Investigating Pedestrian Traffic as a Contributor to Tetracycline Resistance in Public Land Soil Microbiomes

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ABSTRACT. Since the development of the first antibiotic in the early 20th century, antibiotic resistance has become one of the greatest challenges faced by clinicians and public health experts in modern history. Previous research has demonstrated an exchange of antibiotic resistance between livestock and environmental microbiomes, as well as human and livestock microbiomes. The goals of this study were to investigate a similar relationship between human and environmental microbiomes using antibiotic growth media and polymerase chain reaction assays. Soil samples were collected from the Sangre de Cristo Mountains in north New Mexico. Tetracycline resistance assays were used to quantify the percentage of colony-forming units (CFUs) in each sample that demonstrated resistance to tetracycline to determine if collection sites with higher amounts of human foot traffic showed higher levels of resistance. PCR and gel electrophoresis were applied to test for the presence of four common tetracycline resistance genes (*tet*(A), *tet*(B), *tet*(D), and *tet*(E)) in the high-traffic samples. Samples taken from the high and moderate-traffic sites showed statistically significant levels of resistance, while the low-traffic site did not. Additionally, the high-traffic site demonstrated levels of tetracycline resistance that were greater than both moderate and low-traffic sites. Five samples tested positive for *tet*(A), and five tested positive for *tet*(E). No samples tested positive for either *tet*(B) or *tet*(D). These results indicate a correlation between human foot traffic and tetracycline resistance in soil samples.

KEYWORDS. Bacteria; tetracycline; antibiotic resistance; soil microbiome

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INTRODUCTION

When Alexander Fleming discovered penicillin in 1928, it revolutionized modern medicine and paved the way for new advancements in clinical and industrial microbiology. Now, nearly a hundred years later, the scientific community is faced with a new threat posed by rapidly evolving microbes. Bacteria have continued to evolve new methods to protect themselves from antibacterial treatments. Researchers have attempted to combat resistance by developing new drugs and modifying old ones, but these efforts have not been enough to curb the threat of antibiotic resistance (Huemer et al. 2020). Without new and innovative solutions to these trends, the antibiotic resistance problem is on track to becoming one of the greatest public health threats in history.

Part of the ongoing research into antibiotic resistance is determining major sources of resistance genes, and one source of particular interest is environmental bacteria. Because of its vast genetic diversity, it is theorized that the environmental microbiome could already hold resistance genes for any possible antibiotic that could ever be developed (Larsson and Flach 2021). Previous studies have identified cases of environmental bacteria displaying resistance mechanisms to clinical antibiotics like those found in pathogenic bacteria, suggesting a common source (Forsberg et al. 2012; Ekwanzala et al. 2018). The importance of environmental bacteria as a source of clinically significant multi-drug resistant (MDR) pathogens is poorly understood, but these reservoirs may play a much larger role in this public health crisis than previously thought.

The Prevalence of Antibiotic Resistance in the Environment (PARE) project was created by the Tufts University School of Medicine to aggregate data on environmental antibiotic resistance

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reservoirs. The goal of the PARE project is to recruit educators and students to participate in hands-on research of antibiotic resistance in the soil microbiome using the PARE methodology (Genné-Bacon et al., 2018). The results from this study are sent to the PARE database, where findings can be accessed by researchers investigating the antibiotic resistance crisis.

The PARE project methodology relies on tetracycline as a model for studying antibiotic resistance due to its activity against a wide variety of bacteria, its frequent use in agricultural applications (Kyselková et al. 2015), and because tetracycline resistance mechanisms and ecology have been studied extensively and are well understood. Tetracycline and its derivatives are broad-spectrum antibiotics that were first discovered in 1948 after being isolated from *Streptomyces aureofaciens* (Grossman 2016). Their use in clinical treatment began the same year, and by 1953, the first tetracycline-resistant infection was reported (Roberts 1996). Since then, several new derivatives have been synthesized, and bacterial resistance to the drug has increased alongside its use in the treatment of infections. Today, tetracycline resistance is as high as 67% in some species of bacteria (Jones et al. 2011), severely limiting the usefulness of tetracycline-class antibiotics to the treatment of simple infections of the respiratory, urogenital, and GI tracts (Grossman 2016).

