The prevalence and identification of multidrug-resistant bacteria in adjacent ecological systems in the Hocking Hills region of Appalachia

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Abstract

Multidrug resistance in clinical settings is a major threat to human health, but very little is known regarding the prevalence of multidrug-resistant organisms in the natural environment. Studying antibiotic resistance in the environment is important for understanding the transfer of resistance between environmental microorganisms and those found in healthcare settings. In this study, soil samples from seven adjacent ecological zones were evaluated to determine if there were differences in the amount and types of antibiotic-resistant bacteria present. We hypothesized that we would find antibiotic-resistant bacteria in all ecological zones studied and that these bacteria would be unique to their specific niche. Several resistant organisms from each site were also tested for multidrug resistance and subsequently identified through DNA sequencing of the 16S gene. Antibiotic resistance was discovered in all sites at varying percentages. Some forms of bacteria were present at all sites, but there were differences in types of resistant bacteria found between sites. Six different genera of bacteria were identified, and multidrug resistance was found in all the isolates studied. Our findings indicate that multidrug resistance is prevalent in many different types of environments, including those that have never been directly used for agricultural or urban development.

Introduction

Antibiotic resistance (AR) has been declared a global health epidemic (53). Resistant bacteria currently cause over 2 million infections and 23,000 deaths each year in the USA alone (8). Multidrug resistance (MDR) has amplified this threat by making some infections difficult, if not impossible, to treat with antibiotics (33). The escalation of not only antibiotic-resistant organisms, but multidrug-resistant organisms has been linked to overuse and misuse of antibiotics in clinical and agricultural settings (48). This overuse has led to resistance to every class of antibiotics (8) and consequently, loss of antibiotic efficacy for treating common bacterial infections. In addition, lack of pharmaceutical investment in antibiotic discovery (48) has restricted the availability of novel treatment options. Antibiotics have limited effectiveness as introducing them to bacterial populations creates a selective pressure, allowing resistant bacteria to survive and reproduce. AR can be acquired via a random mutation (52), preexisting efflux pumps, or by horizontal gene transfer (HGT). HGT has been linked to the spread of resistance since AR genes are often carried on mobile genetic elements such as plasmids (28), integrons (51), transposons (45), or bacteriophages (9). Through HGT, these genes can disseminate and proliferate both within a population and between different populations of bacteria.

Most research has studied AR in clinical settings, yet there is evidence that some clinical AR genes have environmental origins (50). Naturally occurring resistance is widely abundant but is still not entirely understood (10, 12, 41). Specifically, it is not well understood if resistance is found only in specific environments or in certain groups of bacteria. Previous studies have found antibiotic-resistant bacteria in pristine environments including remote Alaskan soil (2) and multidrug-resistant bacteria in isolated cave systems (5). A more detailed surveillance of AR in the environment could allow us to determine the relationship between environmental reservoirs and clinical resistance.

The Centers for Disease Control and Prevention (CDC) recommends combatting deadly infections and the spread of resistance by tracking AR patterns in all environments (53). Previous research has also suggested there is a need for thorough environmental surveys, especially of those environments affected by human activities (17, 22, 32). These areas are of interest as urban environments with high population and building density often have higher levels of AR (19, 32), possibly due to antibiotic effluent from hospitals or from farms that pollute the surrounding environment and water bodies (26). People living or working in these areas also acquire resistant bacteria into their own microbiome (42), further spreading the prevalence of AR. To address the CDC's recommendation, a project called the Prevalence of Antibiotic-Resistant Bacteria in the Environment (PARE) has created an infrastructure capable of organizing environmental data collection by large numbers of individual researchers (18). This project coordinates undergraduate and high school students across the USA to collect and compile data using identical methodologies. Eventually, the PARE project will make it possible to track resistance over time, identify areas with high AR, and find relationships between resistance and different environments.

