Detection of Antibiotic Resistant Bacteria in the Gallinas River

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Laurel Carr confirming bacterial growth in the New Mexico Highlands University microbiology lab.

Introduction

This study addresses 3 questions:

1. Does antibiotic resistance (AR) increase in bacteria as you move down stream Gallinas River (GR), accruing human and agricultural impact?
    a. This will be answered by calculating the number of antibiotics isolates are found to be resistant to per site.
2. Does multidrug resistance increase in bacteria as you move down stream GR, with increasing human and agricultural impact?
    a. This will be answered by calculating the number of bacterial isolates resistant to each antibiotic per site.
3. Do antibiotic resistant bacteria (ARB) lose AR over time; specifically, in the amount of time it takes to obtain a pure culture?
    a. This will be answered by comparing bacterial resistance to antibiotics per isolate per antibiotic over time.

The goal of this study is to identify the level of antibiotic resistance in gram-negative bacteria isolated from water samples taken from six sites along the Gallinas River. Obtaining samples at the headwaters of the Gallinas watershed allows for comparison of human and agricultural influence from downstream samples. In addition, obtaining samples from the City of Las Vegas, NM Waste Water Treatment Plant (WWTP) allows for analysis of input/output AR values of human isolates. The hypothesis is that antibiotic resistance in gram-negative bacteria (likely *Enterobacteriacea)* isolates from the Gallinas River will increase as water collection sites move closer to human and agricultural influence.

The rise of antibiotic resistant bacteria (ARB) is of particular concern to the medical community as new antibiotic innovations have been slow to gain approval. There is concern that bacteria in the environment containing antibiotic resistant genes will laterally transfer these sections of genes to other bacteria. Providing pure and safe drinking water should include the impact of ARB. Continual or inappropriate exposures to antibiotics select bacteria resistant to antibiotics. Medical and agricultural use of antibiotics accounts for a large percentage of seepage into our water supply (Pruden et al., 2013). According to the World Health Organization,

discovery of new antibiotic classes has ceased since the mid 1980s. It is therefore important to preserve the efficacy of the antibiotics currently available. (WHO, 2014).

The Gallinas River (GR) is located on the eastern side of the southern Sangre de Cristo Mountains and is a tributary to the Pecos River watershed. Throughout its entirety, the GR is exposed to a varying amount of humans and livestock, with limited exposure at its headwaters to increased exposure below the outfall of the city's wastewater treatment plant. Six sites along the GR from the headwaters to beyond the City of Las Vegas WWTP were selected for testing AR of isolates (Figure 1).

