Tetracycline resistance gene *tet*(W) abundances in settled dust of North Carolina residences

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ABSTRACT

As antibiotic resistance has become a notable public health issue, increasing studies have characterized antibiotic resistance genes (ARGs) throughout environments with high risk for antibiotic resistance, particularly hospitals and confined animal feeding operations (CAFOs). However, relatively little research has been devoted to studying ARGs in the residential home, where the risk factors for non-nosocomial antibiotic resistant infections may be assessed. This study tested for ribosomal protection tetracycline resistance genes *tet*(O), *tet*(M), *tet*(Q), and *tet*(W) using PCR on community DNA of 90 homes in North Carolina. Only *tet*(W) was detected. Using quantitative PCR (qPCR), the abundance of *tet*(W) per bacterial genome was determined, and outdoor sample subsets were found to correlate to total livestock density and bacterial families Clostridiaceae, Streptococcaceae, and Bacteroidaceae. However, the central result of this study was the detection of *tet*(W) in the majority of samples but of none of the other genes, contrary to studies that find both *tet*(W) and *tet*(M) throughout environments unaffected by human activity.

KEY WORDS

Antibiotic resistance genes; tetracycline; tet(W); residential microbial community

INTRODUCTION

Antimicrobial agents are considered one of the greatest public health achievements of the 20th century (Gandara et al., 2006). However, the spread of antibiotic-resistant microbes through misuse of such agents in medical institutions and large scale livestock operations has become a source of global concern for the treatment of human and animal diseases. The CDC reports at least 23,000 deaths from antibiotic resistant infections annually in the US alone (U.S. Dept. of Health and Human Services, 2013). Pathogenic bacteria can gain resistance to antibiotics through horizontal gene transfer of naturally occurring antibiotic resistance genes (ARGs) native to other microbes or de novo mutations under environmental pressure (Hoffman 2001). An estimated 16 million kg of antibiotic compounds are used annually in the US alone, with about 70% going to non-therapeutic uses (accurate amounts are hard to obtain due to lack of reporting) (Sarmah et al., 2006, p. 728). The ubiquitous use of antimicrobial agents in hospitals provides strong pressure for multi-drug resistance gene selection, and nosocomial antibiotic resistant infections have become a detrimental public health problem (Schaberg et al., 1991). Over-prescription and under-dosing of clinical antibiotics foster the spread of ARGs.

In the US, confined animal feeding operations (CAFOs) use antibiotics heavily, not only for disease treatment, but also prophylactically and as growth promoters (Khan et al. 2008). These compounds are poorly absorbed and incompletely metabolized in the livestock gastrointestinal tract, thus the usage of antibiotics in CAFOs selects for ARGs both within the livestock microbiome and by accumulating antimicrobial compounds in the environment (Khan et al. 2008). Significantly elevated levels of antibiotic resistant and multidrug resistant bacteria have been found in the wastewater lagoons of CAFOs often used as crop fertilizer (Hölzel 2010; Peak et al. 2007; Koike et al. 2007), in ground and surface water downstream of CAFOs (West et

al. 2011, Koike et. al., 2007; Sapkota et al. 2007), and in air around and downwind of CAFOs (Chapin et al. 2005; Gibbs et al. 2006; Alvarado et al. 2012; McEachran et al. 2015). The role of CAFOs in increasing environmental levels of ARGs in microbes has been established but the transmission of ARGs originating in CAFOs to human communities has been less characterized.

Antibiotic resistant microbes have been found to colonize previously uncolonized farm workers and, to a limited degree, neighboring populations (Levy et al., 1976; Armand-Lefevre et al., 2005; Voss et al. 2005). Antibiotic resistant bacteria have been isolated from commercially available ground meat (White et al., 2001). Due to a lack of research on indoor air in general, there are few studies that characterize the extent of ARGs in microbial communities of residential homes, despite clear evidence for potential health risks from airborne ARGs. Cases of antibiotic resistant infections like MRSA and VRSA are increasingly being acquired outside of higher risk environments, like hospitals and prisons (Herold et al., 1998; Elstrøm et al., 2012; Dufour et al., 2002). However, the work that has studied ARGs in indoor airborne bacteria has found ARGs have significantly higher concentrations indoor than in outdoor air (Rosas et al., 1997; Gandara et al., 2006).

This study seeks to examine the presence of genes for antibiotic resistance to a particularly widely used antibiotic, tetracycline, in the community DNA of settled dust in residential homes of North Carolina. Three main questions are sought to be answered by this study: 1) whether there is a significant difference between indoor and outdoor tetracycline resistance gene abundances, 2) if the amount of tetracycline resistance can be predicted by home location, and 3) whether the abundances of bacterial families by sample can be used to predict the source of tetracycline resistance genes. Characterizing differences between indoor and outdoor levels is important in determining which factors are most relevant to community

exposure to ARGs. Whether the indoor bacterial ARGs, the community that occupants of homes likely have a greater contact to, are driven the outdoor community, that is factors of the location of the home, or driven by behaviors like vocation or purchase of agricultural goods is important in targeting the sources of antibiotic resistant disease.

Methods of molecular biology were used to address these questions – namely quantitative PCR (qPCR) for determination of amount of ARGs and direct PCR and high throughput sequencing of a portion of the 16S rRNA gene to assess bacterial community composition of each collected dust sample. A large portion of previous studies have cultured bacteria to determine the presence of ARGs (Rosas et al., 1997; Gandara et al., 2006), but these methods are not amenable to the efficient characterization of ARGs across a broad range of bacteria, like obligate anaerobes. The molecular method of qPCR includes the entire microbial community and makes gene detection precise and rapid (Koike et al., 2007, p. 4814). Bioinformatic techniques were then be used to efficiently compare sample descriptors with levels of ARGs.

