ANTIBIOTIC RESISTANCE AND VIRULENCE PROFILES OF AEROMONAS POPULATIONS FROM BEACH AND POST-CHLORINATED WASTEWATER COMPARED TO CLINICAL ISOLATES IN MILWAUKEE, WISCONSIN, USA

by

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ABSTRACT

ANTIBIOTIC RESISTANCE AND VIRULENCE PROFILES OF AEROMONAS POPULATIONS FROM BEACH AND POST-CHLORINATED WASTEWATER COMPARED TO CLINICAL ISOLATES IN MILWAUKEE, WISCONSIN, USA

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Under the Supervision of professor Dr. Troy Skwor

Aeromonas spp. are recognized as emerging human pathogens associated with gastroenteritis, wound infections, and bacteremia. Considering its ubiquitous nature in aquatic environments, many of these diseases arise from direct or indirect aquatic exposure. However, comparisons of *Aeromonas* populations among environmental sources compared to clinical populations are rarely reported. Therefore, our objective was to compare environmental *Aeromonas* populations from treated wastewater effluents and neighboring recreational beaches to clinical strains from the same geographical locations to assess the potential health risk. Environmental *Aeromonas* isolates were acquired from beaches and post-chlorinated wastewater (POC) using ampicillin dextrin agar with vancomycin and irgasin (ADA-VI). Antibiotic susceptibility was determined using Kirby-Baur disk diffusion with subsequent analysis of ARGs using PCR. To assess the clinical relevance of environmental isolates, we identified the species and virulence potential. Species were identified using *gyrB* PCR amplification and sequencing of subpopulations of clinical, POC, and beach isolates. Our findings identified clinical and POC populations as the most similar antimicrobial resistance (AMR) profiles except for nalidixic acid resistance (10.5% to 42.9% respectively), while beach isolates had the highest antibiotic susceptibility. Statistical differences among sources ($P<0.05$) were observed for sulfamethoxazole-trimethoprim, tetracycline, gentamicin, overall AMR, and multi-drug resistance. Environmental *Aeromonas* spp. also encoded similar AMR genes (*sul1/2, tetE,*

OXA, and *TEM*) to clinical populations. Among all sources, thirteen species were identified, with the most prevalent beach (*A. veronii)* and POC (*A. hydrophila)* species, resembling *Aeromonas* species common in the clinic. To determine their potential to cause disease, we assessed the presence of ten virulence genes and cytotoxicity of filtered supernatants against human epithelial cells. Although cytotoxic enterotoxin (*act*) and hemolysin (*hlyA*) were more common among clinical populations ($P<0.05$), overall similar virulence profiles existed for clinical, POC, and beach populations. Furthermore, *act* was the only virulence gene strongly associated with the cytotoxic phenotype. In conclusion, strong similarities in antibiotic resistance, species, and virulence factors between environmental and clinical isolates suggest beaches and POC wastewater serve as potential sources of clinical infections.

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Chapter 1: BACKGROUND

I: Introduction

A. Antibiotic resistance:

Once a revolutionary development, antibiotics saved millions of lives and effectively treated infections. Today, however, the escalating prevalence of antibiotic resistance has reached a critical level, prompting the Centers for Disease Control and Prevention (CDC) to designate antibiotic resistance as an urgent threat to public health globally. Worldwide, there were 5 million deaths associated with antibiotic resistance and 1.27 million deaths caused by antibiotic resistance in 2019 (1).

The rise in antibiotic resistance can be attributed to multiple factors including improper disposal of antimicrobials from hospitals, as well as misuse and overuse of antibiotics in the clinic, farming practices, and aquaculture (2-4). The increased quantity, diversity, and interactions of bacteria in wastewater treatment plants also can lead to a rise in antibiotic resistance (5). Additionally, there is a risk of untreated wastewater entering the environment, carrying pathogens harboring antibiotic resistance genes due to sewage leaks, sewer overflows, or absence of wastewater treatment facilities (6).

While in these various environments, bacteria are under increased stress, impacting their metabolic processes and survival due to the antibiotics and disinfectants surrounding them. Encountering these pressures can cause increased horizontal gene transfer between bacteria of the same or different species, leading to the acquisition of antibiotic resistance genes (ARGs) (7, 8). Bacteria can also obtain antibiotic resistance through mutations in specific chromosomal genes (9), and this process can be accelerated in wastewater due to the presence of various mutagens like heavy metals, chemicals, or organic carbon (10).

Diseases caused by these antibiotic resistant organisms will result in more challenging treatments, or potentially rendering them untreatable, leading to higher morbidity and mortality

rates (11-13). With the current trend of antibiotic resistance, it is predicted that by 2050, ten million people may die every year from an antibiotic resistant infection, underscoring the urgency to diminish the prevalence of antibiotic resistance and stressing the need for better solutions to combat these resistant microbes (14).

