoglycosides, chloramphenicol, and sulfonamides. The reduced abundance of *erm*F in the current study may be explained by effects of medicated milk replacer on specific populations of excreted bacteria. For example, Berge et al. (2006b) observed that medicated milk replacer decreased fecal shedding of *Salmonella enterica* by dairy calves. Decreased fecal shedding of certain bacteria could explain the decreased numbers of gene copies of *erm*F with antibiotic feeding; the species of bacteria carrying erythromycin resistance being shed in the feces was unknown in this study. It seems probable that one of the two antibiotics fed in the milk replacer were effective against fecal bacteria carrying the *erm*F gene. Interestingly, the effect of antibiotic treatment was no longer observed when *erm*F was normalized to 16S rRNA genes (**Table 2**). Normalization to the 16S rRNA gene, present in all bacteria, provides an indication of the proportion of the bacterial community carrying the ARG of interest and also aids in accounting for minor variations in sample processing. The similarity in relative abundance (ARGs per 16S rRNA; normalized) of *erm*F between antibiotic fed and non-antibiotic control groups suggests that there was a decrease in overall fecal bacterial shedding in antibiotic-treated groups. Reduced 16S rRNA genes in the manure of antibiotic-treated calves would also be consistent with the *tet*O increase only being significant when normalized to 16S rRNA genes. However, the range of 16S rRNA gene concentrations encountered among the fecal samples was wide, and there was no significant effect found of antibiotic treatment on overall 16S rRNA gene abundance.

ESTABLISHMENT OF ARGs IN CALF FECES WITH TIME

The increased absolute abundance of *tet* and relative *erm*F ARGs with time in all calves in the current study (including calves not fed antibiotics) suggests that the calf gut environment itself was amenable to the establishment of resistance regardless of antibiotic content of the feed. Berge et al. (2006a) similarly reported an age-related increase in antibiotic resistance of *E. coli* (measured phenotypically) even in non-treated calves. On the other hand, no effect of time on *tet* ARGs was observed when data were expressed per unit of 16S rRNA. This suggests that the overall bacterial population density increased in corresponding samples with time and that the bacteria carrying *tet* ARGs were neither negatively nor positively impacted. However, the range of 16S rRNA gene concentrations was again wide and a statistically significant increase with time was not identified as a general phenomenon.

Few studies have specifically characterized the establishment of antibiotic resistance in calf manure with time. Similar to the present study, increased overall resistance to 12 antimicrobials was noted among fecal *E. coli* isolates over the first 4 months of life in calves not receiving antibiotics (Berge et al., 2006a). A recent study by Pereira et al. (2011) examined the antibiotic resistance patterns of *E. coli* isolates on day 2 and day 6 of calves from two farms that did not administer antibiotics in the feed milk as compared to a third farm that administered both chlortetracycline and sulfamethazine. Odds of resistance to most antibiotics observed for the farm administering antibiotics was higher compared to control farms. The inclusion of sulfonamide, rather than neomycin, could have been the driving factor in the effects observed relative to the present study. While neomycin was included in this study because of its widespread implementation in the U.S., *neo* ARGs

(i.e., aminoglycoside phosphotransferases) were not monitored. Nonetheless, as neomycin is rarely used in humans, the present study did provide insight into potential effects of common practice on resistance to three classes of antibiotics of high clinical relevance, in addition to the clinically relevant integron, *int*I1 (Hall and Collis, 1998). Also important to consider, in comparison to the Pereira et al. (2011) study, is that in a study design in which the antibiotic treatments are geographically isolated, the actual established microbiota of the farm, rather than the antibiotic present in the milk, could be an overarching factor. The present study thus has value in its focus of the effects of the three controlled treatments on a single farm.

Of note, as is routine practice in the U.S., monensin was included in the grain fed to all calves. While very little grain was consumed during the early weeks, the monensin-containing grain became the sole source of nutrition post-weaning (7 weeks). *Tet* ARGs did generally increase with time, *tet*G and *tet*W prior to weaning and *tet*X 5 weeks post-weaning. It is not possible to determine from this study whether monensin was related to the observed increases of *tet* ARGs; however, all significant increases with time were lost when normalized to 16S rRNA genes. Therefore, it can be concluded that monensin did not generally suppress the total bacterial density to produce the observed effect. Co-selection of bacteria carrying *tet* ARGs by monensin is one possible explanation for their increase with time; however, acquired resistance of bacteria to monensin has not previously been observed (Butaye et al., 2001). Furthermore, bacteria that are tolerant of monensin have been observed to be no more or less sensitive to medically important antibiotics than monensin-susceptible bacteria (Houlihan and Russell, 2003).

OTHER FACTORS AND THEIR INTERACTIONS

The effects of interactions of time, treatment, gender, and/or breed are not easily explained. They may be attributed to the small number of observations associated with each interaction, although each individual group was comprised of *n* ranging from six to eight. If the effects observed across the gender and breed interactions are indeed real, they highlight the complexity of factors governing the microbial ecology of the bovine gut and the establishment of antibiotic resistance.