Tetracyclines enter bacterial cells by diffusing passively through the OmpF and OmpC porins in the cell membrane, typically while complexed with Mg^{2+} ions (Thanassi et al. 1995). The tetracycline will then bind to the Tet-1 site on the 30S ribosomal subunit, which is located near the A site of the ribosome (Jenner et al. 2013). While tetracycline is bound, tRNA is sterically blocked from binding to the A site and delivering amino acids to the ribosome, inhibiting the synthesis of new proteins (Grossman 2016).

Dozens of tetracycline resistance genes have been identified, all of which can be divided into three categories based on their resistance mechanism. The most common mechanism is the efflux pump. These proteins are found in both Gram-positive and -negative bacteria and are members of the major facilitator superfamily (MFS) of transporter molecules (Chopra & Roberts 2001). There are several variations of these transport pumps, each of which relies on the H^+-coupled antiport of the tetracycline out of the cell and away from the Tet-1 binding site of the ribosome (Grossman 2016).

Several studies on antibiotic use in farm animals have consistently demonstrated that the use of antibiotics for agriculture, both to prevent disease and promote growth in livestock, is commonly linked with reservoirs of antibiotic resistance in the local soil microbiome (Durso et al. 2011; Durso & Cook 2014; Jechalke et al. 2014; Kyselkova et al. 2015). Sustained long-term use of antibiotic administration for veterinary use results in the selection of antibiotic resistance genes in the gut microbiome of farm animals, which can then be excreted in feces, either as part of a bacterial genome (gDNA) or as free environmental material (eDNA; Durso et al. 2011). After excretion, it now becomes part of the soil microbiome, so that resistance genes are free to spread rapidly, either through vertical gene transfer occurring during cell division, or through horizontal gene transfer of transposons or plasmids to native soil bacteria.

Most antibiotics used for veterinary treatment or agricultural practice are the same as those used in the clinical treatment of humans (Manyi-Loh et al., 2018). Extensive use of antibiotics in livestock can foster the spread of resistance in local soil bacteria, and previous studies have implicated environmental bacteria as sources for clinically relevant antibiotic resistance genes, which then can spread to humans and increase the danger of drug-resistant infections (Kyselkova et al. 2015). Although there are large bodies of research examining how animals impact the environmental resistome, and how the environmental resistome impacts humans, limited knowledge exists on the impacts of humans on environmental resistance, particularly through the shedding of resistant bacteria or genes that confer resistance to the environment.

The goal of this study was to examine whether the increased presence of humans in the environment can play a role in spreading resistance genes to soil bacteria. If animals can spread their resistance genes to the environment, humans may have a similar effect through the shedding of their skin bacteria that have developed resistance from past antibiotic exposure. If this relationship does exist, one would predict that areas with highlevels of human activity would show greater levels of resistance in the soil microbiome, and areas with lower levels of activity would show less resistance.

METHODS AND MATERIALS

Sample Collection

Soil samples were collected from the Sangre de Cristo Mountains in northern New Mexico. The collection site was located within a section of the mountains that contained many walking trails and camping sites for visitors. A total of 37 soil samples were collected from the site, and were categorized as either high, moderate, or low-foot traffic samples based on where samples were collected from in relation to hiking trails at the site. The high-traffic samples were taken directly from the trail itself, where visitors would pass through daily. The moderate-traffic samples were collected from soil just off the edges of the trails, ranging from 0–20 ft from the path where visitors would be less likely to walk. The low-traffic samples were taken from soil greater than 20 ft from the edge of the path, where human activity would be much more limited and infrequent. Twelve samples were designated high-traffic, thirteen were moderate-traffic, and twelve were low-traffic. Samples were only taken from trails that prohibited pets; to remove any contribution they may have to the results. Fresh gloves and sterile tubes were used to collect each sample to prevent contamination. Following collection, samples were stored at 4°C until assays were performed.

Tetracycline Resistance Assays

Serial dilutions were performed on each sample, producing five test tubes ranging in concentration from 1/10 to $1/10^5$ g/ml. Each test tube was sterilized with heat before being filled with 9 ml of distilled water using a pipette. In the 1/10 g/mL tube, 1g of soil was added, and the mixture was vortexed for 30 seconds to ensure an even distribution of microbes. Using a micropipette, 1 mL of the mix was then transferred to the $1/10^2$ g/mL tube. This process was repeated until all five tubes contained the proper concentration of soil, and the tubes were vortexed to ensure adequate dispersal of bacterial cells.