Tetracycline resistance genes were chosen as the focus of this study because of the high prevalence of tetracycline in the environment. Tetracycline, a broad spectrum antibiotic, is used for all USDA approved antibiotic use categories in U.S. CAFOs (growth promotion, prophylaxis, and treatment of infections), it is commonly prescribed for human infections, and resistance against it has been well characterized previously (Macauley et al., 2007, p. 1307; Peak et al., 2006). Tetracycline resistance occurs either by a ribosomal protection protein (RPP) or an efflux pump protein (Chopra & Roberts, 2001). RPP encoding genes *tet*(O), *tet*(M), *tet*(Q), and *tet*(W), often found to be more abundant than efflux genes, were quantified with quantitative PCR (qPCR) due to their previously described presence in CAFOs and indoor settings (Macauley et al., 2007; Peak et al., 2006; Koike et al., 2007; Macovei & Zurek, 2006; Wang et al., 2005).

Storteboom et al. (2010) found *tet*(M) and *tet*(W) to be present across a "pristine" environment, CAFOs, and wastewater treatment plants (WWTPs) but in lesser frequencies and with distinct sequences from CAFOs and WWTPs in the pristine environment. *tet*(Q) was found to be highly correlated to CAFOs and *tet*(O) related to WWTPs. Thus, these four particular genes have been shown to be useful indicators the source environments of ARGs.

Hypothesized results of this study were that the indoor samples would have a differently structured bacterial community and higher levels of resistance than the outdoor due to behaviors like shaking bed sheets and drapes and previous findings of higher antibiotic resistance indoors (Gandara et al., 2006). The outdoor samples particularly of homes at a location of high livestock density were expected to have higher overall levels of ARGs with *tet*(Q) presence compared to those in a low density livestock area. Phylotypes having previously been shown to have tetracycline resistance genes are expected to have high abundances in samples with high abundance of tetracycline resistance.

Among the four tetracycline resistance genes screened for, only *tet*(W) was detected. The sample exhibited variable abundance of *tet*(W) per bacterial genome equivalent, from 0 to 2 and outliers around 5 and 6 *tet*(W) copies per bacterial genome. Although indoor and outdoor proportional *tet*(W) abundances were not significantly different across the homes with both data ($n = 31$), they did not exhibit the same trends with environmental factors. The proportional *tet*(W) abundances of the outdoor subset were significantly correlated to total livestock density by county and three bacterial family abundances previously described to have *tet*(W) containing strains, but the same trend was not seen in the indoor subset. Because of the prevalence of *tet*(W) in so called pristine environments as well as those affected by human institutions, it is difficult to

differentiate the possible source of resistance. However, the fact that *tet*(W) alone among *tet*(O), *tet*(M), *tet*(Q), and *tet*(W) was detected is significant and a site for further study.

MATERIALS & METHODS

Sampling. A subset of samples from the Wild Life of Our Homes project (homes.yourwildlife.org), a citizen science project across North America, were used in this study. Over a thousand participants were recruited through the website, social media, and email campaigns from January 2012 to March 2013. Participants instructed to use a microbe sampling kit containing dual-tipped sterile BBLTM CultureSwabsTM and provided a written Informed Consent form approved by North Carolina State University's Human Research Committee (Approval No. 2177). This study focuses on dust samples that participants collected from the upper door trim of an exterior door, one the outside and inside surface. This sampling location was chosen because it is found in every home, unlikely to be cleaned frequently, and serves as a passive collector of aerosols and dust with little to no direct contact from home occupants. Participants returned swabs over the period March 2012 to May 2013 by first-class mail. The swabs were then stored in a -20 ºC freezer until processed. The samples used in this study were the subset from North Carolina because of the state's exceptionally high density of CAFOs – North Carolina is the second state in the US for swine sales (2012 Census of Agriculture), but also because of the large number of participants in North Carolina (n=90 for this study).

A map of sampling locations from which data was obtained is provided in Figure 3. A set of 13 descriptors was compiled: population, livestock density, occupant number, pets, antimicrobial soap use, age of home, and climatic variables (Table 1). Population of humans, hogs, cattle, and

TABLE 1. Sample descriptors investigated

Hogs*
Cattle*
Chickens*
Sum of livestock*
Population
Total occupants
Mean precipitation
Mean temperature
Elevation
Home age
Antimicrobial soap usage
Pet cats
Pet dogs

*Head per square mile by county

chickens for all sampled locations were obtained from the 2012 U.S. Census Bureau data (www.census.gov).

Characterization of Community Tetracycline Resistance. DNA was extracted from swabs using a MoBio PowerSoil-htp Soil DNA Isolation Kit (MoBio Inc., Carlsbad, CA) by placing one of the two swabs in a single bead tube under sterile conditions as previously described (Fierer et al., 2008; Leff & Fierer, 2013). PCR was performed as a screen for ARGs *tet*(O), *tet*(M), *tet*(Q), and *tet*(W) using previously described primers (Aminov et al., 2001) for a marker sequence and amplification programs as follows: 94 °C 5 min, 40 cycles of (94 °C 30 s, annealing temp 30 s, 72 °C 30 s), 72 °C 7 min, 4 °C hold (Table 2). PCR was also performed with 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) primers used to amplify the V4-V5 region of bacterial and archaeal 16S rRNA genes to ensure DNA extraction was successful (Caporaso et al., 2013). The 16S targeting PCR program was as follows: 94 °C for 3 min, 35 cycles of (94 °C 45 s, 50 °C 1 min, 72 °C 1.5 min), 72 °C 10 min, and a 4 °C hold. PCR reactions for *tet* genes were performed with 12.5 μL 2× ProMega GoTaq Colorless Master Mix (ProMega Corp., Madison, WI), 0.5 μL of each appropriate F and R primer, (1 μL total, starting concentration 10 μM), 9.5 μL of PCR-grad water, and 2 μL DNA template, and for 16S with 12.5 μL 2× ProMega GoTaq Colorless Master Mix (ProMega Corp., Madison, WI), 0.5 μL of each appropriate F and R primer, (1 μL total, starting concentration 10 μM), 10.5 μL of PCR-grad water, and 1 μL DNA template. Negative controls were included to test for contamination. After amplification, reactions were visualized on an agarose gel along with negative controls.