ADVANTAGES AND LIMITATIONS OF MOLECULAR-BASED APPROACH

To our knowledge, this study provides the first insight into the establishment of antibiotic resistance in calf feces in the early weeks using qPCR to directly quantify ARGs. This approach is advantageous in that it circumvents biases associated with culturing and thus enables broad quantification of resistance elements, including those harbored by unculturable strains. However, molecular techniques possess their own limitations, which must be considered in interpreting the findings of this study. Firstly, although 10 genes were quantified by qPCR, these likely only scratch the surface of the full array of resistance elements present in calf feces. Indeed, recent examination of the human gut microbiome has revealed a vast array of resistance elements, most of which not previously described (Sommer et al., 2009). Thus, it is quite possible that ARGs not monitored or detectable in this study did in fact respond strongly to antibiotic treatment. Secondly, qPCR is limited by the quality of the DNA extraction. While no ideal DNA extraction method exists, we viewed the FastDNA® Spin Kit for soil to be the best option. While kits specifically formulated to manure are available, head to head comparison has actually indicated downstream detection of a broader range of bacteria when the soil kit was applied to extract DNA from feces (Ariefdjohan et al., 2010). Further, the FastPrep® instrument applied in this study was noted to result in the least DNA damage, relative to other cell lysis techniques. The FastDNA® Spin Kit for soil was successfully applied for qPCR analysis of a range of manures by Layton et al. (2006), although they employed a 1:10 dilution prior to extraction, while our study did not. A potential consequence of undiluted samples is overloading of the extraction column and thus lower overall DNA yield. Normalization to 16S rRNA genes should help account for such variation. Also important to note is that our methods were able to detect significant absolute difference in ARGs in several instances, which would not be expected to be discernible if extraction column overloading was the dominant factor driving the results.

MULTIPLE DRUG RESISTANCE VERSUS ABUNDANCE OF MULTIPLE ARGs

Up to 80% of *E. coli* isolates obtained from mature heifers have been observed to be multi-drug resistant (resistant to three to six drugs), typically displaying resistance to tetracycline, ampicillin, ceftiofur, florfenicol, chloramphenicol, spectinomycin, and streptomycin (Sawant et al., 2007). However, the effect of antimicrobial use on multiple antimicrobial resistance has only recently been studied in calves. Pereira et al. (2011) noted that 81% of the *E. coli* isolates obtained from both medicated and non-medicated calves were resistant to three or more antibiotics already within the first 6 days of life. Berge et al. (2006a) observed that tetracycline and neomycin in the feed were associated with higher levels of multiple antimicrobial resistant fecal *E. coli* in calves monitored up to 4 weeks of age. The same research group (Berge et al., 2010) further noted that fecal *E. coli* were more likely to be resistant to multiple antibiotics in older calves (14- or 28-2) than in newborn calves if calves were fed antimicrobials in milk. In the current study, 8 of the 10 target ARGs were detected in the feces of the experimental calves. Fecal samples from all calves carried multiple types of ARGs within the same sample. Also, feces of all calves carried at least one gene encoding resistance to each of the three classes of antibiotics of interest (tetracyclines, sulfonamides, erythromycins), but class 1 integrons (*int*I1) were not detected in any of the fecal samples. In contrast, bacterial strains carrying class 1 integrons were isolated from dairy manure-impacted soil, but not from a corresponding unimpacted soil (Srinivasan et al., 2008). Class 1 integrons were also detected in 10% of samples from scouring calves (Ahmed et al., 2009); calves in the current study were not diarrheic during sample collections. Wu et al. (2011) observed that sulfonamide use was associated with higher occurrence of *int*I1 genes among tetracycline resistant *E. coli* from beef cattle. That *int*I1 was not detectable but that multiple ARGs were present in feces of all calves is intriguing. It is plausible that the ARGs identified were carried by different bacterial species in the feces sample, but also the analyzed genes may be carried by the same

bacterial species, without linkage to a transferable element such as *int*I1. Thus, conclusive evidence of multiple drug resistance in the calf manure was not obtained in this study. Nonetheless, multiple drug resistance has been observed to be significantly higher in dairy manure-impacted environments, such as soils (Srinivasan et al., 2008), thus the evolution of antibiotic resistance from calf to mature cow to the environment remains an important issue.