B. Wastewater

Wastewater treatment plants (WWTPs) obtain untreated water from a variety of sources including hospitals, households, industries, and storm runoff. The main goal of these WWTPs is to reduce the amount of organic matter before reintroducing treated wastewater to the recipient aquatic environment. This is done through a series of steps including: preliminary treatment, primary treatment, secondary treatment, sometimes tertiary treatment, and disinfection. Preliminary treatment removes large, solid materials from the water by passing it over grates, allowing the water to flow through while preventing the passage of large objects (15). Sewage then passes into sedimentation tanks during primary treatment, where gravity is utilized to remove settable solids, such as sand, gravel, and small rocks (15). Primary effluent water is then transferred into aeration tanks where heterotrophic bacteria break down organic matter during the secondary treatment process (15). Depending on the WWTP, the secondary effluent water may go through tertiary treatment or the disinfection stage. Tertiary treatment of water either utilizes chemical or biological processes to further remove organic compounds, phosphorus, and nitrogen from the water (15). Disinfection of wastewater is typically done through the utilization of ultraviolet light or chlorination, aiming to eradicate the maximum number of bacteria, especially disease-causing pathogens, before being released back into the environment (15). Although significantly diluted in recipient reservoirs, these recipient aquatic environments may be utilized in various ways including recreational purposes for swimming or fishing; thus, it is imperative this water is free or contains low levels of harmful microorganisms.

WWTPs are the main process of minimizing wastewater contaminants before returning it to the environment and facilitate bacterial interactions that may not occur otherwise (16). Pharmaceuticals, biocides, and pesticides are frequently found in wastewater (17), whereby they can act as selective pressures increasing horizontal gene transfer between bacteria (7) as well as mutations aiding their survival (18). Moreover, if WWTPs obtain hospital sewage, it can contain significantly higher concentrations of antimicrobials (19), clinical pathogens (20), and antibiotic resistant bacteria (16, 21) thus supplying key players encouraging exchange of genetic material. One study at Jones Island Reclamation Facility in Milwaukee, WI found 28 potential human and animal pathogens across multiple treatment stages of WWTPs, five of which were detectable in the final wastewater effluent going into Lake Michigan (16). Another study found bacteria associated with the intestinal tract and toxin-producing cyanobacteria in post chlorinated wastewater (22). Although WWTPs effectively reduce the levels of antibiotics and pathogens, residual antibiotics and pathogens can still remain in wastewater effluents. The study in Milwaukee further identified bacterial populations resistant to last-resort antibiotics among post-chlorinated final effluents, including *Aeromonas* among ceftazidime resistant bacteria (16). Given that wastewater effluents constitute the primary pollutant of surface water, potentially used for recreational purposes, the presence of antibiotic resistant pathogens in the wastewater poses a threat to human and environmental health (16).

C. *Aeromonas* species:

Traditionally considered to be an aquatic bacterium, the *Aeromonas* genus consists of Gram-negative, motile, non-spore forming bacilli that are common pathogens in cold-blooded fish and crustaceans. More specifically, *Aeromonas* are primary pathogens capable of causing motile hemorrhagic septicemia, fin or tail rot, and abdominal distention in fish (23). These diseases are most commonly caused by *A. caviae, A. veronii, A. salmonicida,* and *A. hydrophila*

(23). This genus is ubiquitous in aquatic environments and has been discovered across diverse ecological habitats, encompassing soil, fruits, and vegetables, as well as various mammals, including humans, rabbits, dogs, cats, and horses (24).

Globally, *Aeromonas* have been associated with numerous human diseases from gastroenteritis to wound infections. The predominant clinically relevant *Aeromonas* species are *A. caviae, A, hydrophila, A. dhakensis* and *A. veronii* (biovars *sobria* and *veronii*) (25). *Aeromonas* frequently cause traveler's diarrhea, with common symptoms being abdominal cramps, fever, nausea, and vomiting (26). A study analyzed 23,257 fecal samples over approximately a nine-and-a-half-year period and found 1,265 (5.5%) of the samples contained *Aeromonas,* with 70.6% of this group being *A. veronii*, 26.1% being *A. hydrophila*, and 3.3% being *A. caviae* (26). *Aeromonas* have also caused more life-threatening diseases such as necrotizing fasciitis and bacteremia in humans (27). Necrotizing fasciitis, an aggressive soft tissue infection due to the bacteria entering, invading, and causing necrosis in multiple layers of the skin, is rapid, rare, and potentially lethal if not promptly treated. Analyzing patient profiles brought into the emergency department of one hospital over an eighteen-year period, a study found 68 patients were confirmed to have necrotizing fasciitis of a limb due to an *Aeromonas* infection (27). Of these patients, 29.4% died, with *A. hydrophila* accounting for 67.6% of the infections, *A. veronii* biovar *sobria* for 14.7%, and *A. caviae* for 4.4% (27). In addition, clinical *Aeromonas* strains have demonstrated resistance against an array of commonly prescribed antimicrobials (28), as well as last resort antibiotics like fourth generation cephalosporins (29) and carbapenems (30). A comprehensive analysis of clinical, agricultural, and environmental *Aeromonas* isolates exhibited resistance to all 21 antimicrobials analyzed in all sources, suggesting an interconnection between humans, agriculture, and the environment (31). This was further supported by a study in India identifying similarities in the antibiotic resistance genes analyzed from fish, shrimp, insects, chickens, and humans among *Aeromonas* spp. (31).

Most *Aeromonas* spp. are intrinsically resistant to the broad-spectrum antibiotic ampicillin, with a majority of the species showing resistance to first-generation cephalosporins (32). This evolutionary event highlights the further dangers of the complex mobilome, the mobile genetic elements, of *Aeromonas* (33). The ability of these mobile genetic elements to potentially spread resistance between sources is concerning. Additionally, a plasmid containing *catB3* gene in *A. salmonicida*, which encodes chloramphenicol resistance, was able to be transferred into *A. hydrophila* via conjugation (34) supporting intra- and interspecies horizontal transfer events involving *Aeromonas* spp. associated with resistance to heavy metals and antimicrobials. Together, this underscores the imminent health threat *Aeromonas* poses and emphasizes the necessity of implementing a One Health approach to address the challenge of antibiotic resistance effectively.