The Merl and Margaret Primmer Outdoor Learning Center (Primmer), is an outdoor education and research property uniquely suited for a study on environmental drug resistance because of its location away from well-developed areas and its ecological diversity. More than 40 plants and 25 animals have been documented (C. Anderson, Capital University, personal communication), and much of this property has never been developed or used for agricultural purposes (Mark Laughlin, Capital University, personal

communication). The Primmer research property is located in Appalachia, Ohio, a primarily agricultural region. The Hocking River, adjacent to the property, has been historically targeted by the Environmental Protection Agency (EPA) as it had been contaminated by industrial and sewer discharges, as well as mine drainage and agricultural runoff (39). With its seven different ecological zones located adjacent to one another and in proximity to a water source made this property an ideal and unique setting for quantitatively surveying both the prevalence of antibiotic-resistant bacteria in the different ecological zones and the diversity of these antibiotic-resistant organisms. Select isolates were additionally tested for MDR. We hypothesized that antibiotic-resistant bacteria would be found in all ecological zones studied, and also that these bacteria would be unique to their specific niche. Our findings not only supported our hypothesis by showing that antibiotic-resistant bacteria were found in all seven ecological zones studied, but that multidrug resistance can also be prevalent even in the absence of human development.

Materials and Methods

Sample collection

Soil samples were collected from the Primmer research property, located in Logan, Ohio. Fig. 1 shows a map of Primmer and the locations of the 14 collection sites from the seven ecological zones. The distinct ecological zones that have been identified include a grassland (G), a woodland (WL), a wetland (W), a prairie (F), a spring (S) and a riparian zone (R). Since clinical AR genes have been detected at distances up to 15 km from the discharge point (13), samples were also collected from the Hocking River (HR) to assess possible pollution from upstream locations. Soil samples were collected between May and June 2017. GPS coordinates in decimal degrees for each collection site were recorded (Table 1). The sites were primed by loosening up the soil with a rock or piece of wood from the area. Soil samples were isolated an estimated 2.5 cm below the surface from the upper soil horizon, or topsoil since previous studies have showed that bacterial populations are most abundant in this soil horizon (15). Samples were then transported to the laboratory and stored at 4°C overnight.

Ecological Zone	Site	Latitude	Longitude
Spring	S1	39.544504	-82.443202
	S2	39.544272	-82.442057
Woodland	WL1	39.547797	-82.442072
	WL2	39.548157	-82.441953
Prairie	F1	39.547473	-82.442803
	F2	39.546533	-82.443398
Wetland	W1	39.543284	-82.440598
	W2	39.541241	-82.443711
Hocking River	HR1	39.544660	-82.441914
	HR2	39.544387	-82.442431
Grassland	G1	39.548091	-82.439984
	G2	39.548217	-82.440005
Riparian Woodland	R1	39.547007	-82.441353
	R2	39.546853	-82.442282

Table 1. GPS coordinates of sample collection sites.

