Tracking CRE in the Rio Grande: Determining Correlation Between the Appearance of Antibiotic Resistant Bacteria in Surface Waters and Local Infection Rates

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Abstract

Antibiotic resistance is a growing health crisis with fatal consequences. Overuse of antibiotics in clinical and agricultural settings has accelerated the evolution of resistance, but environmental factors may also be responsible for dissemination of these pathogens. The Rio Grande, at nearly 2,000 miles long, could play a role in transporting antibiotic resistant organisms over long distances in New Mexico. The river can easily be contaminated by wastewater effluent and agricultural runoff, both of which can carry carbapenem-resistant *Enterobacteriaceae* (CRE). To determine the role of the Rio Grande in CRE infection rates, water samples were collected every two months for one year, and each sample's bacterial community was analyzed for common carbapenem resistance genes and identifying virulence factors. Three of the five most commonly reported CRE-related genes, as well as two less prevalent ones, have been recovered from the Rio Grande. Two of these genes, *IMP* and *OXA-48*, entered the area during the course of this study. These resistance mechanisms have since been found at multiple sites along the river, spanning a distance of over 120 miles, suggesting that antibiotic resistance genes are spreading via surface water. Furthermore, contamination appears to be linked to local weather patterns. While these genes are currently uncommon in New Mexico CRE cases, this environmental dissemination could increase infection rates and introduce new or uncommon resistance genes to nearby communities.

Background

Antibiotic resistance is a growing public health issue in which bacteria become less susceptible to previously-effective treatments (1). This phenomenon has worsened over time due to overuse of antibiotics in medical and agricultural applications, but development of resistance may be accelerated in nature due to anthropogenic contamination (2,3). Direct environmental contamination can increase transmission of pathogens, but it can also spread resistance due to exchange of resistance genes (4,5). Bacteria that exchange genes have the ability to take up and sometimes express genes from other organisms. This DNA may be free in the environment, or it can be directly inserted into the target organism by a donor cell through conjugation or by a bacteriophage (4,6). When resistant organisms are present in the environment, they have the potential to spread this resistance to other members of the microbial community.

Carbapenem-resistant *Enterobacteriaceae* (CRE) is an emerging group of drug-resistant pathogens which cause approximately one-thousand deaths annually in the United States (7). *Enterobacteriaceae* is a family of enteric bacteria, including such organisms as *Escherichia coli* and *Salmonella enteritidis,* which are found in fecal waste (7,8). Carbapenems are a last-line class of antibiotics that are reserved for otherwise resistant infections (9). Few treatment options exist beyond these drugs. Carbapenem resistance is sometimes a result of the production of extended-

spectrum beta-lactamases (ESBLs), which are enzymes that degrade carbapenem antibiotics (10,11). Currently, the CDC tracks five genes which encode these proteins for epidemiological purposes (12). While the majority of CRE infections in the United States are healthcare-acquired, environmental contamination may increase rates of transmission (M. Sievers, NM DoH, personal communication, 2019). Agricultural runoff containing manure or other livestock wastes could introduce CRE into local surfaces waters (8). Treated wastewater from urban and suburban areas also has the potential to release resistant pathogens into the environment (13,14).

Genes encoding virulence factors, proteins which increase the ability of bacteria to infect eukaryotic cells, can also be useful for epidemiological investigations (15,16). Virulence genes (VGs) are widespread, but they are often specific to certain types of bacteria (15). Enteric VGs are only found in *Enterobacteriaceae*, and many such genes are exclusive to *E. coli* (14,17,18). Comparing which VGs are present in pathogens can provide a link between patients and sometimes lead to a source of infection (16). A similar method may be useful for examining the dissemination of pathogens in surface waters.

New Mexico hosts diverse communities which rely on the Rio Grande for both irrigation and recreational activity, but the river could be responsible for disseminating pathogens throughout the state. Several wastewater treatment plants release their effluent directly into the Rio Grande, and large expanses of land are utilized for agriculture in surrounding areas. These sources of contamination could contribute to the spread of CRE and carbapenem-resistance in the environment, and they could increase infection rates in New Mexico. Water samples were collected from the Rio Grande for approximately one year to monitor the presence of carbapenem resistance genes and virulence factors in order to observe the dissemination of pathogens in the river. These data were then compared to local infection reports from the New Mexico Department of Health.