Due to the ubiquity of *Aeromonas,* this bacterium serves as a potential indicator species to study the interconnectedness of the environment with humans, and animals, otherwise known as the One Health approach (35). The One Health perspective recognizes that the health of humans, animals, and their environments are intimately linked and that changes or disruptions in one domain can have profound effects on the others. Currently, *Escherichia coli* is the most common indicator species used to analyze antibiotic resistance. *Aeromonas,* and their sources would provide more support to assess and develop effective strategies to combat antimicrobial resistance further supporting its use.

D. Virulence factors of *Aeromonas:*

Virulence factors are molecular components that enable bacteria to facilitate adhesion, colonization, and invasion within the host, potentially prompting disease. For a bacterium to cause disease within a host, it must be able to adhere to the host (36). Adherence is normally facilitated by certain virulence factors interacting with the host cell receptors (36). *Aeromonas* can have lateral or polar flagella to aid in facilitating adherence, which can progress to the

development of a biofilm, helping the bacteria evade the host immune response and antibiotics (37). The gene *lafB* encodes a lateral flagellum primarily found in *A. hydrophila,* that gives the bacterium swarming motility and assists with adhesion of the bacterium onto the host cell membrane (38, 39). To cause disease within the host, the bacteria must be able to also evade and penetrate the host chemical and physical barriers, respectively. To achieve this, bacteria can produce enzymes such as hemolysins, lipases, nucleases, proteases, cytotoxic and cytotonic enterotoxins, or utilize other mechanisms encoded by virulence factors, like flagella and secretion systems (36). Once a bacterium invades the host, it can potentially spread throughout the host, leading to systemic infection (36).

While certain bacteria, such as *Corynebacterium diphtheriae,* depend on specific toxins or virulence factors to cause disease, *Aeromonas* spp., eludes a definitive combination of virulence factors required for pathogenicity. One study found that more than 68% of virulence factors were found in both pathogenic bacteria and non-pathogenic *Aeromonas* including exotoxins, type III secretion system proteins, flagella, and capsules (40), suggesting other factors may also be involved in determining infection or disease of the host (41). Virulence factors may be shared among species, but variations in the specific types of these factors may lead to variations in disease presentation (40).

Virulence factors are frequently deployed through a variety of secretion systems into the host cells. There are various types of secretion systems (e.g. T1SS, T2SS, T3SS), each aiding in the transmission of virulence factors into the host or environment from the bacteria (42). T1SSs operate via a single step process where the virulence factor is transported from the bacterial cytoplasm into the extracellular space (43). In contrast, a T2SS involves a two-step process where the virulence factors are translocated from the bacterial cytoplasm to the periplasmic space via the *sec*-dependent pathway and then across the outer membrane into its environment (44). Conversely, T3SSs directly inject the virulence factor into the target host cell (45). Together, secretion systems play a crucial role in the delivery of many virulence factors,

allowing bacteria to interact with host cells and manipulate host responses to promote infection and disease. The virulence factors analyzed in this study were among the most commonly studied in *Aeromonas: hlyA, aero, lafB, lip, nuc, ser, GCAT, act, ast,* and *aexT.*

Some common virulence factors are hemolysins. They can be divided into two classes: α- and β-hemolysins. The virulence factor hemolysin A, *hlyA,* belongs to the α-hemolysin class meaning that it can cause reversible lysis to red blood cells (46), and is secreted via a T1SS (43). Aerolysin, *aero,* a β-hemolysin, utilizes a T2SS (44) and causes irreversible lysis to red blood cells through pore formation targeting the cell membrane (46). Additionally, hemolysins facilitate the ability of bacteria to induce gastroenteritis and are associated with foodborne illnesses in humans (47).

Lipase, *lip,* is a lipolytic enzyme, secreted by a T2SS(48), that hydrolyzes ester bonds in lipids, generating free fatty acids. This enzymatic activity provides a nutritional source for bacteria and contributes to the destruction of leukocyte membranes (46). Whereby nuclease, *nuc,* degrades extracellular traps, allowing the bacteria to escape the host's immune response and the infection site, so it can cause disease throughout the body (49). Extracellular traps are formed by cells such as neutrophils, mast cells (50), eosinophils (51), and macrophages (52)to capture and neutralize pathogens. This virulence factor is also associated with a T2SS (53).

The breakdown of components of the extracellular matrix, the immune system, and signaling molecules can be attributed to serine protease, *ser,* which cleaves peptide bonds of proteins (54). The ability of serine proteases to cleave proteins may also contribute to the activation of glycerophospholipid cholesterol acyltransferase, *GCAT*, and other secreted toxins and enzymes (55). *GCAT*, a bacterial lipase, induces the disruption of red blood cell membranes, leading to cell lysis (55). A T2SS is responsible for secreting both virulence factors (56).

Act is a cytotoxic enterotoxin related to aerolysin, again primarily found in *A. hydrophila* (45). T2SSs facilitate the export of *act,* which can then elicit a range of effects including

hemolytic, cytotoxic, and cytotonic in infected cells (45). *Ast,* a cytotonic enterotoxin, is heat stable and can cause altered fluid secretion from cells (45). *AexT*, an ADP-ribosylating toxin, alters host proteins by adding ADP-ribose to them, disrupting cellular function (45). This toxin is secreted by a T3SS, which approximately 90% of *Aeromonas* species with a T3SS have this virulence factor (45).