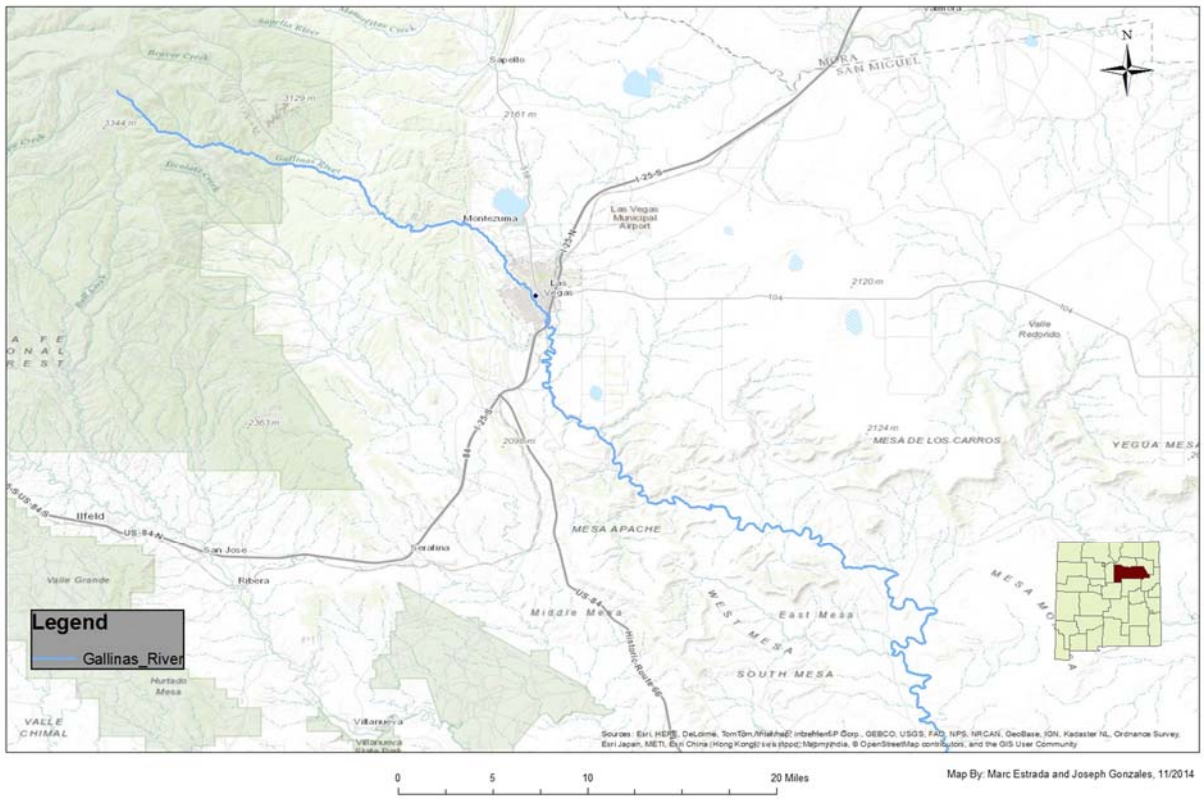

Figure 1. Map of Gallinas River (sites 1-6).

Site 1 (S1) is located above the Santa Fe National Forest boundary in the Pecos Wilderness and represents minimal to no human influence. Several samples over a span of several miles will be taken from feeder streams and the GR. Preexisting antibiotic resistance found in nature will be the primary causes in any resistance found in bacteria isolated from S1. Site 2 (S2) is downstream S1, located before the City of Las Vegas and represents any additional

AR found to be due primarily to agricultural introduction to the GR. Site 3 (S3) is downstream S2, located within the City of Las Vegas. Site 4 (S4) is affluent sewage flowing into the City of Las Vegas Waste Water Treatment Plant (WWTP). S4 represents a culmination of AR primarily from human isolates. S4 is prior to water treatment and reentrance into the GR. Site 5 (S5) is effluent water treated at the WWTP via UV exposure, prior to reentrance into the GR. Site 6 (S6) is downstream the WWTP reentrance pipe by at lease 250 feet. Several samples are taken from areas located beyond the WWTP to monitor changes in ARBs as a direct result of treated sewer (human influence). This data can be compared to other sites in order to isolate human influence. Table 1 provides a summary of S1-S6.

Table 1. Description of Study Sites

Site #	Location	Significance	Filter Size (mL)
1	Pecos Wilderness, SF National Forest	Headwaters, Uninfluenced	5-10
2	Montezuma (prior to City of Las Vegas, NM)	Agricultural Influence (additive of upstream)	5-10
3	City of Las Vegas	City/Human Influence (additive of upstream)	5-10
4	Affluent WWTP, City of Las Vegas, NM	Raw Sewage, Human Influence (isolated)	0.25-0.5
5	Effluent WWTP, City of Las Vegas, NM	UV Treated, Human Influence (isolated)	5-10
6	Downstream WWTP	UV Treated Reentrance into GR (additive of upstream)	5-10

Methods

This study was conducted through a series of procedures involving water sample collection and filtration, bacterial analysis, antibiotic resistance testing, and analysis of numerous subcultures (time-lapse) to measure AR over time (figure 2).

