Survival of carbapenem resistance through wastewater treatment

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Abstract

Background: Wastewater treatment plants (WWTPs) are excellent locations for the development and dissemination of antibiotic resistance (AR) genes and bacteria due to the presence of subtherapeutic levels of antibiotics and other environmental stressors, such as heavy metals and UV radiation.[1–4] Additionally, methods of treatment that are widely employed by WWTPs do not prevent the release of AR genes or bacteria into the environment where AR genes have been isolated from native environmental bacteria.[5–8]

Methods: Samples were taken over a five-month course at the Socorro Municipal WWTP at the influent, aerobic digester, and effluent. Samples were also taken from the low-flow channel into which the WWTP discharged the treated effluent. DNA was extracted from these samples and classical PCR was used to determine the presence of six β-lactamase and carbapenemase genes.

Results: Genes encoding *Klebsiella pneumoniae* carbapenemase (*bla*$_{KPC}$), Gianna extended spectrum β-lactamase (*bla*$_{GES}$) were detected in nearly all samples collected within the WWTP, influent and effluent appeared to be most enriched in the GES genes. *bla*$_{GES}$ and genes encoding Imipenemase (*bla*$_{IMP}$) were detected routinely in effluent and samples collected upstream from the WWTP. *bla*$_{GES}$ was the only gene consistently detected in samples collected downstream from the WWTP.

Conclusions: The methods used by the Socorro WWTP do not entirely remove AR bacteria or genes from the treated wastewater, but instead may select for resistant organisms.

Introduction

Wastewater treatment plants (WWTPs) have often been suspected of harboring and distributing antibiotic resistance into the environment in the forms of mobile genetic elements carrying antibiotic resistance (AR) genes and resistant bacteria, which often survive all but the most intensive treatments. Multiple studies have shown evidence that commonly used treatment methods could select for resistant bacteria, with one study reporting antibiotic resistance profiles in as many as 90% of the bacteria in their treated effluent samples.[9–13] Antibiotic resistance is likely to develop or evolve and proliferate in environments where bacteria are present in a diverse and dynamic populations and are exposed to multiple selective pressures. WWTPs provide an environment conducive to both of these traits, which bring together a dense and varied population of bacteria from many different human and natural origins in an environment where they are exposed to heavy metals, UV radiation, and sublethal doses of multiple antibiotics.[5,9,14–16] Many of the bacteria that are present within WWTPs are rod-shaped (bacillus) and stain Gram-negative and belong either to the Enterobacteriaceae family, which include thousands of common environmental and commensal gut bacteria, and various phyla of common environmental bacteria.[1,17–22] Gram-negative bacilli have become important players in the evolution and dissemination of AR genetic factors due note due to their ability to spread AR genes with a very wide range of similar bacteria via horizontal gene transfer (HGT) through physical contact between two bacteria (conjugation) or through infection by bacteriophage (transduction).[23–25] Antibiotic resistance is a growing concern in public health and safety, but how AR evolves in a microbial community and the dynamics of AR through human communities and the natural environment are not well understood. WWTPs are optimal environments to monitor and study due to their role at the borders of these two worlds.[26–30]

Carbapenem antibiotics are one of the last resort options for treating infections caused by highly resistant Gram-negative bacilli, and increasing microbial resistance to carbapenems and other last line of defense drugs is an urgent concern. Carbapenems are β-lactam antibiotics, so named for their 4:5 member β-lactam active groups, but with an additional ability to inhibit β-lactamases, the bacterial enzymes produced primarily by Gram-negative bacilli to hydrolyze β-lactam antibiotics.[31,32] These powerful drugs are losing their potency as bacteria produce β-lactamases with a broader spectrum of activity against them. Many within the academic community advocate for surveillance of locations known to produce β-lactamases and act as hotspots for HGT.[27,33–39] Currently, the Centers for Disease Control (CDC) monitor carbapenem resistance through surveillance of clinical infections through satellite efforts set up in every US state. There are three major problems with this program, the first being that since the CDC's resources are limited, they can only monitor one city in less populated states, for example, the CDC field office in New Mexico primarily monitors Albuquerque; the second being that they only monitor clinical isolates from reported infections, not potential sources of β-lactamases like WWTPs; the third problem is that the CDC can only screen for five extended-spectrum β-lactamases and carbapenemases that have been determined to be the most clinically significant in the US. These genes are the "Big Five" *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{OXA-48}$, and *bla*$_{KPC}$.[40]

