

# DETECTION OF ANTHROPOGENIC ANTIBIOTIC RESISTANCE INTRODUCED INTO THE GALLINAS RIVER OF LAS VEGAS, NEW MEXICO

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Laurel Carr confirming bacterial growth in the New Mexico Highlands University microbiology lab and collecting water samples from the Gallinas River headwaters located in the Pecos Wilderness.

## Abstract

Understanding the origins of antibiotic resistance is pivotal to decreasing the global health threat of ineffective and insufficient antibiotic treatment. There is much publicity surrounding the impact zoonotic antibiotic resistance has upon human health and lesser amounts of speculation regarding anthropogenic antibiotic resistance (AR) that could, in turn, exacerbate treatment of bacterial infections in humans. This study used the Kirby-Bauer method to compare bacterial resistance in 160 gram-negative isolates from water samples taken from sites along the Gallinas River (GR) and the Las Vegas, New Mexico municipal waste water treatment plant (LVWWTP) influent and effluent. Samples taken in the wilderness from the headwaters of the GR, situated in the Santa Fe National Forest (Site 1), represent sources of bacteria that have minimal-to-no exposure to humans or domestic animals. Sites 2 and 3, taken consecutively downstream Site 1, represent additive human and domestic exposure prior to the LVWWTP. All AR found in sample sites from the LVWWTP influent and effluent (Sites 4 and 5), before treatment and reentering the GR, were identified as anthropogenic. Samples from Site 6, taken approximately 20 meters downstream the LVWWTP effluent, represent combined anthropogenic and environmental contribution of antibiotic-resistant bacteria. Antibiotic-resistant bacteria were isolated from all sites. Samples taken from Site 1 showed the least amount (8%) of resistance, while the highest amount of resistance was found in Site 4 and Site 5 (19% and 15%, respectively). Samples taken from Site 6 showed a statistically significant increase in AR compared to samples taken from Site 1 ( $p < 0.05$ ); whereas, AR patterns between Site 2 and Site 4 showed no statistical differences. Multidrug resistance to three or more antibiotics per bacterial isolate increased in subsequent sites downstream, with statistically significant differences between Site 1 and Site 6 (0% and 18%, respectively;  $p < 0.05$ ). These data suggest anthropogenic introduction of antibiotic-resistant bacteria.

## Introduction

This study addresses four questions:

1. Does antibiotic resistance (AR) increase in bacteria from subsequent sampling sites downstream Gallinas River (GR)?
2. Does multidrug resistance increase in bacteria from subsequent sampling sites downstream GR?
3. Are anthropogenic antibiotic-resistant bacteria introduced into the GR after the wastewater treatment?
4. Are anthropogenic multiple antibiotic-resistant bacteria introduced into the GR after the wastewater treatment?

The goal of this study is to compare antibiotic resistance patterns of bacteria isolated from the headwaters of the Gallinas Watershed to those taken downstream and from the LVWWTP. Obtaining samples at the headwaters of the Gallinas watershed allows for comparison of environmental influence on antibiotic-resistant bacteria. In addition, obtaining samples from the City of Las Vegas, NM Waste Water Treatment Plant (LVWWTP) allows for analysis of input/output AR values of human isolates.

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. 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). Continual or inappropriate exposure to antibiotics lead to the evolution of ARB. Medical and agricultural use of antibiotics accounts for a large percentage of seepage into the water supply (Pruden et al., 2013). Treated municipal wastewater that enters the environment and becomes available for agricultural use should be a consideration of anthropogenic contamination, as there is concern for the spread of ARB from agriculture into human populations.