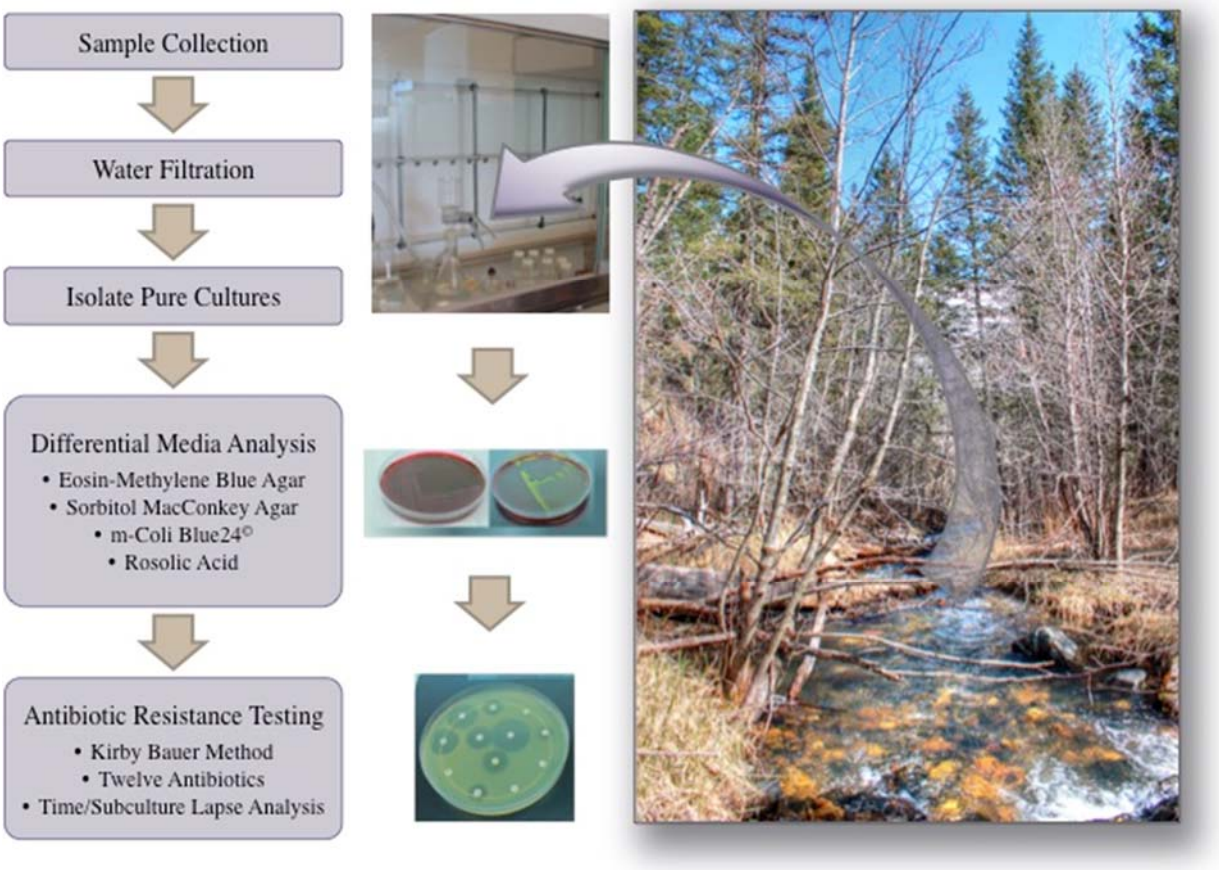

Figure 2. Experimental design depicting sample collection (S1 pictured), water filtration, differential media analysis, and antibiotic resistance testing.

Water Collection & Filtration

A minimum of 100mL of water was collected from each site from the riverbank or from the WWTP sewage lines into plastic collection bottles. All water samples were transported to the lab for processing within 10 hours of collection.

All water filtration was performed as per the *Standard Methods for the Examination of Water and Wastewater*, a joint publication of the American Public Health Association, the American Water Works Association, and the Water Environment Federation. (1998).

Within 10 hours of arrival to the laboratory, samples were filtered according to Table 1 using a 0.2-0.45 micron filter and a water filtration apparatus. After each filtration, 20 mL of distilled water was used to rinse down apparatus. Prior to filtration, apparatus was sterilized in the autoclave.

Bacterial Analysis

E. coli: m-ColiBlue24©:

E. Coli isolation via m-ColiBlue24© Broth (mC) was performed following EPA approved HACH Analytical Procedures (1999). Upon completion of filtration, following aseptic technique, filter paper was placed face up in a small petri dish containing a sterilized growth disc. One mC ampule was used to completely saturate filter. Petri dish was then incubated at 35°C ± 2°C for 18-24 hours. The petri dish was then removed from incubation and *E. coli* colonies were noted (*E coli* isolates appear blue in color, all other isolates fecal coliform appear pink in color).