TABLE 2. *tet* targeting PCR primers

Primer	Sequence	PCR Annealing Temp (˚C)
tet(M)-F*	ACAGAAAGCTTATTATATAAC	55
tet(M)-R	TGGCGTGTCTATGATGTTCAC	
tet(O)-F	ACGGARAGTTTATTGTATACC	60
tet(O)-R	TGGCGTATCTATAATGTTGAC	
tet(Q)-F	AGAATCTGCTGTTTGCCAGTG	63
tet(Q)-R	CGGAGTGTCAATGATATTGCA	
tet(W)-F	GAGAGCCTGCTATATGCCAGC	64
tet(W)-R	GGGCGTATCCACAATGTTAAC	

*F, forward; R, reverse.

To ensure that *tet*(W) was the gene being amplified, a subset (n=17) of PCR amplicons of *tet*(W), the only of the four genes to successfully amplify, were cloned with a TA Cloning kit in TOP10 cells (Invitrogen, Carlsbad, Calif.). Colonies of kanamycin-resistant transformants were screened for presence of *tet*(W) genes using restriction endonuclease with Eco-RI-HF and NEBuffer (New England Biolabs, Ipswich, MA) and agarose gel visualization. Recombinant plasmids were single-pass Sanger sequenced in both directions at Beckman Coulter Genomics (Beckman Coulter Inc., Danvers, MA) using universal primers M13F and M13R. The resulting forward and reverse sequences were aligned with CodonCode Aligner (http://www.codoncode.com/aligner) on default settings, trimmed, and BLAST analysis performed against a downloaded database of the Comprehensive Antibiotic Resistance Database (http://arpcard.mcmaster.ca/) and a database sequence of *tet*(W) from *Bifidobacterium longum (*EU434751*).*

Quantitative PCR (qPCR) was performed on DNA from each swab extraction to determine abundances of *tet*(W), the only *tet* gene to have amplified in regular PCR, and of 16S to normalize the non-quantitative *tet* samples. qPCR standards were prepared from *E. coli* K-12

(for 16S rRNA) and extracted plasmid DNA of a sequenced clone described above (for *tet*). The standards' concentration was determined with a QuantiT PicoGreen dsDNA assay (Invitrogen Life Technologies, Grand Island, NY), and a standard curve for each 96-well qPCR plate was generated with seven 10-fold dilutions. Because of *E. coli* standard, the 16S rRNA gene results are in relation to *E. coli* genome equivalents as an estimate of total bacterial cells to normalize *tet* values. The primers described above were used (Table 2). Each reaction was comprised of 12.5 μL 2× qPCR mix (Absolute QPCR SYBR Green Mix, no ROX, Fermentas Inc., Boston, MA), 1.25 μL of the respective F and R primers (2.5 μL total, 10 μM starting concentration), 5 μL of PCR-grade water, and 5 μL of template DNA. Triplicate qPCR reactions were run for each dilution of the appropriate standard and for each swab sample on a Mastercycler ep realplex thermal cycler (Eppendorf, Hamburg, Germany) in 96-well plates. Cycler conditions for *tet*(W) were as described above with an initial step of 95 °C 15 min and conditions for 16S were as described above with an initial 95 °C 15 min and 40 cycles instead of 35. The estimated copy number of *tet*(W) and genome copy number of 16S was obtained from comparisons to the appropriate standard curve. The lower detection limit of this method was set to 100 copies. This study reports the ratio of *tet*(W) copies to *E. coli* genome copy numbers. Due to loss of sample DNA in thermal cycler malfunctions, the number of samples for downstream analysis was 70 indoor and 40 outdoor.

Determination of Community Taxa. The second of the two swabs for each sample was prepared for high-throughput sequencing with direct PCR technique described previously (Flores, Henley, & Fierer, 2012). Swab tips with appropriate negative controls were loaded directly into 2 mL 96-well plates (Axygen Inc.) and were processed using Extract-N-Amp PCR kit (Sigma-Aldrich, Inc.) using a modified version of the manufacturers' instructions. Following

the addition of 250 μL of the Extract-N-Amp Extraction solution, the plate was sealed securely with a 96 round well Impermamat Silicon Sealing Mat (Axygen, Inc.) and heated at 90 ºC for 10 minutes in a dry bath. Next, extract-N-Amp Dilution solution was added to the wells at a 1:1 ratio to the extraction solution and mixed gently by pipetting. The plate was resealed with the mat and stored at 4 ºC. Then, 20 μL triplicate reactions per sample were conducted using 10 μL of Extract-N-Amp Ready Mix, 1 μL of the forward and reverse primers, 5 μL of PCR-grade water, and 4 μL of the Extract-N-Amp sample solutions from the 96-well plate. High-throughput sequencing methods were used to assess microbial diversity by the variation in marker gene sequences. The same 515F/806R primers described above were used but appropriate Illumina adapters were added as well as an error-correcting 12-bp barcode unique to each sample on reverse primers to permit multiplexing of samples. The PicoGreen dsDNA assay was used to quantify PCR products of all samples. The samples were pooled in equimolar concentrations for sequencing on either an Illumina HiSeq or MiSeq instrument (Illumina Inc., San Diego, CA). Sequencing runs were performed at the University of Colorado Next Generation Sequencing Facility.