MacConkey agar plates with and without tetracycline were used for culturing. Tetracycline (TET) plates contained tetracycline at a concentration of 3 μg/mL, while non-tetracycline (NA) plates were standard MacConkey agar. Assays for each sample utilized 5 NA plates and 3 TET plates. Each of the NA plates corresponded to one of the five test tubes of soil mix. Each of the tetracycline plates corresponded to one of the test tubes ranging from 1/10 to $1/10^3$ g/mL in concentration. Each sample was run in duplicate.

Each plate was inoculated with 0.2 mL of soil mix from their corresponding solution tube. Once the mix had been transferred, sterile disposable inoculation loops were used to spread the mix over the entire surface of the agar. Once both sets had been inoculated, plates were left to dry for 2–3 minutes and were then placed in an incubator at 37°C. Incubation took place for 48 hours before the plates were removed for analysis.

After agar plates were removed from the incubator, plates were selected for analysis if the number of colonies was between 30 and 300. More than 300 colonies were considered too many to count, and less than 30 were considered too few. Once a plate with a countable number of colonies was identified from both the non-antibiotic and tetracycline plate set, colonies were counted and recorded in the datasheet, along with the concentration of the inoculating soil solution. The number of colonies was recorded as the number of colony-forming units (CFUs) within the plated soil mix.

Once colony data was recorded, equation (1) was used to calculate the number of CFUs per gram of soil.

$$\text{CFUs} \times \text{dilution factor} \times 5 = \text{CFUs/g} \quad (1)$$

The dilution factor is equal to the denominator of the concentration on the counted plate. Multiplication by 5 is required to adjust for the use of 0.2 mL, or 1/5th of 1 mL of mixture in the plate inoculation step. Once the CFUs/g were calculated for both the NA plate and TET plate from the same set, equation (2) was used to determine what percent of the CFUs in the soil sample were resistant to tetracycline at a concentration of 3 μg/ml.

$$\text{TET CFUs/g} \div \text{NA CFUs/g} = \%\ \text{resistant CFUs} \quad (2)$$

Results from the two sets were averaged to determine the mean percent resistant CFUs for each sample.

Tetracycline Resistance Genes

The four tetracycline resistance genes selected for this study were *tet*(A), *tet*(B), *tet*(D), and *tet*(E). Previous studies have linked these four genes with environmental tetracycline resistance, particularly with gram-negative soil microbes, and each of the

four genes code for a tetracycline efflux protein (Roberts 2005; Hedayatianfard et al. 2014). To ensure proper amplification of each gene, the 4 genes were combined into 2 groups each having 2genes based on their overall size. Group A consisted of *tet*(A) and *tet*(E), while Group B consisted of *tet*(B) and *tet*(D).

DNA Extraction

Bacteria from the high foot traffic samples were cultured by adding 0.2 g of each sample to 5 ml of Luria broth (LB) and incubating at 37°C for 24 hrs. An inoculation loop was then used to transfer solution from the LB to standard MacConkey agar plates, to select for gram-negative bacteria. Plates were incubated at 37°C for another 24 hrs. DNA extraction was performed on 5–7 colonies taken from each plate using ZymoBIOMICS® DNA Microprep Kit®.

Multiplex PCR

Following DNA extraction, target genes were amplified using reagents from the Qiagen Multiplex PCR Kit and custom primers ordered from Integrated DNA Technologies. The following PCR conditions were used for gene amplification: initial heat activation of HotStarTaq® DNA Polymerase at 94°C for 15 min; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 90 seconds, and extension at 72°C for 60 seconds for Group A and 90 seconds for Group B; and a final extension at 60°C for 30 min for Group A, and 72°C for 10 min for Group B. Primer sequences for each of the genes were derived from the article "Multiplex PCR for the detection of tetracycline resistant genes" (Ng et al. 2001).

Gel Electrophoresis

Agarose gels were prepared using a 1x TAE buffer. Amplified samples were loaded into gel lanes, and 100 V was applied to the gel chamber for 30 minutes. Gels were then removed from the chambers and stained overnight in 1x Fast Blast DNA Stain while undergoing gentle stirring. The following day, gels were removed from the stain and observed for the presence of visible bands.

RESULTS

The results of the colony analysis showed that the soil samples taken from the high-traffic foot path displayed a higher level of resistance than both the moderate and low-foot traffic sample sets. The average percentage of tetracycline-resistant CFUs for each sample and the sample means for the high, medium, and low traffic sets are shown in Table 1.