There is much attention given to identifying agricultural contribution of antibiotic resistance to human populations, but a lesser amount is reserved to study anthropogenic sources of antibiotic resistance. Organizations, such as the Alliance for the Prudent Use of Antibiotics (APUA) and the Centers for Disease Control (CDC), strive to raise public awareness about antibiotic resistance by conducting research and disseminating information on how to control and monitor antibiotic resistance. Consideration of anthropogenic resistance may be of use in their repertoire of reporting measures. For instance, the directionality of antibiotic resistance, as published on websites hosted by the APUA and CDC, does not mention water containing municipal waste entering the agricultural or environmental cycle that could then exacerbate the problem (APUA, 2017; CDC, 2015). Often, the spread of antibiotic resistance is pictured unidirectional, with the cause of human resistance clearly pointing at agricultural contamination. There is little published regarding a bidirectional cycle, rather than linear and unidirectional, that humans could be the source of diversity and prevalence found in zoonotic antibiotic resistance.

## **Methodology**

This study was conducted through a series of procedures involving water sample collection and filtration, bacterial analysis, and antibiotic resistance testing (Figure 1).

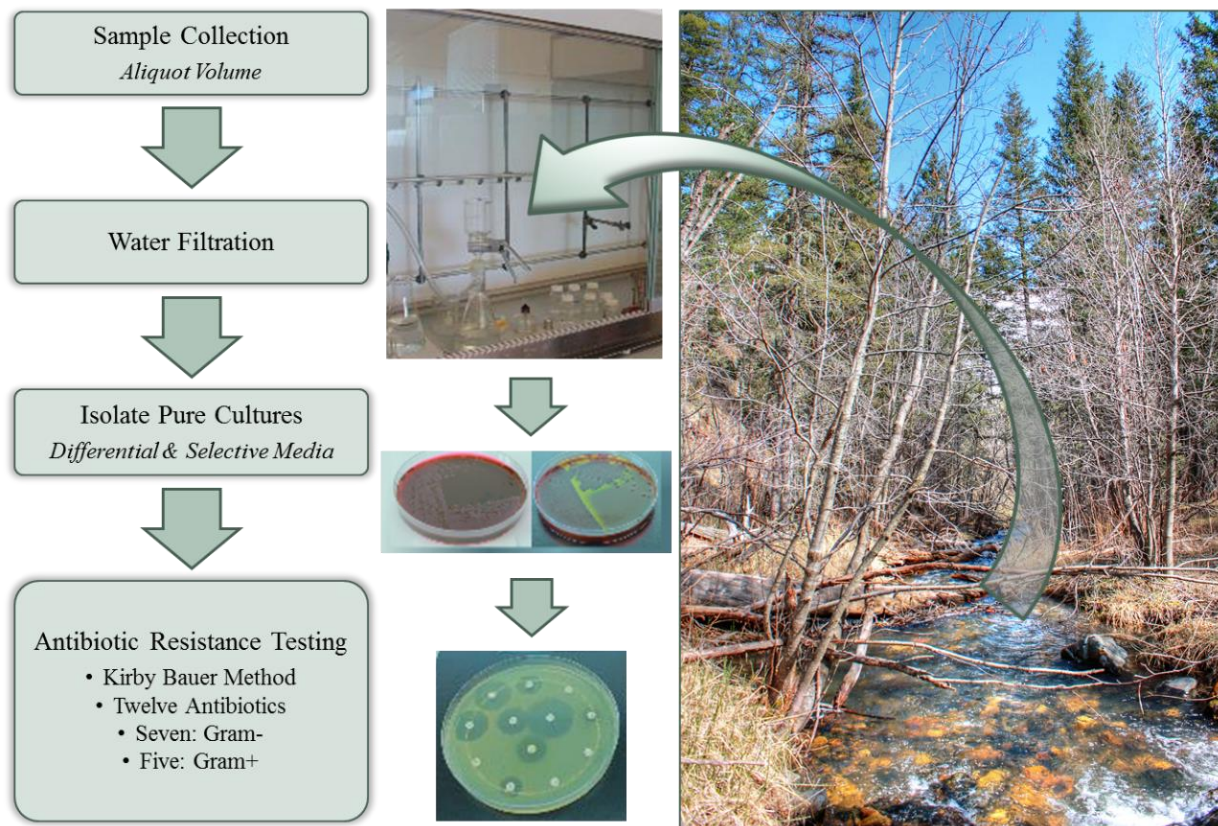


Figure 1. Experimental Design: depicting sample collection (Site 1 pictured), water filtration, differential media analysis, and antibiotic resistance testing.