II: Hypothesis and Specific Aims

Although ubiquitous in aquatic environments, *Aeromonas* species are prevalent in numerous environments, including food, animals, and humans. They can cause numerous diseases in both aquatic cold-blooded organisms and humans with the most common species associated with clinical infections being *A. hydrophila, A. caviae, A. dhakensis* and *A. veronii* (25). WWTPs harbor a wide variety of bacterial species, with *Aeromonas* being a major contributor to the bacterial community. However, despite exposure to chlorine to remove bacteria, some *Aeromonas* are resilient and survive the disinfection process (16, 57, 58). Concurrently, stressors within these environments can contribute to an increase in acquisition of antibiotic resistance genes (10, 59, 60). There is a lack of knowledge regarding the potential health effects of wastewater effluents on their receiving reservoirs. Understanding these effects is vital for public health, especially for people utilizing these recreational waters utilizes as receiving reservoirs. Therefore, this study aims to analyze the antimicrobial resistance of environmental *Aeromonas* populations from final post-chlorinated wastewater effluents to surrounding recreational beaches, compared to local clinical isolates within Milwaukee County, WI, and compare their potential to cause human disease. Previous studies primarily examined environmental sources, wastewater, and clinics individually. However, there has been an absence of *Aeromonas* studies on beaches and minimal assessments of the

interconnectedness between the environment and clinical strains, making it challenging to interpret their findings.

The hypothesis of this proposal is: *Post-chlorinated wastewater and beach isolates will contain similar antibiotic resistance and virulence patterns as clinical isolates suggesting these environments serve as potential sources for Aeromonas clinical infections.*

The following aims will be utilized to test this hypothesis:

1. **Determine the species and genes associated with antibiotic resistance in wastewater effluents and surrounding beaches compared to clinical isolates of *Aeromonas*.** The working hypothesis for this aim is: Wastewater effluents and beaches will contain isolates of the same species and with similar antibiotic resistance genes as observed in clinical *Aeromonas* isolates.
2. **Determine the virulence phenotype and genotype of wastewater effluents and surrounding beaches compared to clinical isolates.** The working hypothesis for this aim is: There will be shared virulence profiles among all sources, indicating the potential of environmental isolates to cause human disease.

Chapter 2: MATERIALS AND METHODS

I. Specific Aim 1:

A. Sources of microbial populations:

Aeromonas populations from wastewater effluents, recreational beaches, and clinical isolates were used to study the phenotypic and genotypic resistance patterns. Beach isolates were collected from foreshore sand of two beaches north of the WWTP (Bradford and Atwater) and one beach south of the WWTP (South Shore) in Milwaukee, WI between June and September 2021 (Figure 1). Wastewater effluent isolates were procured from the final effluent at

Jones Island Water Reclamation Facility between October 2020 and October 2021 (Figure 1). All environmental isolates were acquired before the start of this project by other members of the Skwor lab. Clinical isolates were acquired from Wisconsin Diagnostics Laboratories in the Milwaukee area from various human sources (Table 1).

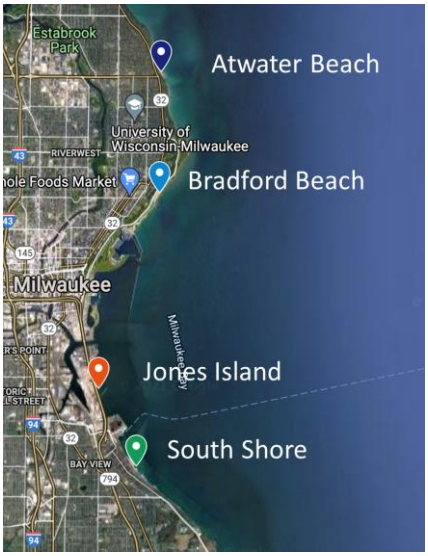


Figure 1. Location of Atwater, Bradford, and South Shore beaches and Jones Island Reclamation facility.

Table 1. Source of *Aeromonas* clinical isolates

Specimen Source	% (N)
stool	73.7 (14)
bone	5.3 (1)
abdomen	10.5 (2)
stent	5.3 (1)
tissue	5.3 (1)

Reference strains of *Aeromonas* were obtained from the American Type Culture Collection (ATCC) to be utilized as positive controls when needed. All protocols performed and proposed in this study have been approved by the University of Wisconsin--Milwaukee Institutional Biosafety Committee.

B. <u>Acquisition and isolation of presumptive *Aeromonas* isolates:</u>

Foreshore sand was collected to obtain *Aeromonas* beach isolates. Briefly, sand was collected from the surface of the berm approximately 6 inches deep, where waves periodically hit. The sand was then weighed and washed with deionized water before being placed onto 0.45µm filter paper at various dilutions. The filter papers were placed on tryptic soy agar (TSA) plates, an all-purpose agar, or ampicillin-dextrin agar with vancomycin and irgasin (ADA-VI) plates, an agar selective for *Aeromonas,* before being incubated for 24 hours at 30°C. After incubation, the plates were counted for colony forming units (CFUs) and the percent of *Aeromonas* was determined. Colonies on the ADA-VI plates were selected for isolation onto new ADA-VI plates and incubated for 24 hours. Presumptive *Aeromonas* isolates, identified by yellow halos on ADA-VI, were obtained from overnight cultures. Single colonies from the plates were then inoculated into Luria broth (LB) and placed into a shaking incubator at 30°C at 150 rpm for 24 hours and made into glycerol frozen stocks.