CONCLUSION

Improved calf health and growth commonly observed with feeding of medicated milk replacers were not observed, likely because the calves in this experiment were maintained on a high plane of nutrition. In situations where management or nutrition is not optimal, health differences may be more apparent. Genes related to tetracycline resistance increased over time regardless of treatment, thus the calf gut itself appears to be an environment conducive to the proliferation of certain ARGs. Genes encoding resistance to antibiotics that were not administered were less abundant, thus co-selection was not observed in this study. However, macrolide and sulfonamide ARGs were still present and persisted in the feces of these experimental calves. Other ARGs not examined were also likely present and may have responded to antibiotic treatment. The results have important management implications, indicating that if nutritional requirements are appropriately managed, the cost and risk of subtherapeutic antibiotic use may not be necessary. Nonetheless, ARGs across three classes persisted and increased with time, even in the feces of calves not administered antibiotics. Thus, comprehensive management strategies that limit cross-dissemination between animals and control transport of manure from farms, in addition to prudent use of antibiotics, are called for.

ACKNOWLEDGMENTS

Financial support was provided by the Virginia Agricultural Council. The authors wish to thank Chad McKinney for technical assistance.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 November 2011; accepted: 23 March 2012; published online: 10 April 2012.

*Citation: Thames CH, Pruden A, James RE, Ray PP and Knowlton KF (2012) Excretion of antibiotic resistance genes by dairy calves fed milk replacers with varying doses of antibiotics. Front. Microbio. **3**:139. doi: 10.3389/fmicb.2012.00139*

This article was submitted to Frontiers in Antimicrobials, Resistance and Chemotherapy, a specialty of Frontiers in Microbiology.

APPENDIX

Table A1 | Gender and breed distribution across antibiotic treatment groups.

Calf	Treatment	Breed	Gender
1	Control	Crossbred	Heifer
2	Control	Crossbred	Heifer
3	Control	Crossbred	Heifer
4	Control	Holstein	Heifer
5	Control	Holstein	Heifer
6	Control	Holstein	Heifer
7	Control	Crossbred	Bull
8	Control	Crossbred	Bull
9	Control	Crossbred	Bull
10	Control	Holstein	Bull
11	Control	Holstein	Bull
12	Control	Holstein	Bull
13	Subtherapeutic	Crossbred	Heifer
14	Subtherapeutic	Crossbred	Heifer
15	Subtherapeutic	Holstein	Heifer
16	Subtherapeutic	Crossbred	Bull
17	Subtherapeutic	Crossbred	Bull
18	Subtherapeutic	Holstein	Heifer
19	Subtherapeutic	Holstein	Bull
20	Therapeutic	Crossbred	Heifer
21	Therapeutic	Crossbred	Heifer
22	Therapeutic	Holstein	Heifer
23	Therapeutic	Holstein	Heifer
24	Therapeutic	Holstein	Heifer
25	Therapeutic	Crossbred	Bull
26	Therapeutic	Crossbred	Bull
27	Therapeutic	Holstein	Bull
28	Therapeutic	Holstein	Bull

Table A2 | Primers and annealing temperatures used in this study.

Primer	Target gene	Primer sequence (5′–3′)	Annealing temperature (°C)	Reference
*sul*1-Fw	*sul*1	CGCACCGGAAACATCGCTGCAC	69.9	Pei et al. (2006)
*sul*1-Rv		TGAAGTTCCGCCGCAAGGCTCG		
*sul*2-Fw	*sul*2	TCCGGTGGAGGCCGGTATCTGG	67.5	Pei et al. (2006)
*sul*2-Rv		CGGGAATGCCATCTGCCTTGAG		
erm(B)-Fw	*erm(*B)	GATACCGTTTACGAAATTGG	58	Chen et al. (2007)
erm(B)-Rv		GAATCGAGACTTGAGTGTGC		
erm(F)-Fw	*erm*(F)	CGACACAGCTTTGGTTGAAC	56	Chen et al. (2007)
erm(F)-Rv		GGACCTACCTCATAGACAAG		
tet(C)-Fw	*tet*(C)	GCGGGATATCGTCCATTCCG	70	Aminov et al. (2002)
tet(C)-Rv		GCGTAGAGGATCCACAGGACG		
tet(G)-Fw	*tet*(G)	GCAGAGCAGGTCGCTGG	64.2	Aminov et al. (2002)
tet(G)-Rv		CCYGCAAGAGAAGCCAGAAG		
tet(O)-Fw	*tet*(O)	ACGGARAGTTTATTGTATACC	50.3	Aminov et al. (2001)
tet(O)-Rv		TGGCGTATCTATAATGTTGAC		
tet(W)-Fw	*tet*(W)	GAGAGCCTGCTATATGCCAGC	60	Aminov et al. (2001)
tet(W)-Rv		GGGCGTATCCACAATGTTAAC		
tet(X)-Fw	*tet*(X)	CAATAATTGGTGGTGGACCC	64.5	Ng et al. (2001)
tet(X)-Rv		TTCTTACCTTGGACATCCCG		
HS463a	*int*I1	CTGGATTTCGATCACGGCACG	60	Hardwick et al. (2008)
HS464		ACATGCGTGTAAATCATCGTCG		
1369F	16S rRNA	CGGTGAATACGTTCYCGG	60	Suzuki et al. (2000)
1492R		GGWTACCTTGTTACGACTT		