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 at the Las Vegas city limits and below the outfall of the LVWWTP. Six sites along the GR from the headwaters to beyond the LVWWTP were selected for water sampling and AR testing of bacterial isolates (Figure 2). Table 1 provides a brief description and the significance of each sampling site.

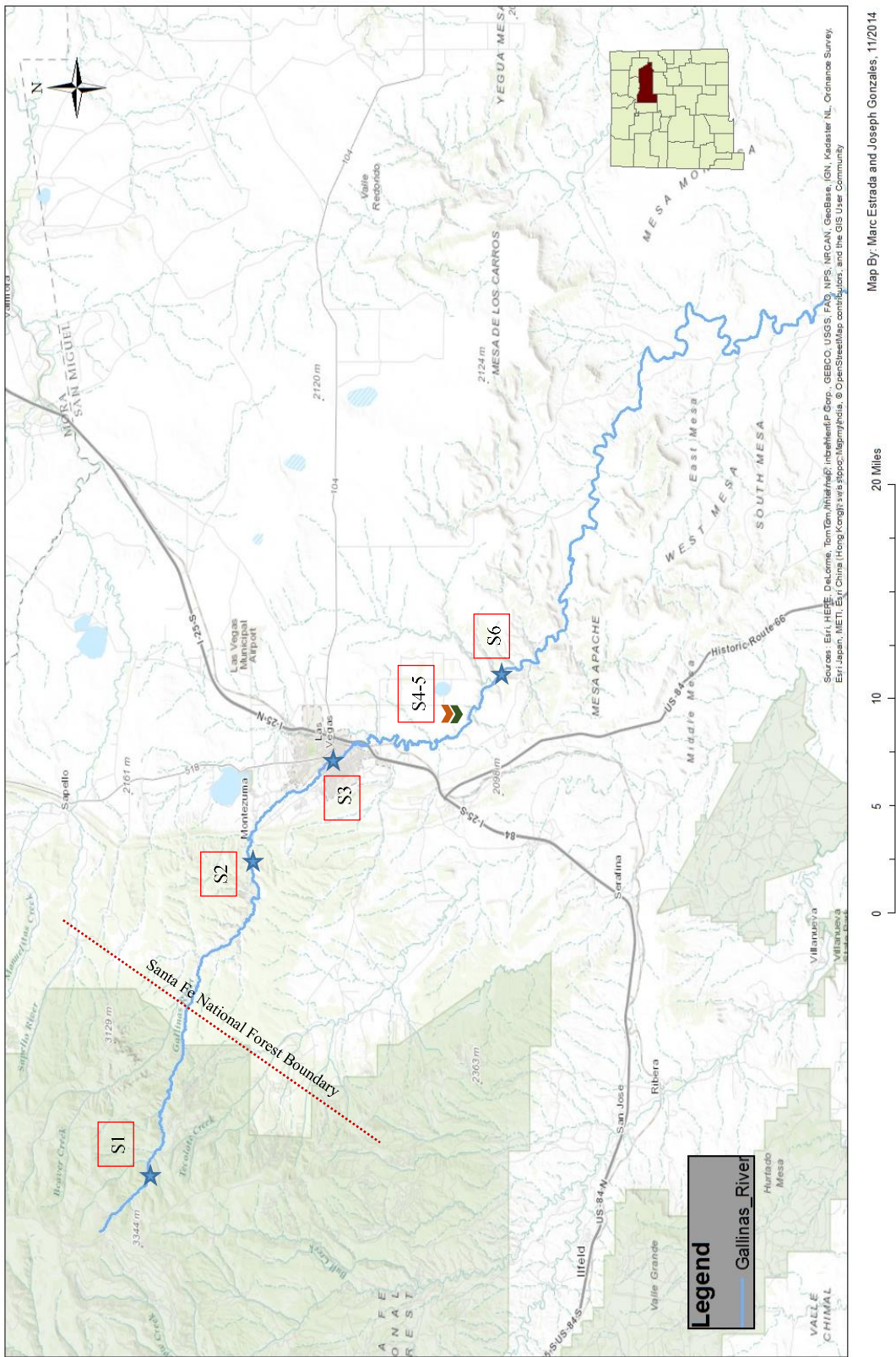


Figure 2. Map of Gallinas River (Sites 1-6)