Methods

Sample Collection and Locations

Quadruplicate 500-mL samples were collected in sterile polypropylene bottles at each sampling site every two months between September 2018 and November 2019. Sites were located at Alameda Open Space (AS), Tingley Beach (TB), and Cottonwood Road in Bosque Farms (BF) (Figure 1). A fourth collection site, the Rio Grande Gorge Visitor Center (RG) in Pilar, was also examined in May, July, and November of 2019. Samples were kept on ice during transport to the lab and processed within 24 hours of collection.

Sample Processing

250 mL of river water was vacuum-filtered using 0.45 μm cellulose membranes to collect bacteria. Community DNA was extracted from membranes using Qiagen DNEasy PowerSoil Pro Kits. 100 μL of water was plated on MacConkey agar, R2A with 4 μg/mL meropenem, and HardyCHROM CRE agar, all incubated at 30°C. DNA was extracted from gram-negative organisms demonstrating antibiotic resistance via freeze/thaw. DNA was extracted from control strains (Table 1) using GeneJET DNA Purification kits (Thermo Fisher Scientific). ESBL controls were

obtained from the CDC Antibiotic Resistance Isolate Bank (AR), and the VG control (EDL-933) was provided by the University of Michigan STEC Center.
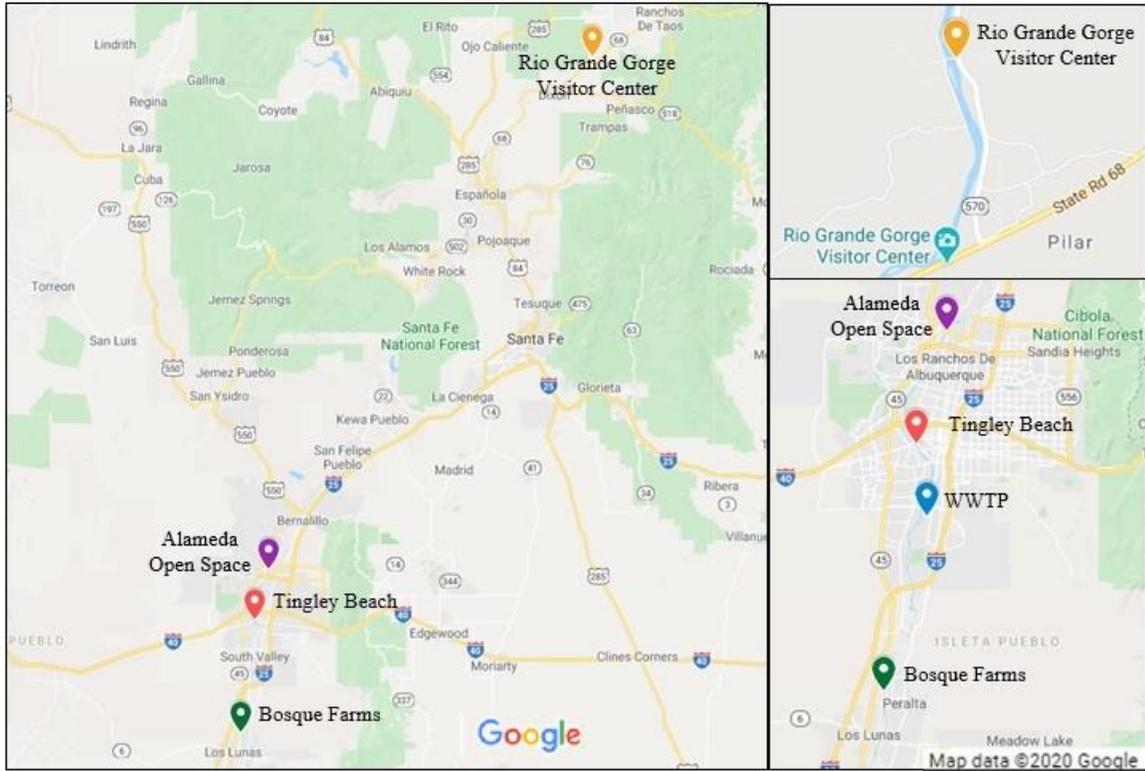

Figure 1: Map of sampling sites. Samples were collected from three sites in central New Mexico every two months during this project. A fourth location in northern New Mexico was also utilized in May, July, and November 2019. Figure generated in Google Maps.