High-Throughput Sequence Processing. A custom Python script (https://github.com/leffj/helper-code-for-uparse), with quality filtering and phylotype clustering, was conducted to demultiplex the 100-bp sequences using the UPARSE pipeline (Edgar, 2013). Prior to phylotype determination, sequences were dereplicated and singletons were removed. Phylotype taxonomy was determined using the Greengenes 13 8 database for 16S rRNA sequences as previously described (McDonald et al., 2012; Flores, Henley, & Fierer, 2012). Direct PCR reagent contamination was controlled for by removing Mycoplasma, Pseudomonas, Serratia, mitochondrial, and chloroplast classified sequences as well as any phylotypes present in

25% or more of the negative controls (Flores, Henley, & Fierer, 2012). To account for potential amplicon sequencing biases, samples with less than 10,000 sequences were removed and the remaining sequences were normalized and rarified to 10,000 sequences per sample as previously described (Paulson et al., 2013).

Statistical Analyses. Comparisons of *tet*(W) abundances to bacterial genome abundances as well as proportional *tet*(W) abundances to sample descriptors were performed. The *tet*(W) abundances were divided by bacterial genome abundances to obtain proportional *tet*(W) abundances. Linear regression analysis was run for *tet*(W) abundance to bacterial genome abundance. A paired *t* test was carried out on the home samples with both indoor and outdoor proportional *tet*(W) abundance data. Linear regression analysis was used to compare each sample descriptors to proportional *tet*(W) abundances for indoor and outdoor samples.

All further analyses were performed in in the R environment (www.r-project.org). Sample locations by proportional *tet*(W) abundances were mapped by inverse distance weighting interpolation using the gstat package (https://r-forge.r-project.org/projects/gstat/). A list of bacterial species found to have the *tet*(W) gene was compiled for a targeted comparison to the bacterial phylotypic abundances (Table 3). A BLAST search of the top 10,000 matches to *B. longum tet*(W) reference sequence (EU434751) was performed using the NCBI database. All sequences that did not match $\geq$ 97% were removed as well as all uncultured representatives and replicates by species. Spearman correlations with a false detection rate correction were run between proportional *tet*(W) abundances and the 11 families known to have *tet*(W) carrying strains.

TABLE 3. Cultured bacterial species found to contain reference *tet*(W) ≥ 97% identity

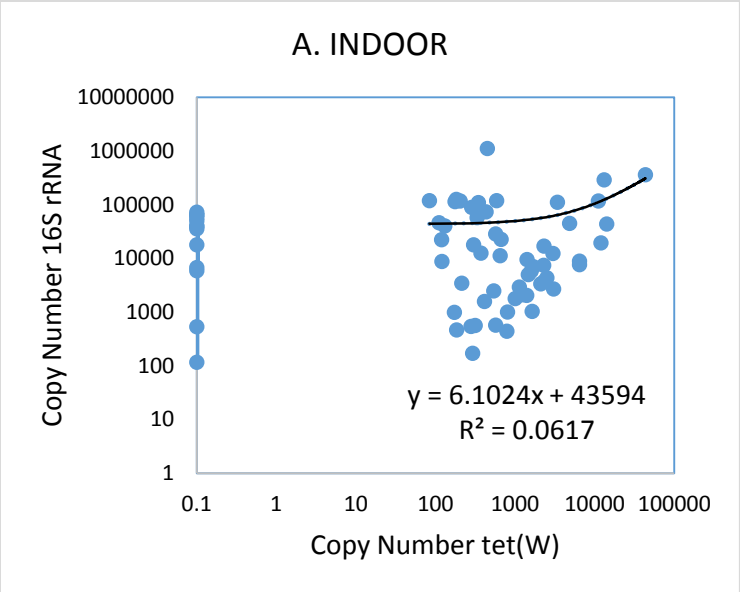
Species	Family
Arcanobacterium pyogenes	Actinomycetaceae
Bifidobacterium adolescentis *Bifidobacterium animalis* *Bifidobacterium bifidum* *Bifidobacterium breve* *Bifidobacterium kashiwanohense* *Bifidobacterium longum* *Bifidobacterium pseudocatenulatum* *Bifidobacterium pseudolongum* *Bifidobacterium thermophilum*	Bifidobacteriaceae
Butyrivibrio fibrisolvens	Lachnospiraceae
Clostridium difficile *Clostridium saccharolyticum* *Faecalibacterium prausnitzii* *Roseburia hominis*	Clostridiaceae
Corynebacterium diphtheriae *Corynebacterium resistens*	Corynebacteriaceae
Eubacterium siraeum	Eubacteriaceae
Lactobacillus acidophilus *Lactobacillus amylovorus* *Lactobacillus reuteri*	Lactobacillaceae
Megasphaera elsdenii *Selenomonas ruminantium*	Veillonellaceae
Mitsuokella multacida	Bacteroidaceae
Streptococcus suis	Streptococcaceae
Treponema succinifaciens	Spirochaetaceae

RESULTS & DISCUSSION

Presence of Tetracycline Resistance Genes. PCR and gel visualization with primers for *tet*(O), *tet*(M), *tet*(Q), and *tet*(W) markers showed successful amplification of *tet*(W) only. In roughly 80% of samples screened for *tet*(W), the expected band for the *tet*(W) marker sequence at about 168 bp was observed. All other samples had no visible bands. The fact that only *tet*(W) genes were identified among four common RPP genes and in such a large percentage of samples is surprising. This is not likely due to differential gene location and DNA extraction bias – i.e. *tet*(W) on the chromosome and the extraction method favoring chromosomal DNA – because *tet*(M) is most often located on the chromosome and *tet*(O) and *tet*(Q) can be chromosomal (Storteboom et al., 2010; Kazimierczak, Flint, & Scott, 2006). The amplification of only *tet*(W) is incongruent with the fact that *tet*(M) has been shown to populate even pristine environments at similar levels to *tet*(W) (Storteboom et al., 2010, p. 1949). The detection of only *tet*(W) certainly calls for further exploration. A subset of the samples that successfully amplified were Sanger sequenced, and BLAST analysis determined all sequenced samples to have between 99 and 100 percent identity with the *tet*(W) reference sequence of *B. longum* and no other ARGs. Storteboom et al. (2010) have shown that *tet*(W) sequence data can be used to differentiate genes of pristine environments, CAFOs, and WWTPs, but the sequences obtained in this study were not of the full gene, only a marker sequence. Therefore, sequencing of the full 1937 bp gene may help elucidate the reason for sole detection of *tet*(W) in future studies of residential tetracycline resistance.