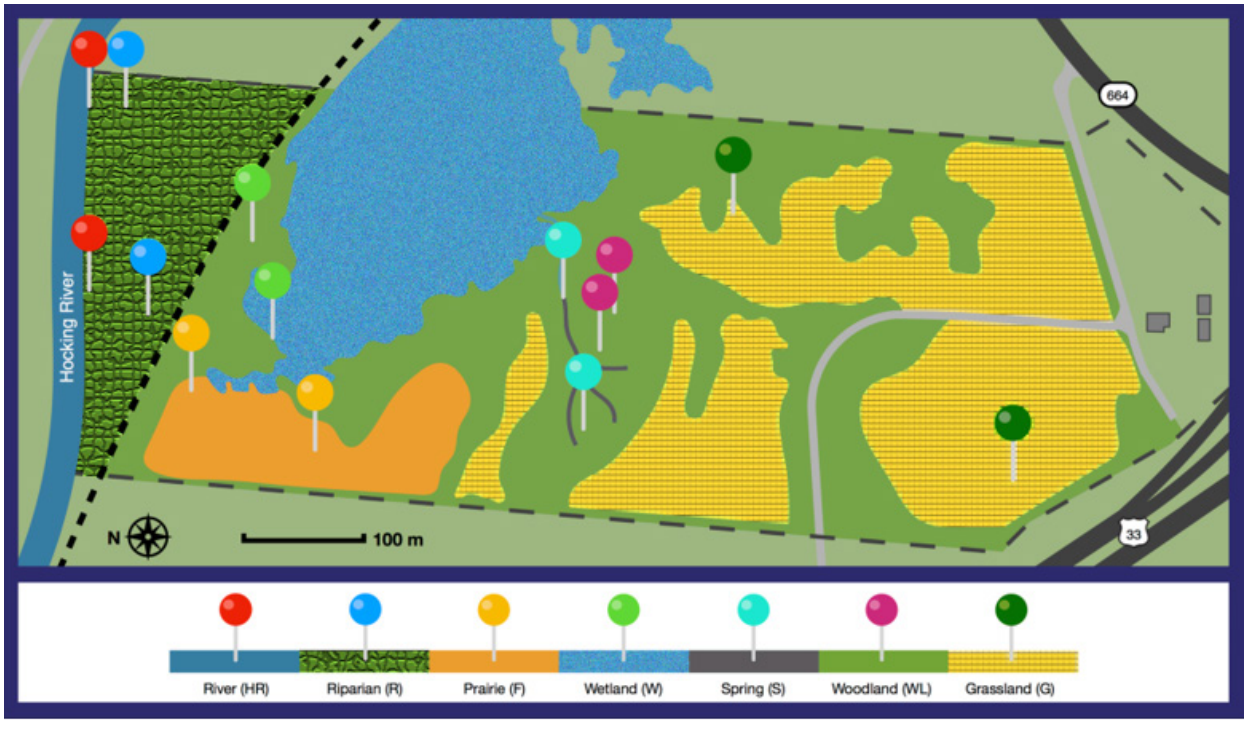

Fig 1. Map of the Primmer Outdoor Learning Center and sample collection sites. Primmer is a 0.3 km^2 private research property with seven unique ecological zones. Modified from the USGS National Map Viewer (2017). Sample collection sites are noted by the pins and correspond to the ecosystems noted in the key.

Determining the prevalence of antibiotic resistance

MacConkey agar and nutrient agar plates without tetracycline (NA), with 3 μg/ml (3tet), or 30 μg/ml (30tet) tetracycline were prepared as per the manufacturer's instructions (BD Biosciences, San Jose, CA). All plates also contained 10 μg/ml amphotericin B to prevent overgrowth on plates by fungi in the soil. One gram of soil was measured, serially diluted for five 1:10 dilutions in sterile distilled water, and 0.2 ml of each dilution were plated on all types of media using sterile glass beads. The plating of bacteria was done in duplicate. Inoculated plates were wrapped in Parafilm and incubated at 28°C for 72 hours. The total number of colony-forming units (CFUs) was determined from countable plates without tetracycline (plates with 30-300 colonies and no overgrowth). The percent tetracycline-resistant colonies were determined by counting CFUs from the plates containing tetracycline and comparing them with the plates lacking tetracycline.

Colony characterization

After incubation, colonies were characterized according to morphological appearances as in Breakwell, ***et al.***, 2007 (7). Colonies of different morphotypes from each ecological zone were selected to test for additional AR. All colonies selected were resistant to either 3 or 30 μg/ml tetracycline.

The Kirby-Bauer test for antibiotic susceptibility was performed as in Hudzicki, ***et al.***, 2017 (24). Tested antibiotic discs (ThermoFisher Scientific, Waltham, MA) contained one of the following: ciprofloxacin (5 μg), ampicillin (10 μg), penicillin (10U), and a tetracycline control (30 μg). These antibiotics were chosen based on their use in human and veterinary medicine (11, 54). The diameter of each zone of inhibition was compared to the standard set by the National Committee of Clinical Laboratory Studies (NCCLS) to determine if the isolate displayed resistance (R), susceptibility (S), or was intermediate (I).