Table 1: Control Strains

Strain Number	Organism	Target Genes	Strain Source
AR239	*P. aeruginosa*	*GES-1, VIM-11*	Imipenem/relebactam Panel
AR252	*P. aeruginosa*	*OXA-2*	Imipenem/relebactam Panel
AR501	*E. cloacae*	*VIM-1*	Imipenem/relebactam Panel
AR502	*E. cloacae*	*IMP-8*	Imipenem/relebactam Panel
AR503	*E. coli*	*NDM-1*	Imipenem/relebactam Panel
AR509	*P. aeruginosa*	*VIM-2*	Imipenem/relebactam Panel
AR516	*P. aeruginosa*	*KPC-2*	Imipenem/relebactam Panel
AR541	*E. coli*	*KPC-3*	Aminoglycoside/tetracycline Panel
AR555	*K. pneumoniae*	*NDM-5*	Aminoglycoside/tetracycline Panel
AR556	*K. pneumoniae*	*OXA-48*	Aminoglycoside/tetracycline Panel
EDL-933	*E. coli*	*stx1, stx2, eae, ehxA, EspP, einV**	University of Michigan STEC Center

EinV was not provided in the original description but was present during screening with previously-verified primers.

PCR and Gel Electrophoresis

Community and single-organism DNA extracts were used as templates for PCR to detect target genes. GoTaq Green (Promega), a premixed PCR formula, was used in conjunction with appropriate primers (Table 2) with the following reaction conditions: 95°C for 3 min; [95°C for 30 sec, T_α for 1 min, 72°C for 1 min] 32 cycles; 72°C for 10 min. PCR products with target bands larger than 200 bp were visualized on 1.5% (w/v) agarose (Fisher Scientific) gels electrophoresed for 25 minutes at 120 V in 1X TAE (Cold Spring Harbor Laboratory 2018). Products smaller than 200 bp were visualized on 2.0% agarose gels electrophoresed for 30 minutes at 120 V.

Table 2: Primer Sequences

Target Gene	Amplicon Size (bp)	Sequence	T_a (° C)	Source
NDM	620	F: GGTTTGGCGATCTGGTTTTC	57	19
		R: CGGAATGGCTCATCACGATC		
KPC	339	F: TGTTGCTGAAGGAGTTGGGC	57	19
		R: ACGACGGCATAGTCATTTGC		
IMP	440	F: GGTTTAATAAAACAACCAC	47	20
		R: GTTTATGTTCATACTTCG		
VIM	247	F: CGCGGAGATTGAGAAGCAAA	57	19
		R: CGCAGCACCGGGATAGAAGA		
OXA-48	435	F: GCGTGGTTAAGGATGAACAC	57	21
		R: CGGAATGGCTCATCACGATC		
OXA-2	569	F: ATTTCAAGCCAAAGGCACGA	57	19
		R: GCCACTCAACCCATCCTACC		
GES	594	F: CTATTACTGGCAGGGATCG	57	22
		R: CCTCTCAATGGTGTGGGT		
eaeA	241	F: TGAGCGGCTGGCATGAGTCATAC	50	23
		R: TCGATCCCCATCGTCACCAGAGG		
ehxA	569	F: AGCTGCAAGTGCGGGTCTG	50	14
		R: TACGGGTTATGCCTGCAAGTTCAC		
espP	397	F: CGGCAGAGTATCATCAAGAGC	50	24
		R: CATTAAATGGAGTTATGCGTC		
einV	140	F; TGGAAAAACTCAGTGCCTCTGCGG	57	23
		R: TTCTGATGCCTGATGGACCAGGAG		
stx1	338	F: TCTCAGTGGGCGTTCTTATG	50	14
		R: TACCCCCTCAACTGCTAATA		
stx2	150	F: GCGGTTTTATTTGCATTAGC	50	14
		R: TCCCGTCAACCTTCACTGTA		

Results/Discussion

Detection of ESBLs

Three genes under CDC surveillance – *VIM*, *IMP*, and *OXA-48*—were detected in water samples from the Rio Grande during this study. Two other ESBLs, *GES* and *OXA-2*, were also consistently present in these samples. *VIM, GES,* and *OXA-2* were all detected at least once in each batch of

samples between September 2018 and November 2019 (Figure 2), suggesting that these genes and the organisms carrying them are endemic to the area. Sources of contamination are likely consistent year-round, and they could be local or widespread. *IMP* and *OXA-48*, however, were first recovered in May 2019.