Because antibiotic resistance is so common and WWTPs likely play a significant role in multiple aspects of AR, the purpose of this study was to determine whether or not clinically significant AR genes could be found at the Socorro Municipal WWTP. The Socorro WWTP serves all of Socorro County NM, and as such receives wastewaters from the general hospital, domestic sewage, and industrial, agricultural, and storm run-off, making it an excellent source of diverse Gram-negative bacilli and different selection factors. This WWTP utilizes extended aeration aerobic digester, secondary digestion and settling, and a final disinfection with sodium hypochlorite before treated wastewater is discharged. The Socorro Municipal WWTP is an excellent example of an unmonitored but potentially significant source of AR into the environment.

Our study sought to determine whether the important carbapenemase and extended spectrum β-lactamase genes that the CDC monitors in the clinic could be found in samples taken from this WWTP. Apart from the "Big Five," one additional gene was added to the testing group: bla_{GES}, which encodes GES (Gianna extended spectrum β-lactamase). This enzyme, while considered to be rare and of little clinical significance in the United States, is interesting in that it exemplifies the dynamic nature of the extended spectrum β-lactamases. GES has over 20 different genetic variants and only around half are known to produce an enzyme capable of hydrolyzing carbapenems.[39,41–44] One reason bla_{GES} was included is that it is often isolated with additional AR genes on the same mobile genetic elements and can be expressed in tandem with those AR factors.[43–46] Additionally, there is evidence that bla_{GES} may be becoming a significant concern in the Southwestern United States. In 2018, two *P. aeruginosa* infections contracted by completely unrelated patients treated in Houston, TX were found co-expressing two GES variants housed on the same mobile genetic element. One variant was found to strongly hydrolyze carbapenems and the other was found to hydrolyze a broad range of antibiotics. This tandem expression caused nearly pan-drug resistance in the *P. aeruginosa* and resulted in the death of one patient.[47] If bla_{GES} is found in the WWTP samples, it could mean that this gene is significant in the Southwest region and should be added to surveillance efforts of clinical isolates.[47–49]

Materials and Methods

Sample locations and collection: Three 500 mL samples were taken at each sample location using sterile Nalgene collection containers. At the Socorro Municipal WWTP, samples were taken from secondary containers and poured into the Nalgene collection containers using aseptic technique, as per the WWTP's own protocol for daily sampling. Samples from the low-flow channel were taken ~1 mi upstream and downstream from the WWTP using a sterile secondary container for each location lowered into the channel and poured into the Nalgene collection containers in the same manner as the WWTP samples. Samples were stored at 4°C within 30 min of collection until processing, which occurred within 24 hrs of collection. Samples were collected in this way from July 2018 to December 2018 in approximately 6-week intervals.

Sample Processing: Three 1.5 mL 40% glycerol stocks were prepared from each sample replicate and stored at -80°C for future use. Next, sterile RO water was used to prepare an appropriate dilution of each sample replicate and vacuum filtered through a 0.45 um gridded mixed cellulose ester membrane filter. This filter was placed into a 47 mm petri dish into which 1 ampoule of mColiBlue indicative media was added to the petripad and incubated as per manufacturer's instructions. Colonies were enumerated the next day and CFUs/100mL were determined for each sample replicate. The remaining volume of all samples was filtered using vacuum filtration through 0.45 um gridded mixed cellulose ester membrane filters and stored at -20°C until DNA extraction.