PCR and sequencing

DNA was extracted from isolates by a boiling lysis protocol as found in Queipo-Ortuño ***et al.***, 2008 (37). For each sample, PCR was performed using one of two different primer pairs for part of the 16S rRNA gene. Primer pairs were: (i): Bakt_341F, (CCTACGGGNGGCWGCAG), and Bakt_805R, (GACTACHVGGGTATCTAATCC) (21, 25) and (ii): pA/27F, (AGAGTTTGATCCTGGCTCAG) (14, 27), and 1492R, (TACGGGTACCTTGTTACGACTT) (27, 44). PCR products were purified using the DNA Clean and Concentrator kit (Zymo Research, Irvine, CA) and then sequenced using the forward primer used for amplification by The Ohio State University Comprehensive Cancer Center Genomics Shared Resource facility. Resulting sequences were compared to previously published sequences using the Basic Local Alignment Search Tool (BLAST) program (National Center for Biotechnology Information).

Results

Percentage of antibiotic-resistant bacteria present in soil

To assess the prevalence of AR in the seven different ecosystems, we collected samples from two different sites from each of the seven ecological zones present within the Primmer research property (Fig 1). Soil samples were serially-diluted and plated onto media with or without tetracycline. Two different types of media and two different concentrations (3 or 30 μg/ml) of tetracycline were used. MacConkey agar was used so that a portion of our results could be contributed to the PARE database, allowing future

comparisons between additional data collected from other sites. MacConkey agar is the current standard media used by the PARE Project as it commonly results in more uniform colony morphology, which makes colony counting more accurate (18). Nutrient agar was used to better assess the diversity of bacteria present. Of the MacConkey agar data, sites F2, HR1, and G1 exhibited the highest percentage of resistance to 3 μg/ml of tetracycline with the number of resistant colonies at greater than 10% (Table 2). Only five sites (S1, WL1, HR1, HR2, and R1) displayed resistance to the higher concentration of tetracycline (30 μg/ml). With the exception of HR2, these sites showed a decrease in the percentage of resistant colonies to the higher concentration of tetracycline. All but one site (S2) showed resistant colonies at some level of tetracycline concentration.

Sample	% resistant to 3 μg/ml tet		% resistant to 30 μg/ml tet	
	MacConkey	nutrient	MacConkey	nutrient
S1	5%<x<10%	5%<x<10%	1%<x<5%	1%<x<5%
S2	ND[a]	>10%	ND	1%<x<5%
WL1	5%<x<10%	5%<x<10%	<1%	<1%
WL2	<1%	5%<x<10%	ND	<1%
F1	1%<x<5%	1%<x<5%	ND	<1%
F2	>10%	1%<x<5%	ND	<1%
W1	<1%	<1%	ND	<1%
W2	1%<x<5%	TM[b]	ND	TM
HR1	>10%	>10%	<1%	<1%
HR2	1%<x<5%	<1%	1%<x<5%	<1%
G1	>10%	5%<x<10%	ND	<1%
G2	1%<x<5%	5%<x<10%	ND	<1%
R1	1%<x<5%	<1%	<1%	<1%
R2	<1%	1%<x<5%	ND	<1%

Table 2. Percentage of bacterial colomes plated on MacConkey agar and nutrient agar media resistant to 3 μg/ml or 30 μg/ml tetracycline.

[a]ND: Not determined due to low coloy counts (<30) present on plates without tetracyline.

[b]TM: Not determined due to high colony counts on plates with no antibiotic.

As expected, overall colony counts were higher on nutrient agar compared to MacConkey, which facilitated the ability to accurately determine the percent resistance for more samples at more sites (Table 2). Every sample of soil showed some level of resistant bacteria. However, the percent resistance for one of the wetland samples (W2) could not be determined due to high colony counts on plates without antibiotic. Both samples from the groundwater spring zone (S1 and S2) exhibited resistance to both concentrations of tetracycline on nutrient agar. S2 and HR1 had the highest percentage of resistant bacteria to 3 μg/ml tetracycline at greater than 10%. Eleven of the fourteen samples showed less than 1% resistance to 30 μg/ml of tetracycline. When comparing results from both MacConkey and nutrient media, samples S1, WL1, and HR1 had identical ranges for each concentration of tetracycline. All other samples, except F2, showed a slightly higher percent of resistant bacteria on nutrient agar compared with MacConkey. These data suggest that naturally occurring AR is even more prevalent than originally expected.