Table 1. Description of Study Sites

| Site # | Location                                       | Significance   | Aliquot Volume (mL) |
|--------|--|--|---------------------|
| S1     | Pecos Wilderness, Santa Fe National Forest     | Isolated Headwaters, Uninfluenced                      | 20-40               |
| S2     | Montezuma (prior to City of Las Vegas, NM)     | Additive Human and Possible Agricultural Influence     | 15-30               |
| S3     | City of Las Vegas, NM                          | Additive Human Influence                               | 5-10                |
| S4     | Municipal Waste, City of Las Vegas, NM         | Isolated Raw Sewage, Anthropogenic Source              | 0.1-0.25            |
| S5     | Effluent Las Vegas Waste Water Treatment Plant | Isolated UV Treated, Anthropogenic Source              | 1-2.5               |
| S6     | Downstream LVWWTP                              | Additive UV Treated Reentrance into the Gallinas River | 2.5-5               |

#### Water Collection & Filtration

Water was aseptically collected from each site from the riverbank or from the LVWWTP sewage lines into collection bottles over a period of months ranging from October 2014-June 2017. Upon arrival to the laboratory, samples were filtered according to Table 1 using a sterile 0.2 or 0.45 micron filter and a water filtration apparatus (APHA, AWWA & WEF, 1998).

#### Bacterial Analysis

*E. coli* and Coliform: m-ColiBlue24<sup>®</sup> Broth

*E. coli* isolation via m-ColiBlue24<sup>®</sup> Broth (mCB) was performed following EPA approved HACH Analytical Procedures (1999). Upon completion of filtration, filter paper was placed in a small petri dish containing a sterilized growth disc. One mCB ampule was used to completely saturate filter and petri dish and 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 appeared blue in color, all other bacterial isolates were identified as coliform and appeared pink in color). As a means of multi-test verification, all bacterial isolates tested from mCB filters were plated to EMB, concurrently, in order to confirm morphology identification.

Gram-Negative Coliform & *E. coli*: Eosin-Methylene Blue

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) 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 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 EMB bacterial isolate, the presence of lactose-fermenting bacteria was confirmed via growth morphology (Figure 3).

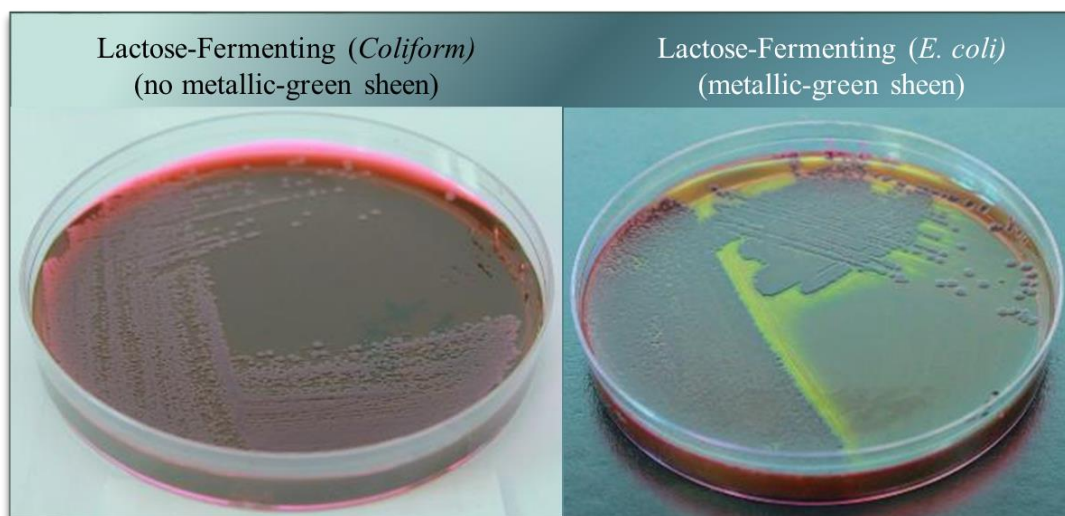


Figure 3. Growth Morphology of Lactose-Fermenting Bacteria on Eosin-Methylene Blue Agar