IMP was initially detected in RG samples, but it also appeared at AS, TB, and BF beginning in July 2019. The gene was recovered in all following samples. *OXA-48* was also first detected in May, but it was present in samples from all four sites. The sudden appearance of organisms containing these two genes may indicate new sources of contamination farther norther than the Rio Grande Gorge Visitor Center, potentially across state lines in Colorado. *IMP*'s patterns of detection demonstrate how antibiotic resistance genes could be spread over long distances via the river, as it was initially found at a single location in northern New Mexico before being detected downriver in central New Mexico two months later. These sample sites are located over 100 miles apart, suggesting major long-distance dissemination. Dispersal of *OXA-48* was more sudden, as it was detected at all four sample sites in May. It is possible that this gene was introduced into the Rio Grande between January and March, allowing more time for it to become established in the microbial population.

Detection of VGs

All six VGs were also recovered at least once, though some were found less frequently than others (Figure 3). The most commonly detected genes were *einV, espP, ehxA,* and *stx1*. The remaining two genes, *eaeA* and *stx2*, were somewhat less common. The gene *eaeA* was first detected in March 2019, after which it was fairly prominent. *Stx2* was only detected during three separate months. In November of 2018 and 2019, the gene was found in samples from central New Mexico (Albuquerque and Bosque Farms), and in May it was detected only in northern New Mexico at RG. This particular spread suggests that the changing microbial population in the Rio Grande is likely due to human activity, potentially related to agriculture. Because this gene was found in
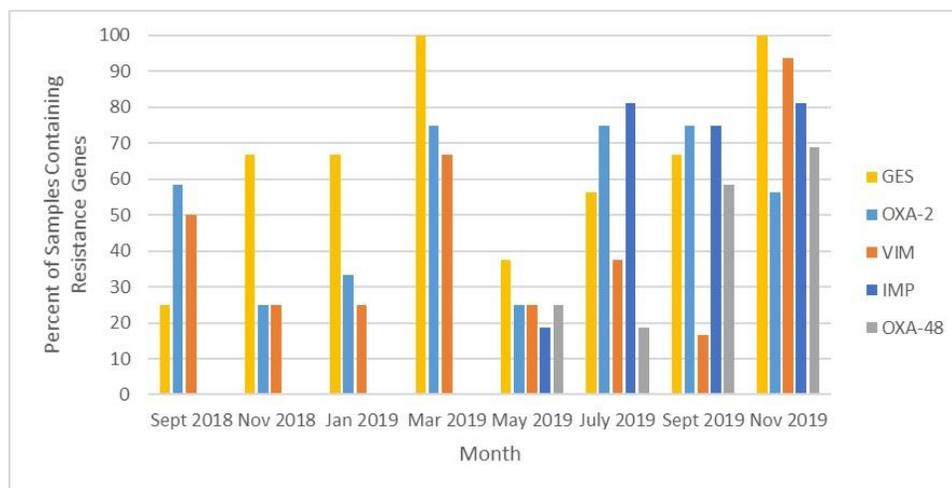

Figure 2: Prevalence of ESBLs in water samples from the Rio Grande. *VIM, GES,* and *OXA-2* were detected in samples during every month of the study, but *IMP* and *OXA-48* were only recovered starting in May 2019.

central New Mexico only during the month of November during both 2018 and 2019, such a distinct occurrence could be due to scheduled changes and not random chance. Tracking the appearance and dissemination of virulence genes which are less prevalent in the local environment could provide a strong indicator of changing conditions and new contamination.
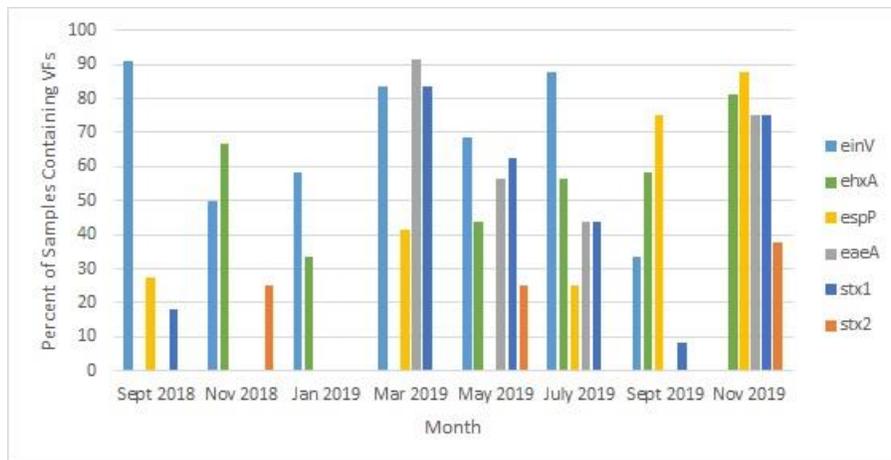

Figure 3: Prevalence of VGs in water samples from the Rio Grande. All six target genes were detected at least once, but *eaeA* and *Stx2* are likely the most representative of changing conditions or contamination in the river due to their rarity.