Identification of isolated antibiotic-resistant bacteria

We next tested our second hypothesis that tet-resistant bacteria would be unique to their particular environment. Tetracycline-resistant colonies on countable nutrient agar plates were analyzed and classified based on their phenotypic characteristics (Table 3). Thirty-six morphotypes were identified from the fourteen collection sites (morphotypes I-XXXVI). Morphotype I, the most common, was found at all 14 collection sites and at percentages ranging from 54.05% (S1) to 98.9% (W2) of the total antibiotic-resistant population. Morphotype I also represented 83.54% of all antibiotic-resistant bacteria. The woodland zone (WL1 and WL2) displayed the highest diversity of morphology with 12 unique morphotypes. These results suggest that, while at low frequencies, adjacent ecological zones contain distinct sub-populations of bacteria.

Morphotype	Form	Elevation	Margin	Size	Color	Site	%[a]
I	Circular	Convex	Entire	Small	White	S1	54
						WL1	75.4
						HR1	73.4
						R1	91.5
						F1	85.9
						F2	96.2
						G1	71
						G2	89.9
						WL2	79.2
						S2*	92.2
						R2	82.1
						HR2*	93
						W1	87.5
						W2	98.9
II	Circular	Convex	Entire	Small	Yellow	S1*	33.8
						WL1	14.8
						HR1*	14.4
						F1	.6
						G1	.8
						WL2	7.8
						R2	.9
						HR2	1.4
III	Circular	Convex	Entire	Small	Orange	G1	3.7
						G2	1.3
						WL2	2.6
						WL1*	2.1
IV	Circular	Convex	Entire	Small	Pink	WL1*	.4

Table 3a. Colony morphology for countable nutrient media plates.

[a]Percentage of antibiotic-resistant colonies with the population at a single soil sample collection site.

*Selected fror isolation

V	Circular	Convex	Entire	Large	White	S1	2
						HR2	2.1
VI	Circular	Convex	Entire	Large	Orange	S1*	2.7
VII	Circular	Convex	Erose	Large	White	HR1	4.2
						WL1	3.5
VIII	Circular	Convex	Erose	Small	White	HR1	4.9
IX	Circular	Crateriform	Entire	Small	White	G1	2.5
						G2	7.6
						WL2	2.1
						R2	11.7
X	Circular	Crateriform	Entire	Small	Yellow	WL1	1.8
XI	Circular	Crateriform	Entire	Large	White	WL1	.4
						HR2	3.5
XII	Circular	Crateriform	Entire	Large	Yellow	F1	2.2
						W2	.4
XIII	Circular	Crateriform	Erose	Large	White	G2*	1.3
XIV	Circular	Crateriform	Undulate	Small	White	G1*	9.1
						WL2	4.7
						S2	1.7
XV	Circular	Crateriform	Undulate	Large	White	G1	.8
XVI	Circular	Crateriform	Lobate	Large	White	G1	.4
XVII	Circular	Raised	Erose	Large	White	WL1	1
						R1	.3
XVIII	Circular	Raised	Erose	Small	White	R1	1
						F2	.9

Table 3b. Colony morphology for countable nutrient media plates.

[a]Percentage of antibiotic-resistant colonies with the population at a single soil sample collection site.

*Selected fror isolation

XIX	Circular	Raised	Entire	Small	White	F2	1.5
XX	Circular	Raised	Lobate	Large	White	F2	.9
XXI	Circular	Raised	Undulate	Large	White	F1*	.5
						W2	.4
XXII	Circular	Flat	Entire	Small	White	F1	6
XXIII	Circular	Flat	Erose	Large	White	S1	3.4
						HR1	2.6
XXIV	Circular	Umbonate	Entire	Small	White	R1	5.8
XXV	Circular	Umbonate	Erose	Large	White	R2*	4.9
XXVI	Circular	Umbonate	Filamentous	Large	White	W2*	.4
XXVII	Irregular	Flat	Lobate	Large	White	HR1	.4
XXVIII	Irregular	Flat	Undulate	Small	White	HR1	.2
						G1	.8
XXIX	Irregular	Raised	Undulate	Large	White	R1	1
						R2	.5
XXX	Irregular	Raised	Erose	Large	White	S1	1.4
XXXI	Irregular	Convex	Filamentous	Large	White	F1	5.4
XXXII	Irregular	Crateriform	Undulate	Small	White	WL2*	3.6
XXXIII	Filamentous	Umbonate	Filamentous	Large	White	S1	2.7
						WL1	.2
XXXIV	Filamentous	Flat	Filamentous	Large	White	WL1	.4
XXXV	Punctiform	Convex	Entire	Small	White	G1	10.7
XXXVI	Rhizoid	Raised	Filamentous	Large	White	W1*	12.5

Table 3c. Colony morphology for countable nutrient media plates.