#### Antibiotic Resistance Testing

Standard Kirby-Bauer technique was used for determination of antibiotic resistance (Bauer et al., 1966). Upon successful isolation of gram-negative bacteria, colonies were plated onto prepared Mueller-Hinton (MH) agar using an aseptic spread technique.

Commercially prepared antibiotic discs containing standardized concentrations of gentamicin 10 $\mu$ g (GM10), oxacillin 1 $\mu$ g (OX1), sulfamethoxazole/trimethoprim 23.75 $\mu$ g/1.25 $\mu$ g (SXT), erythromycin 15 $\mu$ g (E15), tetracycline 30 $\mu$ g (TE30), cephalothin 30 $\mu$ g (CF30), amoxicillin/clavulanic acid 30 $\mu$ g (AMC30), ceftiofur 30 $\mu$ g (XNL), penicillin 10IU/IE/UI (P10), ciprofloxacin 5 $\mu$ g (CIP5), clindamycin 2 $\mu$ g (CC2), and vancomycin 30 $\mu$ g (VA30) were pressed into the agar using an antibiotic disc dispenser. Plates and swabs were then incubated at 35°C  $\pm$  2°C for 18-24 hours. Zone diameters were measured in millimeters (mm) using a ruler. 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).

#### **Results and Conclusion**

##### Antibiotic Resistance

One hundred and sixty bacterial isolates were obtained from six sites located along the Gallinas Watershed and from the Las Vegas Waste Water Treatment Plant and were considered for this study. Of the 12 antibiotics tested, seven are recognized to be effective against gram-negative bacteria and

*Enterobacteriaceae* susceptibility zones were used to measure resistance: GM10, TE30, CIP5, XNL, AMC30, CF30, and SXT. *Enterococci* and gram-positive susceptibility zones were used to measure the remaining five antibiotics: OX1, P10, VA30, E15, and CC2.

The percentages of bacterial isolates displaying resistance per Site, as seen in Figure 4, below, are limited to the seven antibiotics based on gram-negative susceptibility zones. The increase in resistance from Site 1 to Site 4 and from Site 1 to Site 6 is statistically significant ( $p < 0.05$ ).