Fecal Coliform:

Fecal coliform isolation via Rosolic Acid (RA) was performed following EPA approved HACH Analytical Procedures (1999). Upon completion of filtration, following aseptic technique, filter paper was placed face up in a small petri dish containing a sterilized growth disc. One RA ampule was used to completely saturate filter. Petri dish was then incubated at 35°C ± 2°C for 18-24 hours. The petri dish was removed from incubation and fecal coliform colonies were noted (fecal coliform isolates appear blue in color, all other isolates appear colorless).

Gram Negative & E. coli: EMB & SMAC:

Upon completion of filtration of water, following aseptic technique, filter paper was then placed face up in a petri dish containing Eosin-Methylene Blue agar (EMB) or Sorbitol-MacConkey agar (SMAC) and incubated at 35°C ± 2°C for 18-24 hours. Distinct colonies were then collected from the filter and streaked for isolation using aseptic technique on EMB and/or SMAC agar and again incubated at 35°C ± 2°C for 18-24 hours. This process was repeated until results indicate a pure culture of gram-negative bacteria. From the SMAC isolate, the presence of sorbitol-fermenting bacteria was confirmed via growth morphology (Figure 3). From the EMB isolate, the presence of lactose-fermenting bacteria was confirmed via growth morphology (Figure 4).

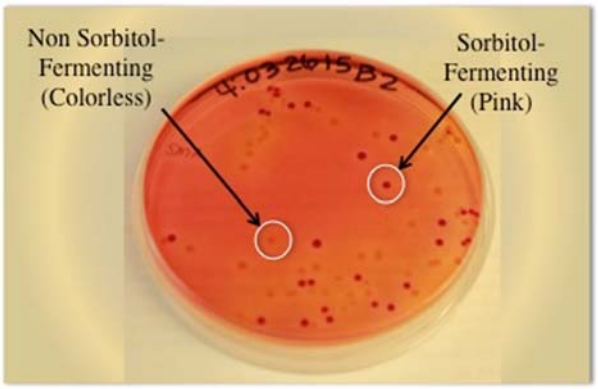

Figure 3. Growth Morphology of Sorbitol-Fermenting Bacteria on Sorbitol-MacConkey Agar.

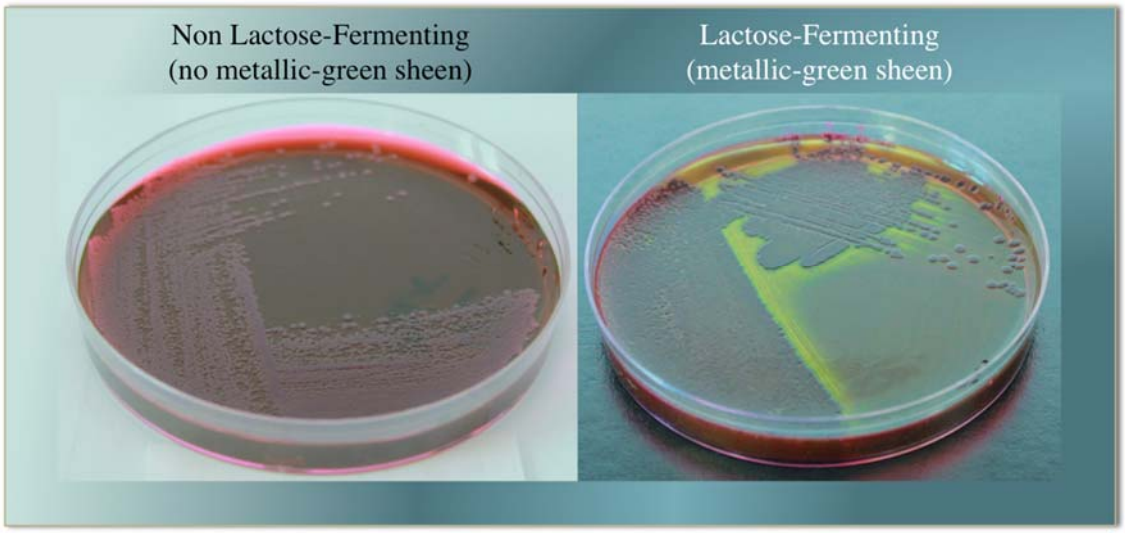

Figure 4. Growth Morphology of Lactose-Fermenting Bacteria (right) and Non Lactose-Fermenting Bacteria (left) on Eosin-Methylene Blue Agar.