Correlating Factors to tet*(W) Abundance.* The estimated *tet*(W) concentrations were compared to bacterial genome equivalents using the results of qPCR for the indoor and outdoor samples respectively to determine if there is a direct relationship between *tet*(W) copies and

bacteria in the settled dust. For the indoor subset, the *tet*(W) copy number was shown to be correlated to the number of bacterial genome equivalents in each home (Pearson's R = 0.26, p = 0.04). The trend can be roughly visualized in Figure 1. There was not a significant correlation among the outdoor subset between *tet*(W) copy number and bacterial genome equivalents (Pearson R = 0.2, p = 0.2). Due to thermal cycler malfunctions, a large portion of samples were lost during qPCR without enough DNA to perform another amplification. Thus, the indoor sample set happens to be larger than the outdoor set (n = 70 and 40 respectively), and the indoor set may be better suited for statistical analysis. In the indoor sample set certainly, greater abundances of all bacteria are correlated to greater abundances of *tet*(W), suggesting the ubiquity of *tet*(W).



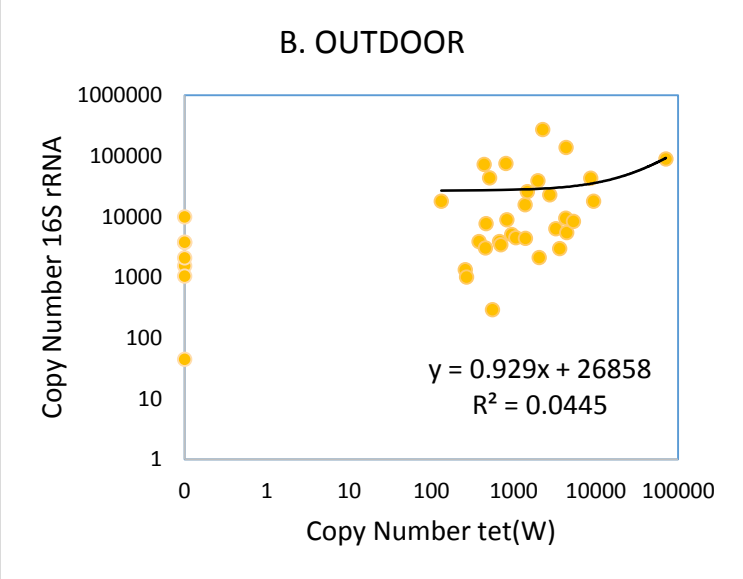


FIGURE 1. Quantitative PCR (qPCR) measurements of bacterial abundances vs. *tet*(W) abundances per home on a log scale with linear trendline for visualization in (A) indoor and (B) outdoor trim dust samples.

For all further analyses, the ratio of *tet*(W) abundance to bacterial abundance was used such that the non-quantitatively obtained samples could be compared quantitatively. Homes exhibited a

wide range of proportions of *tet*(W): from no *tet*(W) genes to almost two for every bacterial genome, with outliers at about five to six genes for every bacterial genome (Figure 3). As far as indoor versus outdoor abundances, the *tet*(W) abundance per bacterial genome was not significantly different or correlated over the limited number of homes with data from both indoor and outdoor trim (n = 31, paired *t* test, $p = 0.4$). Potential *tet*(W) sources, indoor or outdoor factors, cannot be hypothesized based on the proportion of *tet*(W) genes in the community from this study. This contradicts the patterns of multidrug resistance in homes found by Gandara et al. (2006) in which concentration of resistant bacteria was significantly higher inside the home than outside. However, Gandara et al. (2006) cultured bacteria to characterize resistance rather than quantifying ARGs across the bacterial community. It cannot be determined how many bacteria are antibiotic resistant with this study, only the amount of genes across the community, so these results can't be definitively compared to those of Gandara et al. However, this discrepancy may indicate that methods of culturing miss a large portion of bacteria that have the potential for transferring ARGs.

A selection of environmental factors were tested for correlation to proportion of *tet*(W) (Table 1). The only factor to yield a significant correlation was the outdoor sample's total head of livestock per square mile by county (Pearson's R = 0.579, $p = 0.001$), the trend approximately visualized in Figure 2A. The indoor sample set showed insignificant correlation (Pearson's R = 0.1, $p = 0.4$), also in Figure 2A. However, both correlations are highly driven by a single outlier, and without the outlier both appear to have a relationship to high livestock density – the median and mean proportional *tet*(W) abundance are higher in the high livestock density samples – although the correlation is not significant (Figure 2B). Total livestock density is therefore a good candidate for further study on sources of residential ARGs. To further explicate this possible