DNA and Classical PCR: DNA was extracted from each total sample community. DNA from the influent and aerobic digester samples was extracted using the Qiagen DNEasy PowerFecal kit, while DNA from the effluent, upstream, and downstream samples was extracted using the Qiagen DNEasy PowerWater kit for clearwater samples and the Qiagen DNEasy PowerSoil Pro kit for the turbid samples due to the high amount of clay silt present. The manufacturer's instructions were used for each kit. DNA from AR control strains used to show a positive result for the PCR reaction, shown in Table , was isolated by suspending a colony in 100 uL of sterile RO water and boiling for 10 minutes after which the extracts were centrifuged for 5 min at 13,000 RPM.[50–53]

Table 1: CDC AR Panels used for classical PCR controls, strains and the genes they carry

Aminoglycoside/tetracycline Resistance (ATR) Panel		*Pseudomonas aeruginosa*		Imipenem/relebactam Panel		Enterobacteriaceae Carbapenemase Diversity Panel	
0559	bla_{NDM}	0231	bla_{KPC}	0502	bla_{IMP}	0135	bla_{VIM}
0555	bla_{NDM}	0239	bla_{GES}	0509	bla_{VIM}	0137	bla_{NDM}
0545	bla_{KPC}		bla_{VIM}	0525	bla_{KPC}	0138	bla_{NDM}
0541	bla_{KPC}	0241	bla_{IMP}			0155	bla_{KPC}

Classical PCR was performed for each of the *bla* genes mentioned using the primers found in Table 2. Each of these reactions was performed utilizing the following reaction conditions: 10 min at 94°C and 30 cycles of amplification consisting of 30 s at 94°C, 40 s at 55°C, and 50 s at 72°C, with 5 min at 72°C for the final extension.

Table 2: Sequences of primers used for the detection of ARGs in community samples and isolates.

Primer	Primer Sequences	Target Amplicon Size (bp)	Annealing Temp (^{o}C)	Source
bla_{NDM}	F: 5'– GGTTTGGCGATCTGGTTTTC–3' R: 5'– CGGAATGGCTCATCACGATC–3'	621	55	[50]
bla_{KPC}	F: 5'– CGTCTAGTTCTGCTGTCTTG–3' R: 5'– CTTGTCATCCTTGTTAGGCG–3'	798	55	[50]
bla_{OXA-48}	F: 5'– GCGTGGTTAAGGATGAACAC–3' R: 5'– CATCAAGTTCAACCCAACCG–3'	438	55	[50]
bla_{VIM}	F: 5'– GATGGTGTTTGGTCGCATA–3' R: 5'– CGAATGCGCAGCACCAG–3'	390	55	[50]
bla_{IMP}	F: 5'– GGAATAGAGTGGCTTAAYTC–3' R: 5'– TCGGTTTAAYAAAACAACCACC–3'	232	55	[51]
bla_{GES}	F: 5'– CTATTACTGGCAGGGATCG–3' R: 5'– CCTCTCAATGGTGTGGGT–3'	594	55	[52]

Amplification would be verified using 1% agarose gel electrophoresis and a 100 bp molecular weight marker to verify the size of the fragment.

Characterizing CREs and their AR genes: For the samples that were positive for the AR genes of interest, isolates were cultured from the long-term storage glycerol stocks on MacConkey agar to select for Gram-negative enteric bacilli and isolates were maintained on same. Once several phenotypically distinct colony types could be isolated from every sample, each isolate was inoculated into one well of a 96-well microtiter plate containing 150 uL of Muellar-Hinton (MH) broth and 5 ug/mL concentration of meropenem to select for CREs and incubated at 37^{o}C overnight. The CREs were recovered from the microtiter plates and streaked onto MacConkey agar to ensure purity of the isolate. The isolates were cultured in 1.5 mL MH broth containing 7 ug/mL concentration of meropenem and incubated under the same conditions. DNA was extracted from these isolates by spinning the cells out of solution with in a low-power bench-top microcentrifuge, resuspended in sterile RO water and lysed using the boiling lysis method outlined above. The extract was used as the template for a second round of classical PCR to determine the β-lactamase genes carried by the CRE isolates. Further characterization of the CRE isolates would be done using culture- dependent methods, such as Bd BBL Enterotube^{TM} II test tubes, and distinct cultures would be with identified using 16S rRNA sequencing. The gene variants would be determined by purifying and sequencing the PCR products.