Antibiotic Resistance Testing

Standard Kirby-Bauer technique was used for determination of antibiotic resistance. Upon successful isolation of gram-negative bacteria, colonies were plated onto Müeller-Hinton (MH) agar using an aseptic spread technique. A sterilized loop was used to gather the colony and then streak onto MH agar. A sterilized swab was then used to spread the streak across the entire plate. This swab was then placed into nutrient broth in preparation for preservation.

Antibiotic discs containing standardized concentrations of gentamicin (10 µg), oxacillin (1 µg), sulfamethoxazole/trimethoprim (23.75 µg/1.25 µg), erythromycin (15 µg), tetracycline (30 µg), cephalothin (30 µg), amoxicillin/clavulanic acid (30 µg), ceftiofur (30 µg), penicillin (10

IU/IE/UI), ciprofloxacin (5 µg), clindamycin (2 µg), and vancomycin (30 µg) were pressed into the agar using an antibiotic dispenser. Plates and swabs were then incubated at 35°C ± 2°C for 18-24 hours. Zone diameters were measured in millimeters (mm) using a ruler (Table 2). Susceptibility zones were recorded following recommended guidelines as published in the *Performance Standards for Antimicrobial Susceptibility Testing* and the BD BBL™ Sensi-Disc™ Antimicrobial Susceptibility Test Discs Inserts (CLSI, 2012 & BD BBL, 2007). Plates and swabs were then moved to the refrigerator to inhibit further growth.

Table 2. BD BBL™ Sensi-Disc™ Antimicrobial Susceptibility for Enterobacteriaceae (Zone Diameter, nearest whole mm). ****Gram positive susceptibility zones utilized.***

		Enterobacteriaceae Zone Sizes		
Antibiotic	**Symbol**	**Resistant**	**Intermediate**	**Susceptible**
amoxicillin/clavulanic acid 30 µg	AMC30	≤ 13 mm	14-17 mm	≥ 18 mm
ceftiofur 30 µg	XNL	≤ 17 mm	18-20 mm	≥ 21 mm
cephalothin 30 µg	CF 30	≤ 14 mm	15-17 mm	≥ 18 mm
ciprofloxacin 5 µg	CIP5	≤ 15 mm	16-20 mm	≥ 21 mm
clindamycin* 2 µg	CC 2	≤ 14 mm	15-20 mm	≥ 21 mm
erythromycin* 15 µg	E15	≤ 13 mm	14-22 mm	≥ 23 mm
gentamicin 10 µg	GM10	≤ 12 mm	13-14 mm	≥ 15 mm
penicillin* 10 IU/IE/UI	P10	≤ 14 mm	NA	≥ 15 mm
oxacillin* 1 µg	OX1	≤ 10 mm	11-12 mm	≥ 13 mm
sulfamethoxazole/trimethoprim 23.75 µg/1.25 µg	SXT	≤ 10 mm	11-15 mm	≥ 16 mm
tetracycline 30 µg	TE30	≤ 14 mm	15-18 mm	≥ 19 mm
vancomycin 30 µg	VA30	≤ 14 mm	15-16 mm	≥ 17 mm

Time-Lapse Analysis

Concurrently, once a pure culture was obtained, random isolates were then be repeatedly regrown and preserved in the refrigerator in order to compare resistance patterns over time by referencing initial resistance patterns on MH agar. This was repeated for a variety of different time lapses/isolate subcultures.

Results

Results shown thus far are in support of the hypothesis; however, additional isolates are required for sufficient statistical determination. The goal of this study, in order to support statistical deviation, is to collect 25-50 isolates from each site. Figure 5 indicates increased levels of AR due to human influence (isolated sewage) in comparison to uninfluenced headwaters. Antibiotic resistance and multidrug resistance increased from Site 1 to Site 4. As more samples are collected from Site 6, direct correlation can then be reported with human impact on AR in the Gallinas River.

Figure 6 depicts the increase in antibiotic resistance per site. The number of isolates (n) should be noted when interpreting this graph, especially for sites 2 and 6, which have low n values. Figure 6 provides a trend of increasing AR moving downstream.

Of the isolates identified for time-lapse analysis thus far, there has been no indication of isolates losing resistance over time.