Individual Organisms

Several carbapenem-resistant organisms were cultured from Rio Grande water samples. Most of these isolates were identified as *P. aeruginosa* due to their production of pyocyanin. However, despite their resistant qualities, no target genes were detected in any of these organisms. This discrepancy is likely due to only low amounts of CRE being present in the river. Culturing bacteria from 100 μL of a water sample would not provide nearly as much coverage as extraction of DNA from 250 mL.

Infection Rates

CRE is a reportable condition in New Mexico as of June 2016, meaning known cases must be reported to the New Mexico Department of Health (25). All five ESBLs under CDC surveillance have been reported in New Mexico CRE patients since 2016, and *KPC* is the most commonly detected gene in these infections (M. Sievers, NM DoH, personal communication, 2019). *VIM* and *IMP* infections thus far are not correlated to any environmental contamination in areas surrounding the Rio Grande. Both genes have been detected in infections in Doña Ana County that occurred before the beginning of this project, and all cases have been linked to international travel or healthcare (M. Sievers, NM DoH, personal communication, 2019). Infections containing *OXA-48* have been identified in Santa Fe, Bernalillo, and Valencia Counties in northern and central New Mexico (M. Sievers, NM DoH, personal communication, 2019). Exact dates of these infections are not currently available, though they are known to have occurred before September 2019. It is possible that some of these cases may be linked to environmental contamination, though there is not enough evidence to confirm any relationship.

OXA-2 and *GES* are not currently tracked by the CDC or the state of New Mexico, so it is unknown whether or not there have been CRE infections containing either ESBL or if they would be related to environmental factors. Thus far, only two CRE infections have been reported in the United States with these genes. Both cases arose in Texas, and they contained two *GES* variants as well as *OXA-2*, resulting in serious complications which warranted further investigation (26). It is possible that other cases involving these genes have occurred under less severe circumstances, preventing further investigation or reporting. However, this study does indicate that both genes are present in organisms in the Rio Grande, suggesting they could become an issue in environmentally-transmitted CRE infections.

Infections occurring in counties which are not adjacent to the Rio Grande have the potential to be environmentally-acquired, as infections are reported in the patient's county of residence which is not necessarily where the disease was contracted (M. Sievers, NM DoH, personal communication, 2019). Although there is currently no definitive link between CRE infections and environmental contamination, investing more time into such research could present such links in the future. Environmental changes examined in this study may not produce any clinical results for several months and may instead serve as a predictor for future outbreaks. Foodborne illness may be another factor to consider. While there have been no recorded cases of foodborne CRE thus far, it has been predicted that some crops could be contaminated with the pathogen (27,28). The Rio Grande is often used for irrigation in New Mexico, and CRE in the river could theoretically affect the safety of local products.

Weather

Different weather patterns appear to have an effect on pathogenic contamination in the Rio Grande. The number of samples containing target genes as well as the diversity of these genetic elements varied throughout the year. Samples collected during cold, dry months (September 2018, November 2018, and January 2019) were less likely to contain ESBLs or VGs, but samples from warmer months like May or July contained more. However, precipitation also appears to be a factor. Following rainstorms, accumulating water can drain into surface waters, carrying bacteria and other pollutants with it. Snowmelt can have similar effects.

While temperatures did increase between January and March of 2019, the notable spike in target gene detection can likely be attributed to snowmelt in southern Colorado and northern New Mexico. An intense storm system also affected the region in mid-March, bringing heavy rain and snow just days before samples were collected (29). November 2019 presents a similar anomaly, as samples taken during this month contained significantly more target genes than those from November 2018 (two-sample T-test, $p<0.01$). However, the former was New Mexico's second wettest November on record, and samples were collected only a few days after a heavy rainstorm (30). This comparison is compounded further by the increasing diversity of target genes detected throughout the study. Initially, only three different ESBLs were detected (*VIM, GES,* and *OXA-2*), but two more were also found later in the study (*IMP* and *OXA-48*). ESBLs only demonstrated an increase in diversity, meaning genes detected in samples from one month were always found in the next set of samples as well.