Table 1
Percent of CFUs in soil samples taken from high-, medium-, and low-foot traffic areas of the Sangre de Cristo Mountains, New Mexico, that were resistant to tetracycline.

Sample #	High-Traffic % Resistant	Medium-Traffic % Resistant	Low-Traffic % Resistant
1	30.1%	24.4%	0.420%
2	19.0%	2.00%	0.00%
3	1.50%	0.00%	0.00%
4	12.6%	10.0%	0.0300%
5	8.30%	11.7%	0.00%
6	9.10%	0.00%	0.00%
7	62.0%	0.00%	4.00%
8	25.8%	1.90%	0.0300%
9	7.80%	0.00%	0.00%
10	12.0%	0.800%	0.0200%
11	30.6%	5.30%	0.00%
12	27.0%	1.10%	0.140%
13	-	1.30%	-
Mean	20.5%	4.50%	0.387%
p-value	0.000576	0.0209	0.133

One-tailed t-tests for comparison of means were conducted on each of the three sample sets. The high-traffic set showed a statistically significant number of tetracycline-resistant colonies ($t(11, 0.05) = 4.4$, $p < 0.05$), as did the moderate-traffic set ($t(12, 0.05) = p < 0.05$). The number of tetracycline-resistant colonies in the low foot traffic set was not statistically significant ($t(11, 0.05) = 1.2$, $p = 0.133$).

An ANOVA test was also conducted on the data, which showed statistically significant difference within the data set ($F = 13.04$, $p < 0.05$). The high-traffic samples showed greater resistance compared to the moderate-traffic ($Q = 5.49$, $p < 0.05$) and the low-traffic ($Q = 6.90$, $p < 0.05$). The moderate-traffic set did not show a significant difference when compared to the low-traffic set ($Q = 1.41$, $p = 0.58$) (Fig. 1).

Gel electrophoresis on the high foot traffic samples showed 5 samples were positive for *tet*(A), and 5 were positive for *tet*(E), with 4 samples testing positive for both Group A genes. No samples tested positive for either *tet*(B) or *tet*(D) (Table 2).

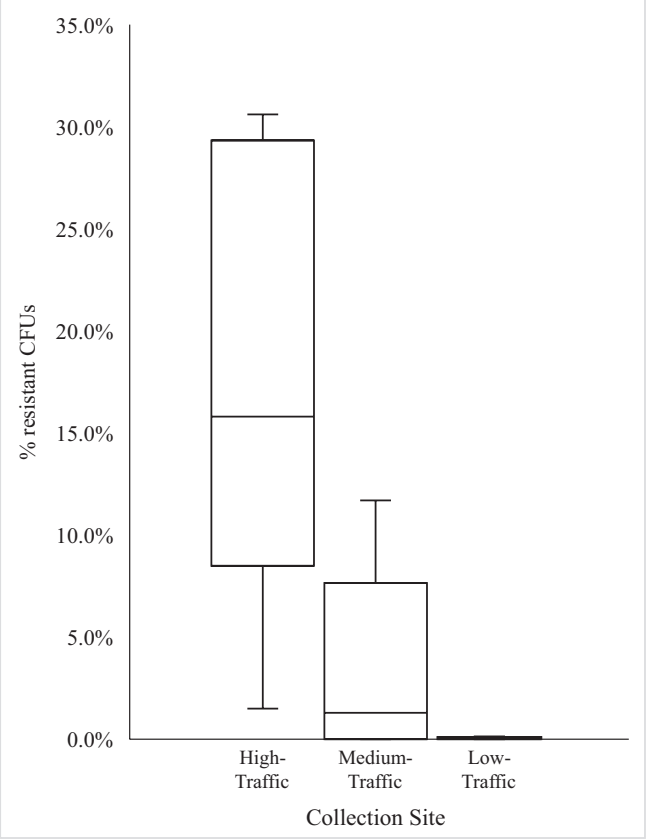

Figure 1. Boxplot of percent of CFUs that showed resistance to tetracycline from high-, medium-, and low-foot traffic collection sites. The middle bar indicates the median, whereas the top and bottom of the box indicate the 75th and 25th percentile, respectively. The tails of each box indicate extremely high or low values.