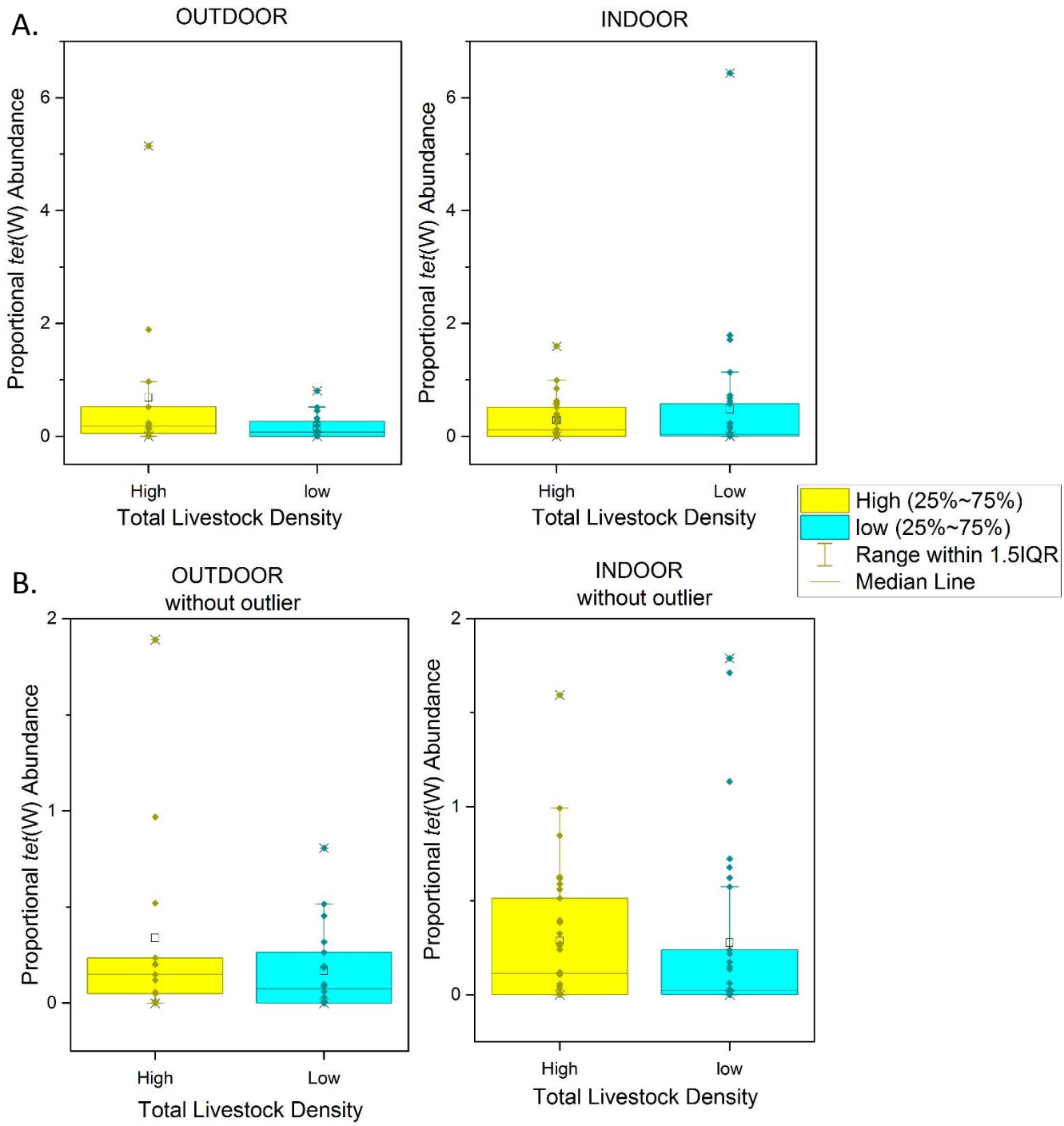


FIGURE 2. *tet*(W) gene abundance per bacterial genome in high and low total livestock density of the outdoor and indoor sample sets (A) with a single outlier above outlier above 5 *tet*(W) genes per bacterial genome and (B) without said outlier. For this figure, high livestock density is set as greater than 100 heads of livestock per square mile and low is less than 50 heads of livestock per square mile.

relationship, data on individual home proximity to farms rather than by county, data on whether livestock are raised in CAFOs or organic farms, and possibly vocational data of occupants in relation to farms may all be useful in addition to sequencing of the entire *tet*(W) gene as suggested above. Comparing the maps representing proportional *tet*(W) abundance (Figure 3) and the map of hog farm operations (Figure 4) (Wing, Cole, & Grant, 2000) shows that no sample in this study was located in a county with high hog farm density comparatively, hogs being North Carolina's main livestock contributor. Samples from these counties may be necessary to illuminating the relationship between residential proportional *tet*(W) abundance to North Carolina's livestock density. Besides this, the maps do not show a strong geographical pattern of proportional *tet*(W) abundances.