Conclusions

The presence of carbapenem-resistance genes and virulence genes in the Rio Grande suggest CRE contamination. Five ESBLs were detected in water samples; while the environmental appearance of *VIM* and *IMP* can not currently be linked to clinical CRE cases, there may be a relationship between such occurrences of *OXA-48*. The increasing diversity of resistance genes in the Rio Grande suggests new sources of contamination are influencing water quality, and environmental transmission may become a greater risk. Enteric-specific VGs were also detected frequently in samples from the Rio Grande, though some genes were not as common as others. Genes appearing less often, including *stx2* and *ehxA*, could be indicative of new or changing sources of contamination. The detection of both resistance and virulence genes over time is likely related to local weather patterns. Higher temperatures, rainfall, and snowmelt prolong bacterial survival and increase fecal contamination in the river, potentially increasing the range over which these pathogens can spread in the environment.

References

1. CDC. About Antibiotic Resistance [Internet]. 2019 [cited 2020 Feb 18]. Available from: https://www.cdc.gov/drugresistance/about.html

2. Codjoe F, Donkor E. Carbapenem Resistance: A Review. Med Sci. 2017 Dec 21;6(1):1.

3. Shaikh S, Fatima J, Shakil S, Rizvi SMD, Kamal MA. Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. Saudi J Biol Sci. 2015 Jan 1;22(1):90–101.

4. Lorenz MG, Wackernagel W. Bacterial gene transfer by natural genetic transformation in the environment. Microbiol Rev. 1994 Sep;58(3):563–602.

5. Mell JC, Redfield RJ. Natural competence and the evolution of DNA uptake specificity. J Bacteriol. 2014 Apr 15;196(8):1471–83.

6. Griffiths AJ, Miller JH, Suzuki DT, Lewontin RC, Gelbart WM. Transduction. In W. H. Freeman; 2000.

7. CDC. CRE - Healthcare Acquired Infections [Internet]. 2019 [cited 2020 Feb 20]. Available from: https://www.cdc.gov/hai/organisms/cre/index.html

8. Webb HE, Bugarel M, den Bakker HC, Nightingale KK, Granier SA, Scott HM, et al. Carbapenem-Resistant Bacteria Recovered from Faeces of Dairy Cattle in the High Plains Region of the USA. Gao F, editor. PLoS One [Internet]. 2016 Jan 29 [cited 2020 Feb 18];11(1):e0147363. Available from: https://dx.plos.org/10.1371/journal.pone.0147363

9. Papp-Wallace KM, Endimiani A, Taracila MA, Bonomo RA. Carbapenems: Past, present, and future. Antimicrob Agents Chemother. 2011 Nov;55(11):4943–60.

10. Antunes NT, Lamoureaux TL, Toth M, Stewart NK, Frase H, Vakulenko SB. Class D β-lactamases: Are they all carbapenemases? Antimicrob Agents Chemother. 2014 Apr 1;58(4):2119–25.

11. Naas T, Poirel L, Nordmann P. Minor extended-spectrum β-lactamases. Clin Microbiol Infect. 2008 Jan 1;14(SUPPL. 1):42–52.

12. CDC. Carbapenemase Producing Carbapenem-Resistant Enterobacteriaceae (CP-CRE) | 2018 Case Definition [Internet]. 2018 [cited 2020 Mar 30]. Available from: https://wwwn.cdc.gov/nndss/conditions/carbapenemase-producing-carbapenem-resistant-enterobacteriaceae/case-definition/2018/

13. Hoelle J, Johnson JR, Johnston BD, Kinkle B, Boczek L, Ryu H, et al. Survey of US wastewater for carbapenem-resistant Enterobacteriaceae. J Water Health [Internet]. 2019 Apr 1 [cited 2020 Feb 18];17(2):219–26. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30942772

14. Garcia-Aljaro C, Muniesa M, Blanco JE, Blanco M, Blanco J, Jofre J, et al. Characterization of Shiga toxin-producing Escherichia coli isolated from aquatic environments. FEMS Microbiol Lett [Internet]. 2005 May 1 [cited 2020 Feb 18];246(1):55–65. Available from: https://academic.oup.com/femsle/article-lookup/doi/10.1016/j.femsle.2005.03.038

15. Cross AS. What is a virulence factor? Crit Care. 2008;12(6):196.

16. Croucher NJ, Harris SR, Grad YH, Hanage WP. Bacterial genomes in epidemiology- present and future. Philos Trans R Soc B Biol Sci. 2013 Mar 19;368(1614).