DISCUSSION

Data from the tetracycline resistance assays show that the soil collected from high-traffic sites contained bacteria with higher levels of tetracycline resistance than those taken from less traveled areas of the park. In addition, although the medium and low-traffic sites were not statistically different from one another in their rates of resistance, the medium-traffic site had a significant level of resistance, while the low traffic site did not. Both sites had lower levels than those of the high-traffic sites, demonstrating a positive correlation between the two variables. In addition, some samples from the high-traffic set showed high resistance to tetracycline in antibiotic assays but tested negative for all four genes in this study. This means that other genes conferring resistance to tetracycline are present within the high-traffic samples. Dozens of other genes conferring resistance to tetracycline have been identified and studied (Ng et al. 2001), and future studies could focus on examining a wider array of genes.

Most research into the human impacts on environmental antibiotic resistance focuses on environmental pollutants. Human or animal sewage (Wang et al. 2020), agricultural contamination (Taylor & Reeder 2020), and improper handling of pharmaceutical waste (Anwar et al. 2020) are just a handful of ways in which human activity can contribute to the transfer of antibiotic resistance to the environment. In some cases, proliferation occurs through contamination by the antibiotics themselves, which act as a selective pressure for the evolution of new resistance genes within the native environmental bacteria (Larsson 2014; Kraupner et al. 2021). Other studies have found that horizontal gene transfer can occur among bacteria found in waste such as human feces, and environmental bacteria (Klümper et al. 2015), also resulting in the rise of resistant microbes.

Given that livestock are known to shed antibiotic-resistant bacteria and resistance genes from their own microbiomes (Durso & Cook 2014; Jechalke et al. 2014; Kyselkova et al. 2015), it is not unlikely that the increased tetracycline resistance seen in the high traffic samples could have in part come from humans through a similar mechanism. Humans shed bacterial cells from their skin regularly to their surrounding air (Hospodsky et al. 2012; Meadow et al. 2015), water (Elmir et al. 2007; Plano et al. 2011), and surfaces (Brooke et al. 2009; Meadow

Table 2
Results of PCR assays to detect four common tetracycline resistance genes in soil samples taken from high-foot traffic areas of the Sangre de Cristo Mountains, New Mexico.

Sample #	Group A		Group B	
	tet(A)	*tet*(E)	*tet*(B)	*tet*(D)
1	-	-	-	-
2	-	-	-	-
3	+	-	-	-
4	+	+	-	-
5	-	-	-	-
6	+	+	-	-
7	+	+	-	-
8	-	-	-	-
9	+	+	-	-
10	-	+	-	-
11	-	-	-	-
12	-	-	-	-
Total	5	5	0	0

et al. 2014). Some of these bacteria would likely harbor genes that confer resistance to any number of antibiotics, and could then begin to colonize the surrounding environment, becoming a new reservoir of resistant bacteria and genes.

Regardless of their origin, drug-resistant microbes can be transmitted from the environment to humans through food or water (Larsson & Flach, 2022). Contaminated water sources used for drinking water, recreation, or aquaculture can all be sources of antibiotic-resistant pathogens which can be transmitted to humans upon exposure. Livestock that are exposed to contaminated soil or water and then consumed can similarly become a source of resistant infections (Wellington et al. 2013). Fresh produce is also capable of harboring drug-resistant pathogens or genes, which can cause infections when consumed (Blau et al. 2018).

The role that non-livestock animals play in spreading antibiotic resistance through the environment is unclear. Future research could focus on sampling the microbiomes of native fauna and comparing microbial and resistance profiles to that of the environmental microbiome to assess correlation. Additionally, while this study focused on trails that prohibited pets, examining the impact that pet microbiomes play in spreading resistance to soil bacteria is also a topic for further study.

More research is required to determine whether humans can shed their antibiotic resistance from their bodies into the environment at a great enough rate to significantly impact the resistance levels of local soil bacteria. Applications of this methodology to other regions and types of antibiotics would allow for a clearer understanding of the dynamics between the human and environmental resistomes. If future studies do support the proposed mechanism of transfer, it could suggest that humans are capable of passively contaminating their surroundings with antibiotic resistance genes found within their microbiomes.

The successful detection of *tet*(A) and *tet*(E) in several samples shows that this method can identify antibiotic resistance genes (ARGs) found within environmental bacterial samples. This procedure should be applied to a variety of different resistance genes for different antibiotics, as well as to different environmental samples, to further test its validity. Additionally, further research should examine whether differences exist between the ARGs found within high- and low-traffic collection sites, and whether the ARGs found within high-traffic sites are like those found in human microbiota.

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