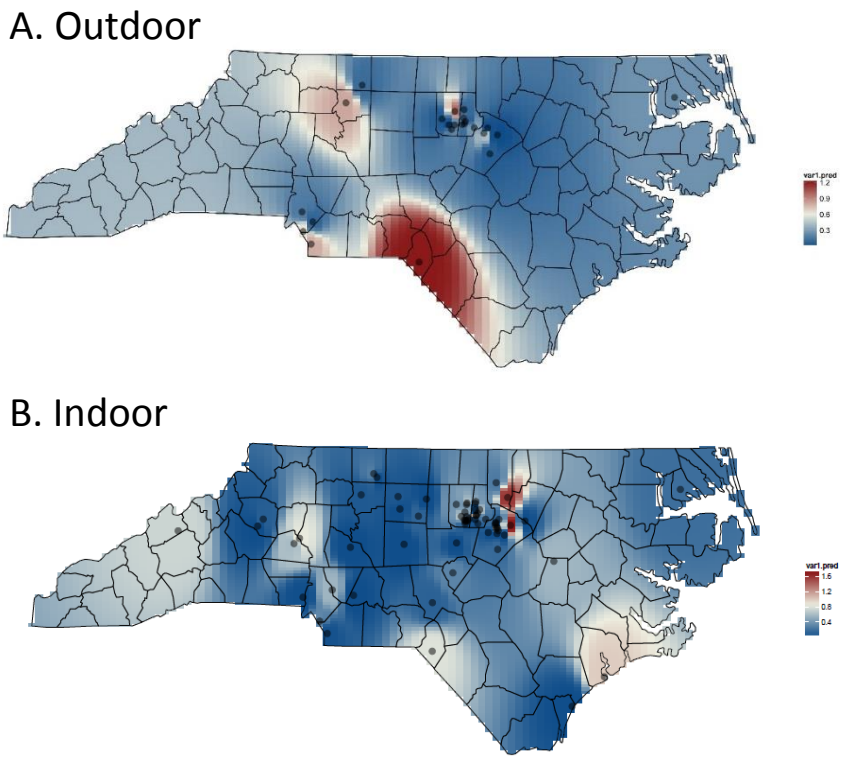


FIGURE 3. Sample locations from which data for *tet*(W) abundance per bacterial genome was obtained in (A) outdoor sample subset (n = 39) and (B) indoor sample subset (n = 69). Outliers are removed for better visualization. Proportional *tet*(W) abundances are represented by inverse distance weighting interpolation using the gstat package. Red represents high proportional *tet*(W) abundance (~0.9-1.2), white medium (~0.4-0.8), and blue low (~0-0.3).

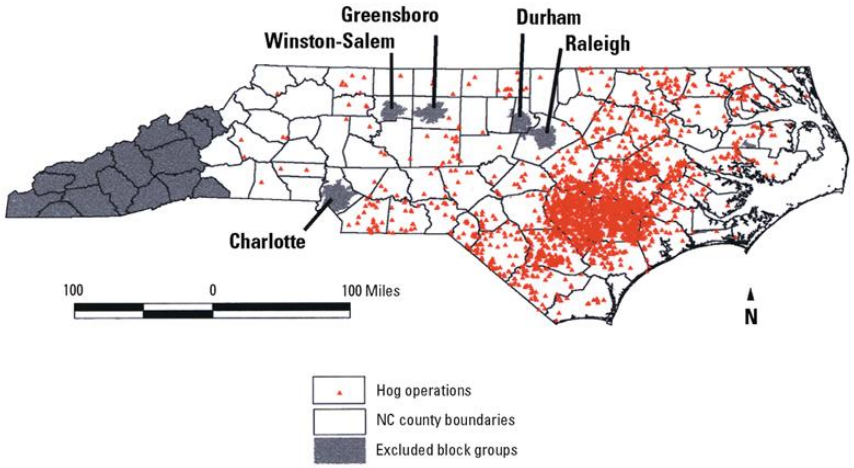


FIGURE 4. Hog farming operations in North Carolina in 2000, still relatively accurate (Wing, Cole, & Grant, 2000; Nicole, 2013).

A test for correlation across all bacterial families to proportional *tet*(W) abundances was performed and yielded no significant correlations. Thus the pool of families tested for correlation was narrowed to the abundances of bacterial families of species previously shown to carry a *tet*(W) gene (Table 2) such that expected correlations are not eliminated by the larger false detection rate correction (Figure 5). In this analysis, the outdoor sample set showed significant correlation to Clostridiaceae, Streptococcaceae, and Bacteroidaceae families (p = 0.009, 0.009, and 0.004 respectively) – all p values shown in Table 4. These three significantly correlated families are generally associated with animals and have notable pathogenic species like *Clostridium difficile* and *Streptococcus suis*. Figure 5A suggests a relationship between the abundance of the families in Table 2 and *tet*(W) proportional abundance for the outdoor sample set. However, the trend is not replicated by the indoor sample set (Figure 5B), nor do the p values between outdoor and indoor sample sets appear to have a correlation (Table 4).

TABLE 4. Spearman correlation after false detection rate correction p values between proportional *tet*(W) abundances and expected family abundance. Outliers are removed as to not drive the correlations.

	Outdoor p values	Indoor p values
Bacteroidaceae	0.004	0.4
Clostridiaceae	0.009	0.4
Streptococcaceae	0.009	0.9
Corynebacteriaceae	0.1	0.4
Lactobacillaceae	0.1	0.5
Veillonellaceae	0.1	0.4
Lachnospiraceae	0.2	0.4
Spirochaetaceae	0.3	0.5
Actinomycetaceae	0.8	0.4
Bifidobacteriaceae	0.8	0.9

Analyses of the outdoor sample set show the amount of *tet*(W) per bacterial genome may be predictable by a home's proximity to livestock and the type of bacteria in the settled dust on homes. However, the indoor sample set did not yield such hypotheses. This may suggest that indoor *tet*(W) abundance levels are tied more to unaccounted factors such as hospital stays and surgeries, occupant vocation, or agricultural goods in the home. Nonetheless, indoor samples would be expected to correlate to expected bacterial families at least. Certain species of

Pseudomonas exhibit intrinsic tetracycline resistance, which were removed from the sequencing results due to large amounts of *Pseudomonas* contaminants in the method of direct PCR (Chopra & Roberts, 2001). Indoor proportional *tet*(W) abundances may be explainable by *Pseudomonas* or other bacterial families that have *tet*(W) genes but have not been proven to by sequencing.