Wastewater was collected in autoclaved bottles at the discretion of the Jones Island Wastewater Treatment facility staff. Wastewater was then placed on filter papers at various dilutions and processed in the same manner as beach samples.

C. <u>Alkaline lysis to acquire genomic DNA:</u>

Alkaline lysis was performed to obtain the DNA from all effluent wastewater, beach, and clinical isolates from overnight cultures. A 100µL sample of the overnight culture was centrifuged for 2 minutes at 10,000 rpm to create a pellet. After discarding the supernatant, the pellet was resuspended in PBS and centrifuged for 10 minutes at 6,000 rpm. Carefully removing the supernatant to not disturb the pellet, 20µL of 0.1 M KOH was added with subsequent incubation in a 100°C water bath for 15 minutes. Samples were then cooled to room temperature before addition of 30µL of 0.1 M HEPES and stored in the -20°C freezer until utilized in later genomic analysis.

D. Kirby Bauer disk diffusion assay:

To determine the phenotypic antibiotic resistance patterns of the effluent wastewater, beach, and clinical *Aeromonas* isolates, Kirby Bauer disk diffusion was used by previous members of the Skwor lab following a published protocol (61). Briefly, the overnight cultures created were diluted in phosphate buffered saline (PBS) to obtain a 0.5 McFarland standard (BD Biosciences) across all samples. Each dilution was spread onto 150mm Mueller-Hinton agar plates stamped with antibiotic disks for amoxicillin/clavulanic acid, meropenem, chloramphenicol, sulfamethoxazole/trimethoprim, aztreonam, ciprofloxacin, tetracycline, gentamicin, cefotaxime, nalidixic acid, tigecycline, cefazolin. Plates were then incubated at 35°C for 24 hours, and zones of inhibition were subsequently measured. The Clinical Laboratory Standards Institute (CLSI) M45 3rd edition was used to determine the antibiotic susceptibility of each antibiotic for each isolate or CLSI 2020 M100 for *Enterobacterales* if not found in the M45.

E. *GyrB* PCR and sequencing to determine species:

To confirm that the alkaline lysis was successful with no PCR inhibitors and to identify the *Aeromonas* species by PCR, amplification of the *gyrB* gene of all isolates was performed. *GyrB* is a housekeeping gene encoding the β-subunit of DNA gyrase, which is a type II topoisomerase enzyme common among bacteria (62). Previous studies have demonstrated that while all housekeeping genes, including *gyrB*, have their limitations, *gyrB* is still fairly effective at distinguishing between *Aeromonas* species (63). Green 2x PCR MasterMix (Promega, Madison, WI, USA) was combined with genomic DNA and the *gyrB* gene primers for amplification of the *Aeromonas* isolates. The primers used were the following sequences: Forward: 5'TCCGGCGGTCTGCACGGCGT-3' and Reverse: 5' TTGTCCGGGTTGTACTCGTC-3' (62). A negative control, substituting water for DNA, was analyzed to confirm the validity of the results by ensuring any amplification was due to the presence of the target gene and not due to contamination. All PCR reactions were performed on an Eppendorf X50 thermocycler. A

subsample of 5µL of the 50µL PCR reactions in addition to a 1kB ladder (ThermoFisher Scientific) was run on a 1.0% agarose gel with ethidium bromide at 100V to assess successful amplification. Positive bands appeared at 1130 bp (62).

To obtain purified *gyrB* amplicons for subsequent sequencing, the QIAquick PCR purification kit (Qiagen) was utilized following manufacturer's instructions. Briefly, the remaining 45 µL of the PCR reaction was added to an epitube with 5 times the remaining volume of PB buffer. This solution was placed into a QIAquick column and centrifuged for 1 minute at 15,000 rpm before the collection tube was decanted. Next, 750µL of PE buffer was added to the column and centrifuged for 1 minute at 15,000 rpm. Following the collection tube being decanted, the column was centrifuged again at the same settings. The columns were then placed into an epitube and 40µL of molecular grade water was added to the center of the column, which incubated for 1 minute before being centrifuged again for 1 minute at 15,000 rpm. The purified DNA was then analyzed on the nanodrop to determine the concentration, as well as the 260/280 ratio and 260/230 ratio to verify its purity before storing it at -20°C. Once all isolates were purified, DNA samples were sent in for sequencing by Eurofin Genomics (Louisville, KY, USA).

F. Determining *Aeromonas* species from *gyrB* sequences:

DNA purification was performed for all isolates exhibiting intermediate or full resistance (Table 5), as well as a random subpopulation of beach isolates, and all clinical and wastewater effluent isolates. Chromatogram peaks were analyzed to ensure there was a clear peak associated with each nucleotide. If there were nucleotides unable to be deciphered by the software program, it was manually analyzed, and where possible the correct nucleotide recorded. Upon obtaining the chromatograms from these isolates, the sequences were shortened at the beginning and end so that each isolate started and ended at the same nucleotide sites. Following these modifications, the entire sequence was approximately 871

nucleotides long. The sequence was placed into nucleotide PubMed BLAST and species with greater than a 98% similarity was reported as the identity of the isolate. Sequences of reference genomes were acquired from NCBI and Mega 11 Muscle was used to align followed by a Neighbor-Joining method of the 871-nucleotide sequence among all isolates to make a phylogenetic tree.