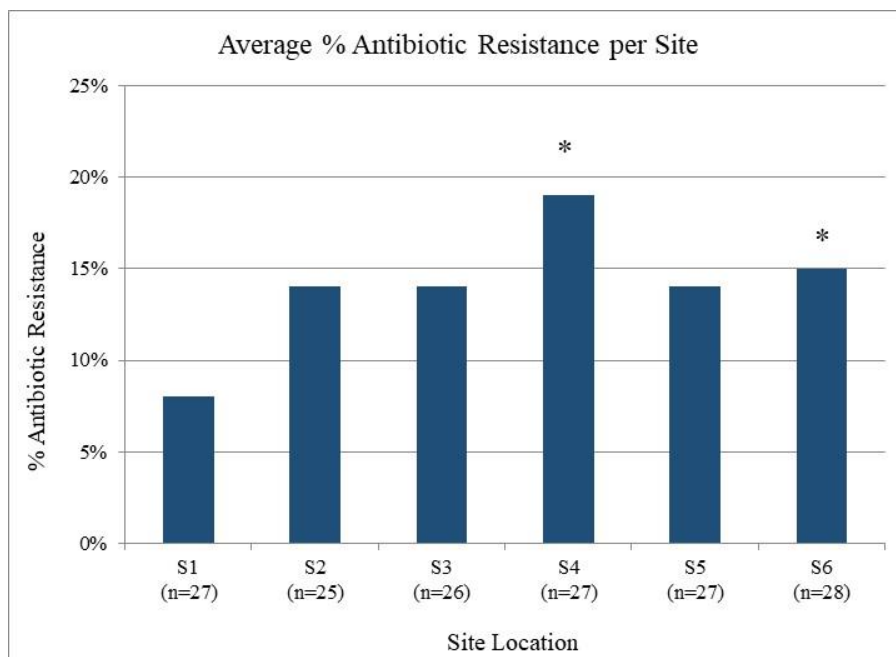


Figure 4. Average % Antibiotic Resistance per Site, (\* $p < 0.05$  vs. Site 1)

At least one bacterial isolate from the entire data pool showed resistance to each antibiotic, however, some samples taken displayed no resistance patterns. The highest resistance exhibited was to ceftiofur (54%) at Site 3, followed closely by amoxicillin clavulanic acid (41%) at Site 4. There were no significant differences in resistance towards XNL or AMC between sites. Figure 5 indicates significant increased levels of AR due to human influence (isolated sewage) in bacterial isolates sampled from Site 4 in comparison to Site 1. Antibiotic resistance and multidrug resistance increased from Site 1 to Site 4. Antibiotic resistance increased in Site 6 in comparison to Site 1, as shown in Figure 6. Figure 7 shows Site 4 and Site 5, combined, to isolate human impact on AR entering the Gallinas River.



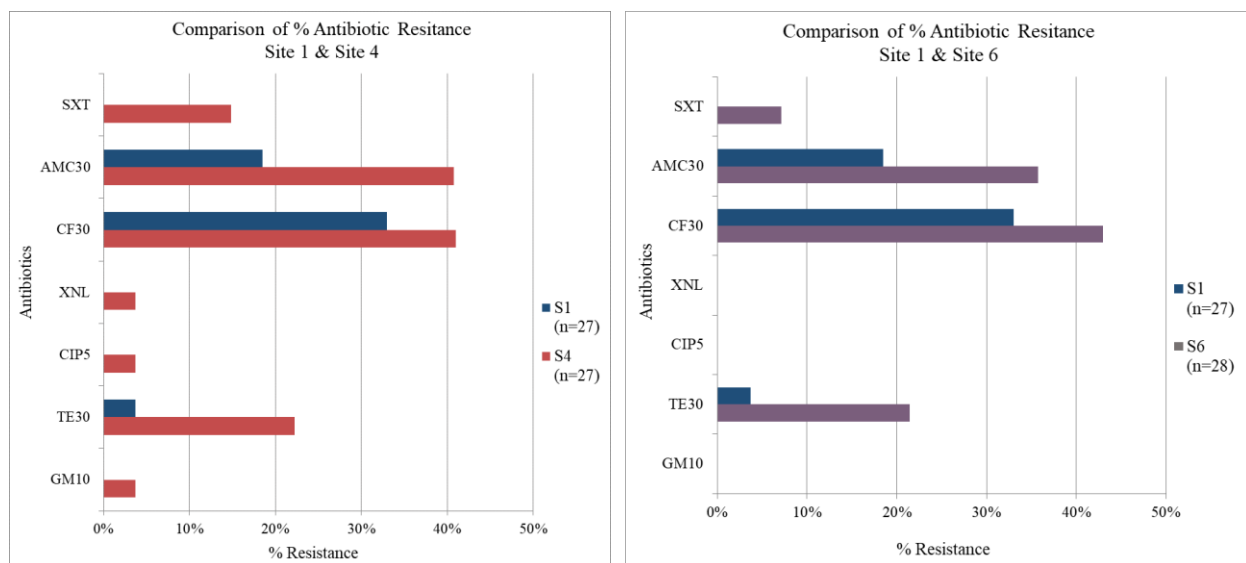


Figure 5 (left). Differences in Antibiotic Resistance between Site 1 and Site 4,  $p < 0.05$ ; Figure 6 (right). Differences in Antibiotic Resistance between Site 1 and Site 6,  $p < 0.05$