Results and Discussion

Fecal coliform enumeration using the mColiBlue plate method: Fecal coliforms were present in all samples taken during the sampling period for this project. The quantities of fecal coliforms in each sample aligns with what is expected under these conditions.

Table 3: Fecal coliforms detected in each sample in CFUs/100 mL

Influent	Aerobic Digester	Effluent	Upstream	Downstream
> 216K *	> 44K *	<< 150 †	> 150 *	> 250 *

*Lowest averages recorded – one sampling event † Highest average recorded – one sampling event

It is noteworthy that the treated effluent rarely exceeded and was usually well below the EPA limits for recreational water, 126 CFU/100 mL.[54]

PCR amplification: Once the community DNA extractions for each sample replicate had been amplified using classical PCR, several *bla* genes were detected. Figure 1 shows the *bla* genes that reliably amplified in at least two of three sample replicates, indicating that those genes were very likely present in that sample.

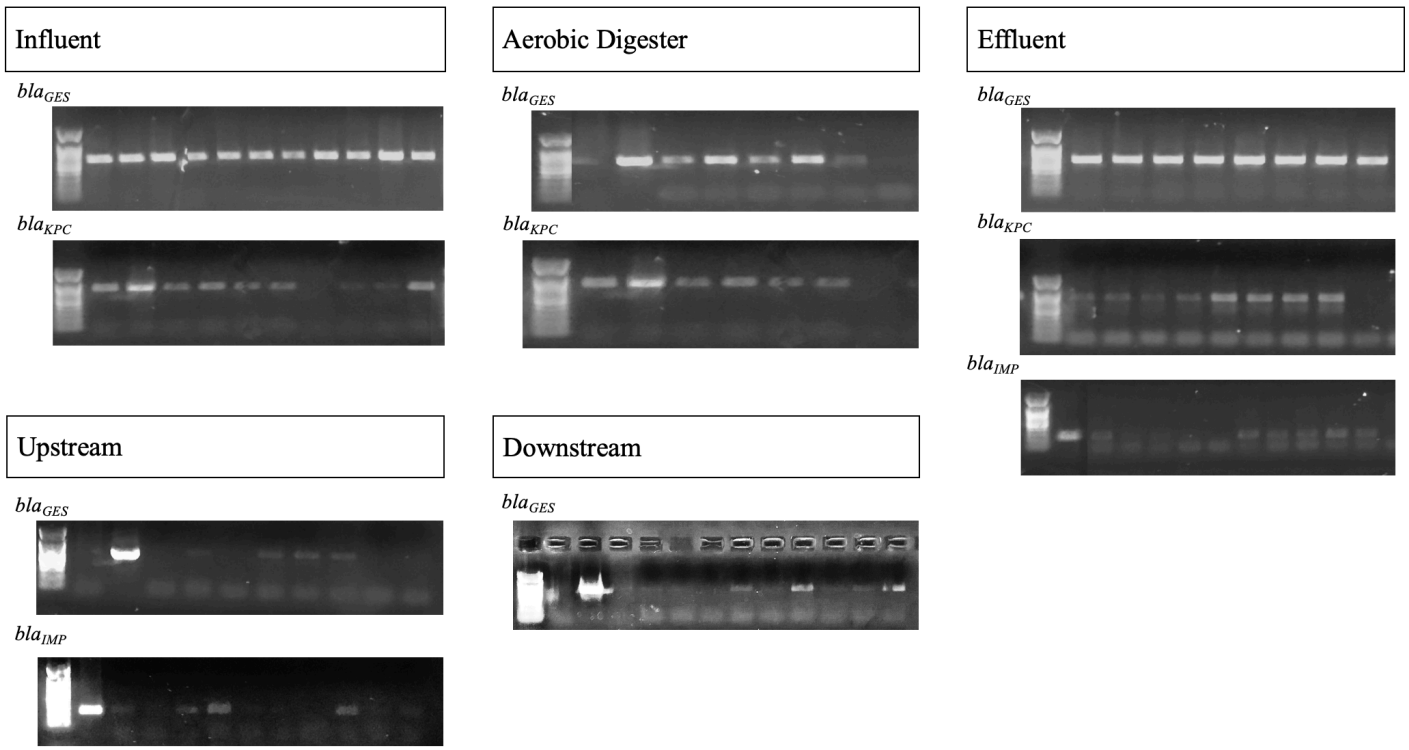

Figure 1: PCR products of the AR genes detected in each sample for each sample type.

Although the effluent samples had the fewest number of fecal coliforms and DNA yields were typically near those of the upstream and downstream samples, the AR gene diversity is consistently the highest in these samples over the entire course of the sampling period. These results are supported by similar results from other studies. The treatment and disinfection methods commonly used in the United States are not enough to remove AR genes and bacteria entirely and may in fact be enriching the effluent with them, as can be noted by the appearance of the bla_{IMP} gene in the effluent where it is not at a detectable level in the influent or aerobic digester samples. Also of note, the AR genes that were detected in the WWTP were also detected in the upstream samples, indicating the WWTP is not influencing the resistome for those samples and there are additional active sources of AR in the environment, which can be expected because this area is home to dairy, beef, and small animal agriculture and small-scale crop production. Strangely, the only AR gene that is present in every sample type in nearly every sampling event was the bla_{GES} gene.

Characterizing the CREs: Since AR genes were detected in nearly all sample sets, every sample set was cultured to determine the organisms carrying these genes. Currently, bacteria resistant to 5 ug/mL meropenem have been isolated from all sample sets and CREs resistant to 7 ug/mL have been isolated from influent and effluent samples. The species of bacteria present and the AR genes they are carrying have not yet been confirmed, but are currently being analyzed.