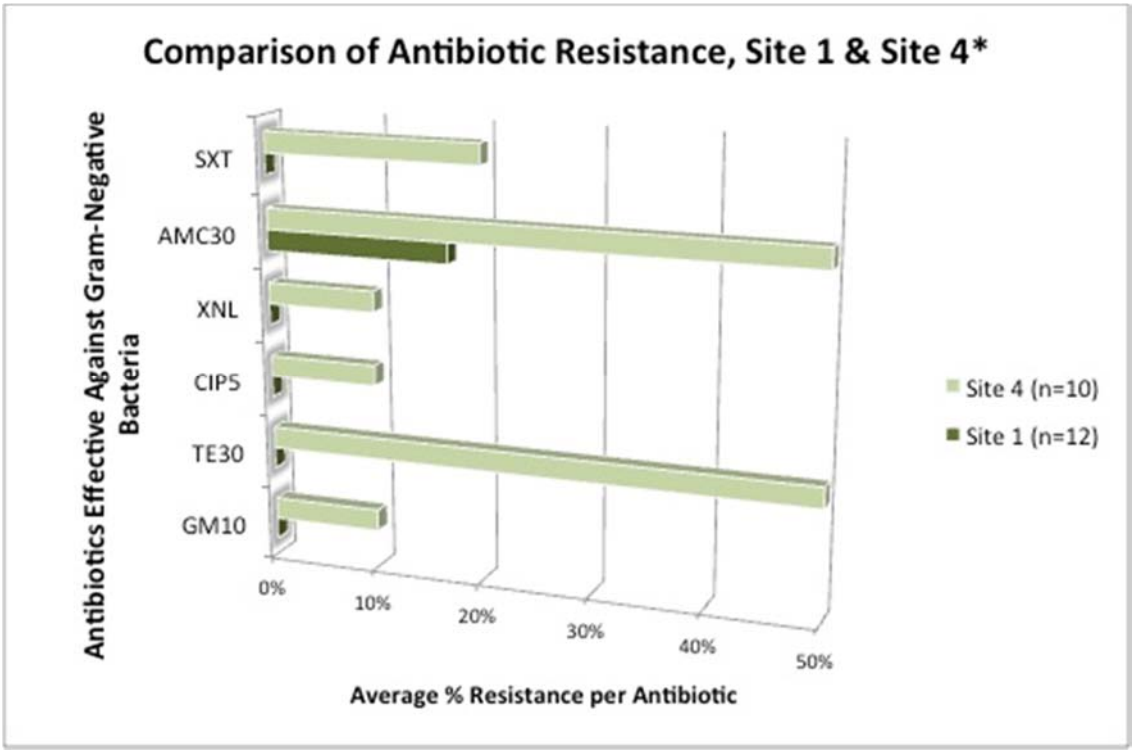

Figure 5. Differences in antibiotic resistance between Site 1 (headwaters, uninfluenced) and Site 4 (affluent WWTP, human influence). **Excluding AB effective primarily against gram-positive bacteria. 6 of the 12 antibiotics tested shown to be effective against gram-negative bacteria.*