[a]Percentage of antibiotic-resistant colonies with the population at a single soil sample collection site.

*Selected fror isolation

To determine if the resistant bacteria were unique to their ecological zone, we selected a subset of colonies from different ecosystems and of different morphology. Some colonies were selected from 3tet plates while others were selected from 30tet plates. We performed PCR on these isolates using primers designed to amplify the 16S ribosomal RNA gene. Sequence analysis of these genes allowed identification at the genus level. Surprisingly, of the ten bacterial colonies successfully sequenced (Table 4), nearly all of the bacteria identified were of different genera. As expected, ***Streptomyces***, which is prevalent in soil (23)**,** was identified in four different ecological zones (G, WL, S, and HR). Morphotype XXVI from the wetland zone (W2) and morphotype XXV from the riparian zone (R2) were identified as ***Bacillus*** with 99% confidence. The duplicate genera suggest that antibiotic-resistant bacteria are not entirely specific to their ecological zone. These data showed that while some species of antibiotic-resistant bacteria were present in multiple zones, there were some that were specific to different zones, supporting our initial hypothesis. These data also showed that a variety of different bacteria are capable of possessing AR.

Antibiotic susceptibility test

Since all of our isolates were found to be resistant to tetracycline, we wanted to know if they were resistant to other antibiotics. To test this, we performed a Kirby-Bauer disk diffusion test allowing us to test other antibiotics used in human and veterinary medicine. The antibiotics chosen for testing were ciprofloxacin, which has a synthetic origin (49), and ampicillin and penicillin, which are both naturally occurring β-lactam antibiotics (1, 16). Some of our isolates selected from 3tet plates were sensitive to the disk with 30 μg of tetracycline (isolates B, E, H, and I), showing that their resistance is concentration-dependent. Since all isolates were resistant to some concentration of tetracycline and one or more antibiotics, 100% of the isolates were multidrug-resistant (Table 5). Interestingly, every isolate displayed resistance to penicillin. Isolates A, D, F, K, and N (36% of isolates) were resistant to all four antibiotics. Discovery of MDR in each ecological zone indicates that there must be some selective advantage for this, even in naturally occurring environments.

Isolate Designation	Site	Morphotype	Tetracycline Concentration	Identity	% Identity
A	S1	II	30tet	ND[a]	ND
B	S1	VI	3tet	ND	ND
C	WL1	III	30tet	*Flavobacterium*	91
D	HR1	II	3tet	ND	ND
E	F1	XXXI	3tet	*Chitinophaga*	99
F	G1	XIV	3tet	ND	ND
G	G2	XIII	3tet	*Streptomyces*	97
H	WL2	XXXII	3tet	*Streptomyces*	82
I	S2	I	3tet	*Streptomyces*	87
J	W2	XXVI	3tet	*Bacillus*	99
K	W1	XXXVI	3tet	*Burkholderia*	99
L	HR2	I	30tet	*Streptomyces*	99
M	R2	XXV	3tet	*Bacillus*	99
N	WL1	IV	3tet	*Mucilaginibacter*	100

Table 4. Designation for identification

[a]ND: Not determined

Isolate	Tetracycline		Ciprofloxacin		Ampicillin		Penicillin	
	ZOI[a]	RES[b]	ZOI	RES	ZOI	RES	ZOI	RES
A	7	R[c]	7	R	7	R	7	R
B	19	S[d]	9	R	7	R	7	R
C	10	R	11	R	16	S	11	R
D	8	R	12	R	7	R	7	R
E	23	S	13	R	12	I	8	R
F	7	R	7	R	7	R	7	R
G	16	I[e]	23	S	8	R	7	R
H	21	S	21	S	12	I	7	R
I	21	S	22	S	7	R	7	R
J	8	R	23	S	8	R	7	R
K	12	R	11	R	7	R	7	R
L	7	R	10	R	12	I	7	R
M	13	R	30	S	14	S	11	R
N	7	R	7	R	7	R	7	R