Conclusions

The results from this project support the notion that wastewater treatment methods in a typical municipal WWTP are not enough to remove AR genes and bacteria from effluent and could in fact be enriching them. Additionally, the detection of the bla_{GES} gene is a concern. Right now, surveillance efforts in New Mexico offered by the CDC only monitor clinical isolates within the Albuquerque area and not any of the smaller towns. Due to their proximity to livestock and their handling of hospital wastewater, there is a very significant chance for the bacteria within the Socorro WWTP to be exposed to AR genes and organisms and with the presence of other selection factors, such as heavy metals. The Socorro WWTP is likely to be a site conducive not only to HGT of existing AR genes, but could support the development and distribution of new combinations of AR genes into the environment, where other sources of AR genes evidently also reside.

The presence of bla_{KPC} and bla_{IMP} is not surprising, considering both are part of the CDC AR surveillance efforts, which monitor only clinically significant AR factors, and bla_{KPC} has been reported in New Mexico clinical isolates.[40] However, to the knowledge of the authors, the gene detected in nearly every sample taken over the course of this study, bla_{GES} is not currently monitored by the CDC or any other agency due to its assumed weak carbapenem hydrolysis and relative rarity in comparison to the others. Given that this gene is commonly found on the same mobile genetic cassettes

on conjugative plasmids, and that small amino acid substitutions can completely change the carbapenemase activity if the GES ESBL, more research into the genetic variants that were found in this project are currently underway to determine whether the variants that were isolated during this project have carbapenemase activity and what kinds of genetic elements they are associated with to determine its relative mobility in the environment. Additionally, screening of clinical samples from the local hospital as well as from CDC samples would give important data as to whether the *bla*$_{GES}$ gene variants found in this study are clinically significant.

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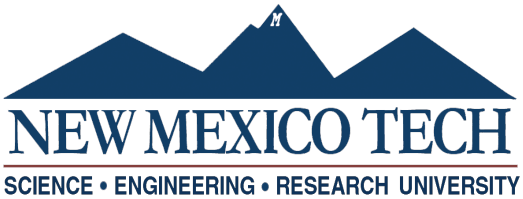

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