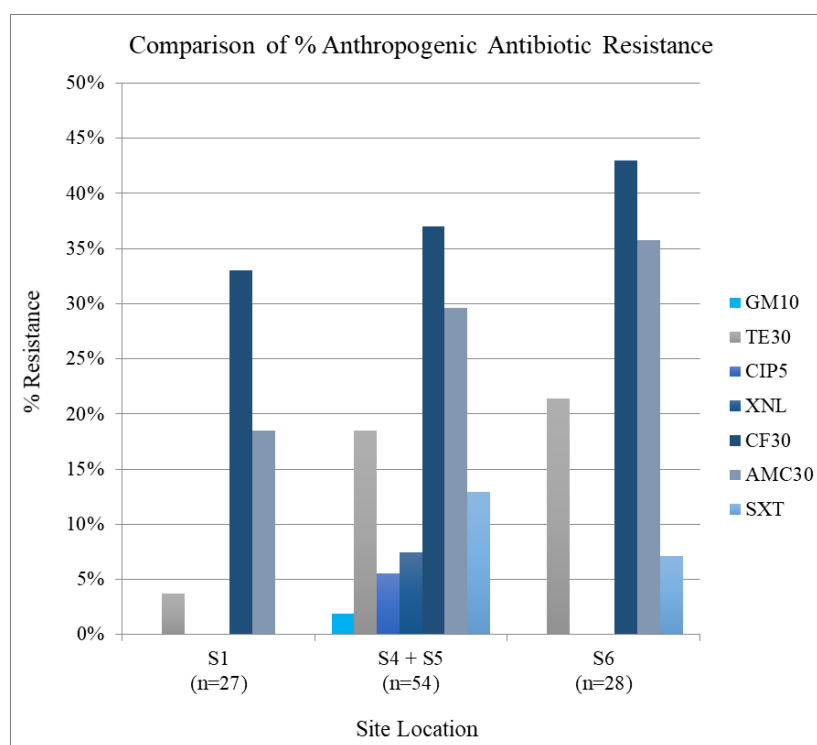


Figure 7. Comparison of Anthropogenic Antibiotic Resistance: Site 1, Sites 4 and 5, and Site 6

### Multiple Antibiotic Resistance

Most of the resistant bacterial isolates showed resistance to multiple antibiotics. Of the bacterial isolates tested ( $n=160$ ), 75 (47%) were sensitive to all antibiotics tested, 85 (53%) were resistant to at least one antibiotic, and, 50 (59%) of those isolates were resistant to two or more antibiotics (Table 2).

For this study, bacterial isolates that depicted resistance to three or more antibiotics were considered to display multiple antibiotic resistance (MAR). The increase in multiple antibiotic resistance to three or more antibiotics between Site 1 and Site 6 was significant. There were zero of the 27 (0%) bacterial isolates tested from Site 1 that showed resistance to three or more antibiotics and five of the 27 (18%) bacterial isolates tested from Site 6 that showed resistance to three or more antibiotics. Figure 8 shows the percentage of bacterial isolates found to display MAR to three or more antibiotics per Site.

| Sites (n) | No Antibiotics (%) | One+ AB (%) | Two+ AB (%) | Three+ AB (%) |
|-----------|--------------------|-------------|-------------|---------------|
| S1 (n=27) | 18 (67%)           | 9 (33%)     | 6 (22%)     | 0 (0%)        |
| S2 (n=25) | 10 (40%)           | 15 (60%)    | 8 (32%)     | 2 (8%)        |
| S3 (n=26) | 10 (38%)*          | 16 (62%)*   | 8 (31%)     | 1 (4%)        |
| S4 (n=27) | 11 (41%)           | 16 (59%)    | 11 (41%)    | 3 (11%)       |
| S5 (n=27) | 13 (48%)           | 14 (52%)    | 8 (30%)     | 2 (7%)        |
| S6 (n=28) | 13 (46%)           | 15 (54%)    | 9 (32%)     | 5 (18%)*      |
| Totals    | 75 (47%)           | 85 (53%)    | 50 (31%)    | 13 (8%)       |