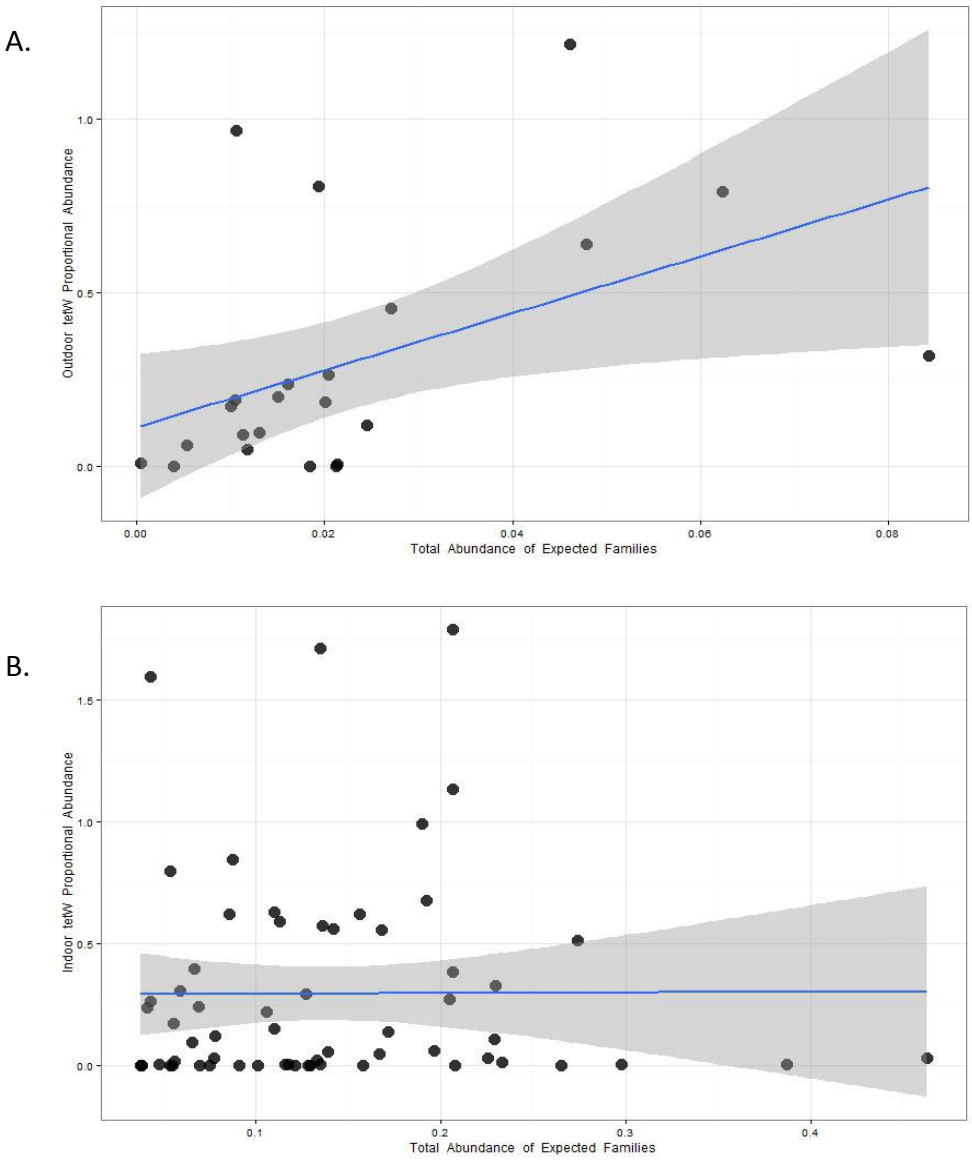


FIGURE 5. Proportoinal *tet*(W) abundance to the sum of the abundances of bacterial families previously shown to carry *tet*(W) in (A) outdoor samples (n = 23) after removal of samples with <10,000 sequences) and (B) indoor samples (n = 62), with one outlier above 5 *tet*(W) genes per bacterial genome removed from each sample set.

CONCLUSION

This study observed the presence of detectable levels of tetracycline resistance gene *tet*(W) in the majority of 90 North Carolina residential settled dust samples from inside and outside door trim, and the absence of detectable levels of *tet*(O), *tet*(M), and *tet*(Q). The amount of *tet*(W) per bacterial genome was shown to vary across samples with a number of samples having one *tet*(W) gene per bacterial genome. Among the environmental factors studied (Table 1), only the total livestock density of outdoor samples with an outlier suggested a significant correlating factor. A significant correlation was found between the abundance of Clostridiaceae, Streptococcaceae, and Bacteroidaceae families and the proportional *tet*(W) abundances for the outdoor samples alone as well.

Future directions of this project would seek to elucidate the nearly ubiquitous detection of *tet*(W) and the total absence of *tet*(O), *tet*(M), and *tet*(Q). Perhaps the best way to approach this could be through sequencing of the full, 1937 bp *tet*(W) gene as did Storteboom et al. (2010) such that it can be determined if the residential *tet*(W) is due to the naturally occurring resistance in some bacteria or if there are instances of human activity's influence. It would also be valuable to run test qPCR screens for *tet*(O), *tet*(M), and *tet*(Q), because qPCR is a much more sensitive detection system than visualized PCR, along with other RPP tetracycline resistance genes like *tet*(S), *tet*(T), and *tet*B(P).

Another necessary direction to the continuation of this project would be the addition of samples from areas of the greatest hog farm concentration to get a more complete picture of North Carolina residences. The two outliers above five *tet*(W) genes per bacterial genome continually removed from analyses in this study may prove to be examples of the influence of human antibiotic use above the baseline of this study's samples. If this is the case, the samples

from the high livestock density counties of North Carolina would be hypothesized to look more like the outliers found in this study. In addition, data on occupant vocations, time in a hospital, and usage of antibiotics should be collected which could elucidate the cause of the outliers as well.

Tetracycline is a first-line antibiotic and resistance has become widespread. Characterization of the abundance and sources of other, less benign resistance to last-resort antibiotics is a field that could be explored in the air-borne bacterial community of residences to combat the annual number of deaths from antibiotic resistant infections. The residential air environment is understudied considering the fact that it is where most Americans live out a significant portion of their lives, and there is much to be explored in this environment's bacterial communities.

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