G. Genomic analysis of antibiotic resistance genes:

Isolates determined to have antibiotic resistance were further analyzed for resistance genes associated with their corresponding resistance phenotype. PCR was used to identify whether strains resistant to tetracycline had the genes *tetA, tetB, tetC, tetD,* and *tetE*. The genes *sul1* and *sul2* were analyzed for isolates with resistance to sulfamethoxazole trimethoprim (SXT). Isolates exhibiting intermediate or complete resistance to meropenem, aztreonam, and cefotaxime were analyzed for extended-spectrum beta lactamase genes CTX-M1 subgroup, CTX-M2 subgroup, CTX-M9 subgroup, OXA, and TEM. Every set of PCR reactions contained a negative control with the primers, MasterMix, and water to ensure there was no DNA contamination in the reagents causing a false positive. Additionally, a positive control was also included containing ARG primers, MasterMix, and positive control DNA obtained from the CDC & FDA antibiotic resistance isolate bank (ARISOLATEBANK -CDC) to ensure the PCR worked properly. The ARG primers were selected from previous research (Table 2). Identification of ARGs occurred by running 10µL of the PCR products on a 1.0% agarose gel with ethidium bromide and a 100bp ladder (ThermoFisher Scientific) at 100V for 1 hour.

Table 2. List of PCR primers for ARGs

Target gene	Primer	Sequence (5' → 3')	Products size (bp)	Annealing temperature	Reference

tetA	tet(A)-F	TTGGCATTCTGCATTCACTC	494	60	(64)
	tet(A)-R	GTATAGCTTGCCGGAAGTCG			
tetB	tet(B)-F	CAGTGCTGTTGTTGTCATTAA	571	60	(64)
	tet(B)-R	GCTTGGAATACTGAGTGTAA			
tetC	tet(C)-F	CTGCTCGCTTCGCTACTTG	897	55	(65)
	tet(C)-R	GCCTACAATCCATGCCAACC			
tetD	tet(D)-F	TGTGCTGTGGATGTTGTATCTC	844	60	(66); modified by Skwor lab
	tet(D)-R	CAGTGCCGTGCCAATCAG			
tetE	tet(E)-F	TATTAACGGGCTGGCATTTC	544	60	(64)
	tet(E)-R	AGCTGTCAGGTGGGTCAAAC			
sul1	sul1-F	CGGCGTGGGCTACCTGAACG	433	60	(67); modified by Skwor lab

	sul1-R	GCCGATCG CGTGAAGT TCCG			
sul2	sul2-F	GAATAAATC GCTCATCAT TTTCGG	810	60	(68); modified by Skwor lab
	sul2-R	CGAATTCTT GCGGTTTC TTTCAGC			
CTX-M1 subgroup	CTX-M1-F	GGTTAAAAA ATCACTGC GTC	873	55	(69)
	CTX-M1-R	TTGGTGAC GATTTTAGC CGC			
CTX-M2 subgroup	CTX-M2-F	ATGATGACT CAGAGCAT TCG	886	55	(70)
	CTX-M2-R	TGGGTTAC GATTTTCGC CGC			
CTX-M9 subgroup	CTX-M9-F	ATGGTGAC AAAGAGAG TGCA	863	55	(70)
	CTX-M9-R	CCCTTCGG CGATGATTC TC			
OXA-1	OXA-F	ATATCTCTA CTGTTGCAT CTCC	619	54	(71)
	OXA-R	AAACCCTTC AAACCATCC			
TEM	TEM-F	ATCAGCAAT AAACCAGC	516	54	(71)

	TEM-R	CCCCGAAGAACGTTTTC			
Int1	Int1-F	CCTCCCGCACGATGATC	280	60	(72)
	Int1-R	TCCACGCATCGTCAGGC			

II. Specific Aim 2:

A. <u>Virulence genes:</u>

To determine the virulence genes of each isolate, PCR was performed against ten virulence genes (Table 3): *lafB, nuc, aero, ser, GCAT, lip, act, aexT, hlyA,* and *ast.* Every PCR reaction contained a negative control with the primers, MasterMix, and water to ensure there is no DNA contamination in the reagents causing a false positive. PCR was performed to determine the existence of the genes (Table 3), followed by running the PCR products on a 1.5% agarose gel with ethidium bromide and a 100bp ladder at 100V for 75 minutes.

Table 3. List of PCR primers for virulence factors

Target gene	**Primer**	**Sequence (5' → 3')**	**Products size (bp)**	**Annealing temperature**	**Reference**
Lateral Flagellum B	lafB-F	GACCAGCAAGGATAGTGGGTTGGAG	624	64°C	(73)
	lafB-R	AAGCACCATCGCGTTGGTATAAGG			
Nuclease	nuc-F	CAGGATCTGAACCGCCTCTATCAGG	504	64°C	(73)

	nuc-R	GTCCCAAGCTTCGAACAGTTTACGC			
Aerolysin	aero-F	GAGCGAGAAGGTGACCACCAAGAAC	417	64°C	(73)
	aero-R	TTCCAGTCCCACCACTTCACTTCAC			
Serine protease	ser-F	ACGGAGTGCGTTCTTCCTACTCCAG	211	64°C	(73)
	ser-R	CCGTTCATCACACCGTTGTAGTCG			
GCAT	GCAT-F	CATGTCTCCGCCTATCACAACAAGC	339	64°C	(73)
	GCAT-R	CCAGAACATCTTGCCCTCACAGTTG			
Lipase	lip-F	GACCCCCTACCTGAACCTGAGCTAC	155	64°C	(73)
	lip-R	AGTGACCCAGGAAGTGCACCTTGAG			
act	act-F	AGAAGGTGACCACCAAGAACA	232	64°C	(74); modified by Skwor lab
	act-R	AACTGACATCGGCCTTGAACTC			
AexT	AexT-F	ATGCAGATTCAAGCAAACAC	226	54°C	(75)