Table 5. Zone of inhibition diameter measurements of selected bacterial colonies

[a]Zone of inhibition diameter measured in mm

[b]Resistance determined by diameter standard (CLSI, 2013)

[c]Resistant

[d]Susceptible

[e]Intermediate

Discussion

AR is naturally occurring, even in environments away from anthropogenic selection pressures. Studying this naturally occurring AR is important for understanding and dealing with the growing resistance of pathogenic microorganisms in clinical settings. Previous evidence of natural AR has been found in remote soil and isolated cave systems (2,5). While the Primmer research property is not as isolated as a cave system**,** our samples were collected from a site that has never been developed. Because resistant organisms were found in each of the ecological zones tested, our results support what previous research has found, that AR occurs naturally in bacterial populations.

Not much is known yet regarding how frequently AR gene transfer takes place in natural environments. MDR can occur through various mechanisms, one of which is efflux of the drugs by membrane transport proteins. Efflux pumps are able to transport specific substrates or expel cytotoxic compounds (6, 29). While there is evidence that efflux pumps are physiologically important (35), they commonly mediate MDR. This has led to the occurrence of microorganisms intrinsically resistant to multiple antibiotics (33). Our data support this claim, as resistance was found to ciprofloxacin, despite a seemingly absence of evolutionary pressures. Interestingly, more of our isolates exhibited resistance to penicillin than ampicillin, yet both are β-lactam antibiotics. This could be attributed to the fact that penicillin has natural origins whereas ampicillin is semi-synthetic. Overall, our results suggest that MDR in a bacterial population is a naturally occurring phenomenon. HGT of naturally occurring resistance genes to pathogens could also be a factor for the acceleration of resistance found in clinical and agricultural settings.

To characterize the diversity of the bacterial populations present in the different ecological zones, we analyzed colony morphology present on our non-selective media plates and selected antibiotic-resistant isolates to be sequenced. Previous research has shown a wide variety of taxonomic groups to have AR and MDR bacteria (31) and also that bacteria are capable of transferring AR genes either between bacteria of the same species or between different species (4). We found a high diversity of colony morphologies (Table 3) and identified six unique bacteria genera (Table 4) from a small subset of total colonies. Since all selected isolates were found to be multidrug-resistant, it is possible that HGT of AR genes occurs naturally at some frequency. These data support our hypothesis that unique bacteria would be found in the different ecological zones, and more importantly, that antibiotic-resistant bacteria can be from a highly diverse group.

Soil is rich in microbial abundance and species diversity. It has been estimated that a single gram of soil can have up to 10^{10} bacterial cells and more than 4×10^{3} different bacteria species (20, 34, 36, 40, 46, 47). In addition, bacteria populations can differ between geography and altitude (30). Using a site with seven adjacent ecological zones gave us the unique ability to directly compare both the prevalence and diversity of antibiotic-resistant organisms in these different ecosystems. We found a large range in the overall percentages of resistant organisms in each of the ecological zones. The highest percent resistant organisms were found in the spring (S), woodland (WL), and river (HR) zones. High percentages in the woodland areas could be due to availability of nutrients around the rhizosphere, an active region around a plant root that microorganisms inhabit (3). The river and spring zones could have high resistance from upstream pollution sources, especially since the Primmer research property is located within an agriculturally focused region. This is not surprising as other bacteria have been previously found to survive

in water for a long time, such as those from the genera ***Pseudomonas*** (38, 43).

In conclusion, the differences we found between the adjacent sites show that while there is a relationship between ecology, prevalence of antibiotic-resistant organisms, and types of bacteria present, multidrug-resistance was found in all sites and all types of bacteria tested and is likely more common in the environment than we thought. Additional surveillance of the resistome present in different ecological locations will likely be essential for developing novel antibiotic treatments.

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