Table 2. Percentages of Susceptibility and Resistance per Bacterial Isolate by Site, (\* $p < 0.05$  vs. Site 1)

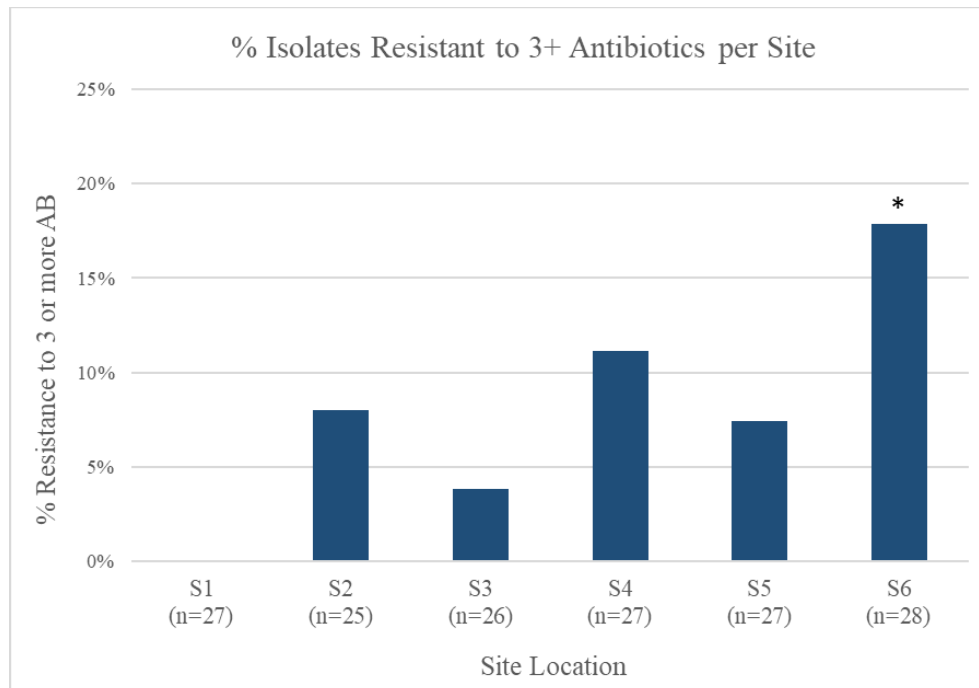


Figure 8. Percentage of MAR in Bacterial Isolates per Site, (\* $p < 0.05$  vs. Site 1)

The results obtained from this study showed statistically significant increases in AR between Site 1 and Site 6, suggesting that both antibiotic resistance and multiple antibiotic resistance increased in the environment as a direct result of treated human influence on the Gallinas River. This study indicated an overall trend of increased antibiotic resistance and multiple antibiotic resistance in gram-negative bacterial isolates tested from subsequent sampling sites downstream as human influence increased.

Considerations for continued research include decreasing the number of sample sites while increasing the number of samples per site to provide more statistically relevant data, exploring the sole use of m-ColiBlue24© Broth to isolate *E. coli*, and genotyping isolates in order to verify bacterial strain and identify antibiotic resistant genes. As this study did not fully explore sorbitol-fermenting bacteria, further studies could be conducted in order to differentiate the occurrence of pathogenic *E. coli* (O157:H7) introduced into the environment from human sources (WWTP outfall).

## References

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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 and water treatment facilities worldwide as a plausible means for monitoring prescription cycles of AB classes.

### **Poster Presentations and Publications**

- 59<sup>th</sup> Annual New Mexico Water Conference, NMWRRRI, 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).
- NMHU Thesis, September 2017
- Journal Submission: In Progress

### **Special Recognition, Awards, and Notable Achievements**

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

### **Acknowledgements**

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