	AexT-R	TTGCCGATCCACTCTTTGAT			
hlyA	hlyA-F	GGCCGGTGGCCCGAAGATACGGG	579	66°C	(76); modified by Skwor lab
	hlyA-R	GGCGGCGCCGGACGAGACGGG			
ast	ast-F	ATGCACGCACGTACCGCCAT	260	66°C	(77); modified by Skwor lab
	ast-R	ATCCGGTCGTCGCTCTTGGT			

B. <u>Cytotoxicity of Bacterial Isolates:</u>

Bacterial supernatants were acquired to determine the presence of secreted cytotoxic compounds to lyse human epithelial cells, as previously described (61). To create supernatants of all isolates, an overnight culture was created by inoculating individual LB test tubes with wastewater effluent, beach, and clinical isolates. Additionally, one LB test tube was inoculated with no bacteria to serve as a negative control. The test tubes were then shaken at 150 rpm at 35°C for 24 hours. Test tubes were then observed to ensure turbidity after 24 hours. Following this, 1000µL of the overnight culture was transferred to an epitube, which then underwent centrifugation for 10 minutes at 15,000 rpm. The supernatant was passed over a 0.22µm filter to ensure the absence of bacteria, and the filtered supernatants were then frozen at -20dC until further analyzed.

C. <u>Culturing and subculturing HeLa cells:</u>

To study cytotoxic effects of all wastewater effluent, beach, and clinical isolates, HeLa cells were exposed to each filtered supernatant from the *Aeromonas* isolates. HeLa cells were

chosen due to their human epithelial cell nature and their ease to work with. The HeLa cells were obtained from the American Type Culture Collection (ATCC). All protocols performed in this study were approved by the University of Wisconsin--Milwaukee Institutional Biosafety Committee.

The HeLa cell line was stored in liquid nitrogen with the following operations performed in the biosafety hood. The cells were thawed in a 37°C water bath and transferred from the original vial to a conical tube with Complete Dulbecco's Modified Eagle Medium (C-DMEM), which is Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Bovine Serum with 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 µg/mL amphotericin B. The conical tube was centrifuged for 5 minutes at 1,000 rpm to pellet the cells followed by aspiration of the C-DMEM. The pellet of cells was then resuspended in warmed C-DMEM and dispensed into the culture flask and incubated at 37°C with 5% CO_2. When cells reached 80-90% confluency, they were subcultured. To do so, the C-DMEM was aspirated from the culture flask with a sterile Pasteur pipette and Hanks HBSS 1X was added to the flask, with volume changing depending on the size of the flask. This was then gently washed back and forth over the bottom of the flask before being aspirated from the flask. Trypsin was added to detach the cells from the culture flask, volume again depending on the size of the flask, before being gently moved back and forth over the cells. The flasks were then incubated around 5 minutes in 37°C with 5% CO_2. C-DMEM was added to the flask and gently pipetted up and down before transferring the cells to a conical tube and centrifuging at 1,000 rpm for 7 minutes. The supernatant was then aspirated, and the pellet was resuspended with C-DMEM. Cells were counted via a hemocytometer and then diluted in C-DMEM to $1x10^5$ cells/mL, which was then placed into a culture flask and incubated at 37°C with 5% CO_2.

D. <u>Cytotoxicity of HeLa cells:</u>

Cytotoxicity was determined utilizing a 96 well plate MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay following a previous protocol (78). Briefly, 100µL of $1x10^5$ cells/mL of HeLa cells in the stationary phase were plated and incubated for 24 hours at 37°C with 5% CO_2. Using the filtered *Aeromonas* supernatants, 25µL of the supernatant was added to a well containing HeLa cells and performed in triplicate. This was repeated for all selected wastewater effluent, beach, and clinical *Aeromonas* isolates. LB alone served as the negative control, where 25µL was plated for every plate in triplicate and supernatants from *Aeromonas hydrophila* ATCC7966 served as a positive control. Plates were then incubated for 18 to 24 hours under the same conditions. After incubation, cytotoxicity was measured by adding 5µL of MTT solution (5mg/ml in PBS) to each well and incubated for 2 to 4 hours. The media was then aspirated from every well and 50µL of MTT solvent (90% isopropanol, 10% Triton X-100, pH 4.0) was added and the plates were shaken for 1 minute to solubilize insoluble purple crystals. The plate was read at 570nm while subtracting the 630nm, using a plate reader and the average of the triplicates was recorded for each *Aeromonas* isolate. Cytotoxicity was determined by comparing values to the negative control. An absorbance less than 50% of the negative control was considered cytotoxic.