17. Brunder W, Schmidt H, Karch H. EspP, a novel extracellular serine protease of enterohaemorrhagic *Escherichia coli* O157:H7 cleaves human coagulation factor V. Mol Microbiol [Internet]. 1997 May 31 [cited 2020 Feb 18];24(4):767–78. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1046/j.1365-2958.1997.3871751.x

18. Lorenz SC, Son I, Maounounen-Laasri A, Lin A, Fischer M, Kase JA. Prevalence of hemolysin genes and comparison of ehxA subtype patterns in Shiga toxin-producing Escherichia coli (STEC) and Non-STEC strains from clinical, food, and animal sources. Appl Environ Microbiol. 2013;79(20):6301–11.

19. Mlynarcik P, Roderova M, Kolar M. Primer evaluation for PCR and its application for detection of carbapenemases in Enterobacteriaceae. Jundishapur J Microbiol. 2016 Jan 1;9(1).

20. Hujer KM, Hujer AM, Hulten EA, Bajaksouzian S, Adams JM, Donskey CJ, et al. Analysis of antibiotic resistance genes in multidrug-resistant Acinetobacter sp. isolates from military and civilian patients treated at the Walter Reed Army Medical Center. Antimicrob Agents Chemother. 2006 Dec;50(12):4114–23.

21. Hosseinzadeh Z, Sedigh Ebrahim-Saraie H, Sarvari J, Mardaneh J, Dehghani B, Rokni-Hosseini SMH, et al. Emerge of bla NDM-1 and bla OXA-48-like harboring carbapenem-resistant Klebsiella pneumoniae isolates from hospitalized patients in southwestern Iran. J Chinese Med Assoc. 2018 Jun 1;81(6):536–40.

22. Maina D, Revathi G, Whitelaw AC. Molecular characterization of multidrug-resistant Klebsiella pneumoniae and Escherichia coli harbouring extended spectrum beta-lactamases and carbapenemases genes at a tertiary hospital, Kenya. Microbiol Medica. 2017 Dec 30;32(4).

23. Pass MA, Odedra R, Batt RM. Multiplex PCRs for identification of Escherichia coli virulence genes. J Clin Microbiol. 2000;38(5):2001–4.

24. Gerrish RS, Lee JE, Reed J, Williams J, Farrell LD, Spiegel KM, et al. PCR versus hybridization for detecting virulence genes of enterohemorrhagic Escherichia coli. Emerg Infect Dis. 2007;13(8):1253–5.

25. Landen MG. Epidemiology of Carbapenem-resistant Enterobacteriaceae and Pseudomonas aeruginosa in New Mexico, 2015-2017 [Internet]. Santa Fe, NM; 2017 [cited 2020 Feb 28]. Available from: https://nmhealth.org/data/view/report/2040/

26. Khan A, Tran TT, Rios R, Hanson B, Shropshire WC, Sun Z, et al. Extensively Drug-Resistant Pseudomonas aeruginosa ST309 Harboring Tandem Guiana Extended Spectrum β-Lactamase Enzymes: A Newly Emerging Threat in the United States. Open Forum Infect Dis [Internet]. 2019 [cited 2020 Feb 28];6(7). Available from: https://academic.oup.com/ofid/article-abstract/6/7/ofz273/5512661

27. Zhou X, Qiao M, Wang FH, Zhu YG. Use of commercial organic fertilizer increases the abundance of antibiotic resistance genes and antibiotics in soil. Environ Sci Pollut Res. 2017 Jan 1;24(1):701–10.

28. Liu BT, Zhang XY, Wan SW, Hao JJ, Jiang R De, Song FJ. Characteristics of carbapenem-resistant Enterobacteriaceae in ready-to-eat vegetables in China. Front Microbiol. 2018 Jun 1;9(JUN).

29. NOAA. March 2019 Weather Summary. NOAA's National Weather Service;

30. NOAA. November 2019 Climate Summary. Albuquerque; 2019 Dec.