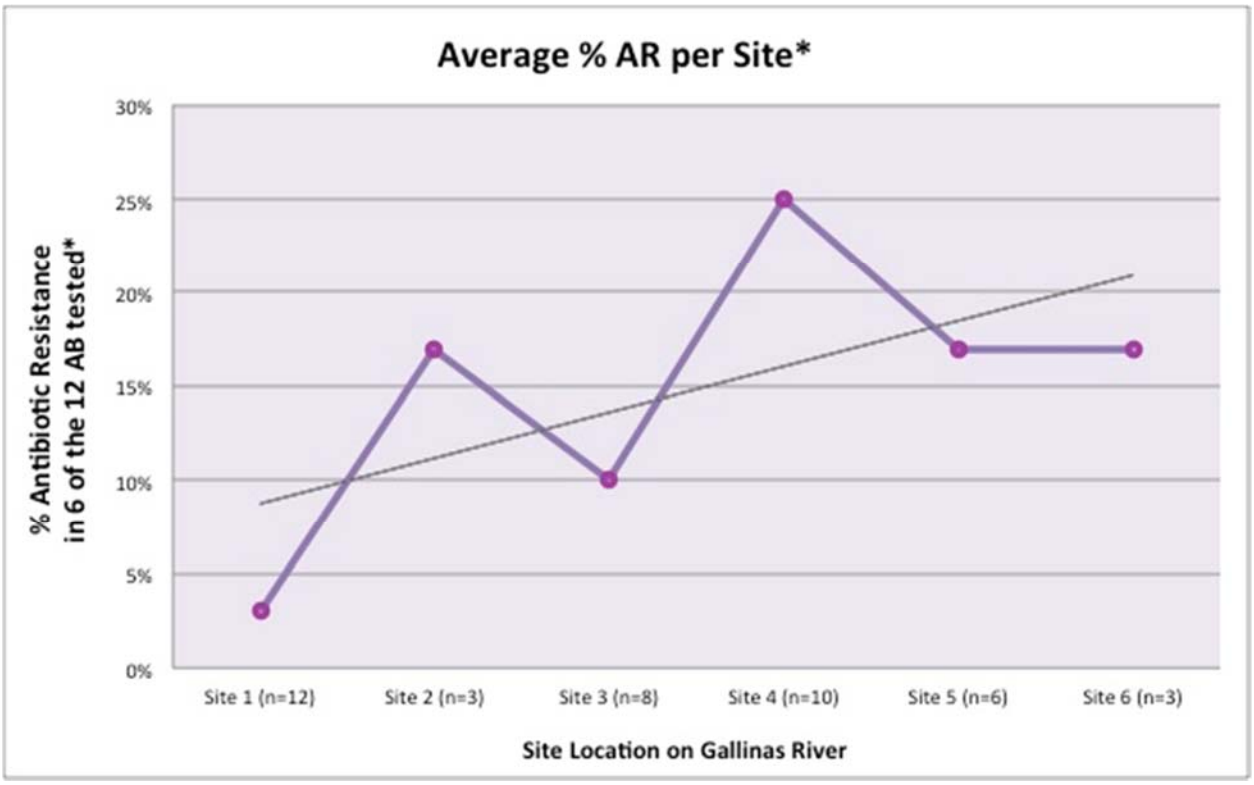

Figure 6. Average percentage of antibiotic resistance found in isolates per site. **Excluding AB effective primarily against gram-positive bacteria. 6 of the 12 antibiotics tested shown to be effective against gram-negative bacteria.*

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Beneficiaries of Research

The City of Las Vegas, NM and other municipalities may benefit from the results of this research, as well as water agencies such as the New Mexico Water Resources Research Institute and the University Counsel of Water Resources. The published results will be made available to those in research and will provide a better understanding of AR found in watershed areas with minimal to no exposure to human or agricultural influence to the general scientific community. This model of research could be of interest to the medical community as a plausible means for monitoring prescription cycles of AB classes. Water treatment facilities worldwide should be aware of the effectiveness of water treatment techniques (i.e. UV exposure, chlorination, etc.) in treating ARB in order to collaborate with community health and environmental programs designed to protect the interests of public health.

Poster Presentations

- 59th Annual New Mexico Water Conference, NMWRRI, Santa Fe, NM (11/19/15).
- First International Conference on Antibiotic Resistance, Lisbon, Portugal (01/26/15-01/28/15).
- New Mexico Highlands University Research Day (04/10/15).
- University Counsel on Water Resources, Las Vegas, NV (06/15/15-06/18/15).
- *Planned:* 60th Annual New Mexico Water Conference, NMWRRI, Taos, NM (10/08/15-10/09/15).

Publications

- NMHU Thesis, May 2016
- Report to NMWRRI, June 2015
- Report to the City of Las Vegas, May 2016
- Submission of Summary to the *Journal of Contemporary Water Research and Education - University Council on Water Resources*, Planned (not yet submitted)
- Submission of Summary to the *Journal of Global Antimicrobial Resistance*, Planned (not yet submitted)

- Submission of Summary to *The New England Journal of Medicine*, Planned (not yet submitted)

Other students or faculty members who assisted on the project

- NMHU Faculty: Dr. Edward Martinez for guidance with the Gallinas watershed and support with water collection techniques.
- NMHU Students: Mr. Joseph Gonzales & Mr. Marc Estrada for GIS site map contribution and Miss Rachael Lucero for showing me the ropes.
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Special recognition, awards and notable achievements

- NMHU News, December 2014
- Las Vegas Optic, Front Page, December 2014
- New Mexico Water eNews Newsletter, March 2015

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