III: Data Analysis

The data obtained from our experiments was analyzed utilizing GraphPad Prism 10. Significance for antibiotic resistance, multidrug resistance (MDR), and antimicrobial resistance (AMR) between clinical, beach, and POC isolates was determined utilizing a Fisher's exact test. For MAR index, a one-way ANOVA was utilized, with significance determined by a *P*-value of ≤ 0.05. Significant for virulence genes and cytotoxicity was determined utilizing a Fisher's exact test, with significance determined by a *P*-value of ≤ 0.05.

Chapter 3: RESULTS

i. Specific Aim 1

Comparison of antimicrobial resistance profiles among beaches and post-chlorinated wastewater isolates to clinical isolates.

Table 4. Antibiotic resistance in beach sources

	% (no.) of isolates with full (R) or intermediate (I) resistance								
	Bradford Beach (n=57)			South Shore Beach (n=53)			Atwater Beach (n=48)		
Antibiotic	R	I	R + I	R	I	R + I	R	I	R + I
Cell wall									
AMC*	14.0 (8)	33.3 (19)	47.4 (27)	28.3 (15)	38.7 (20)	66.0 (35)	33.3 (16)	27.1 (13)	60.4 (29)
Aztreonam	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Cefazolin	68.4 (39)	14.0 (8)	82.5 (47)	67.3 (35)	24.5 (13)	92.5 (49)	79.2 (38)	10.6 (6)	91.7 (44)
Cefotaxime	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Meropenem	0.0 (0)	1.8 (1)	1.8 (1)	0.0 (0)	7.5 (4)	7.5 (4)	0.0 (0)	0.0 (0)	0.0 (0)
Protein Synthesis									
Chloramphenicol	0.0 (0)	1.8 (1)	1.8 (1)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Gentamicin	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Tetracycline	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Tigecycline	0.0 (0)		0.0 (0)	0.0 (0)		0.0 (0)	0.0 (0)		0.0 (0)
DNA synthesis									
Ciprofloxacin	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Nalidixic Acid	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Folic Acid synthesis									
SXT*^^	7.0 (4)	5.3 (3)	12.3 (7)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
MDR	1.8 (1)			0			0		
MAR index	0.081			0.089			0.102		
AMR	71.9 (41)			73.6 (39)			85.4 (41)		

MAR index: Median multiple antibiotic resistance index
MDR: % (no.) resistant to 3+ classes of antibiotics
AMR: % (no.) resistant to 1+ antibiotic
AMC: Amoxicillin-Clavulanic Acid
SXT: Sulfamethoxazole-Trimethoprim

*p < 0.05, resistant isolates
**p < 0.01, resistant isolates
^p < 0.05, resistant + intermediate isolates
^^p < 0.01, resistant + intermediate isolates
x: CLSI does not provide intermediate breakpoint for cefazolin or tigecycline

The prevalence of antibiotic resistance amongst bacterial populations from three Milwaukee beaches was determined to evaluate the potential health risk posed. Two of the beaches, Atwater (n=48) and Bradford (n=57) are located north of the effluent discharge site from Jones Island Reclamation Facility, while South Shore (n=53) is south of the wastewater treatment plant (Figure 1). Notably, AMR characterization of *Aeromonas* from recreational beaches has never been performed to our knowledge. We found Atwater beach had the highest AMR (85.4%), while South Shore and Bradford beach had similar levels (Table 4: 73.6% and 71.9% respectively). Interestingly, Bradford beach contained the only SXT resistant isolates and an intermediate chloramphenicol resistant isolate among the beach isolates, as well as a MDR isolate. This is particularly intriguing because it is the most populated beach over the summer. Overall, minimal differences were observed between the beaches, irrespective of location in

proximity to wastewater effluent site, except for SXT resistance, which was exclusive to Bradford (Table 4: R+I 12.3%, *P*<.01).

Table 5. Prevalence of antibiotic resistance among clinical, recreational beaches, and post-chlorinated wastewater isolates

	% (N) of isolates with full (R) or intermediate (I) resistance								
	Clinical (n=19)			Beaches (n=158)			POC Wastewater (n=56)		
Antibiotic	R	I	R + I	R	I	R + I	R	I	R + I
Cell wall									
AMC**^^ (R:13, I:18)	89.5 (17)	10.5 (2)	100 (19)	24.7 (39)	32.9 (52)	57.6 (91)	64.3 (36)	25.0 (14)	89.3 (50)
Aztreonam (R:17, I:20)	5.3 (1)	0.0 (0)	5.3 (1)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Cefazolin** (R:19, I:22)	89.5 (17)	10.5 (2)	100 (19)	71.5 (113)	20.9 (33)	88.6 (140)	92.9 (52)	1.8 (1)	94.6 (53)
Cefotaxime (R:22, I:25)	5.3 (1)	0.0 (0)	5.3 (1)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Meropenem (R:19, I:22)	0.0 (0)	10.5 (2)	10.5 (2)	0.0 (0)	3.2 (5)	3.2 (5)	0.0 (0)	1.8 (1)	1.8 (1)
Protein Synthesis									
Chloramphenicol (R:12, I:17)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.6 (1)	0.6 (1)	0.0 (0)	0.0 (0)	0.0 (0)
Gentamicin*^ (R:12, I:14)	5.3 (1)	0.0 (0)	5.3 (1)	0.0 (0)	0.0 (0)	0.0 (0)	1.8 (1)	0.0 (0)	1.8 (1)
Tetracycline*^^ (R:11, I:14)	5.3 (1)	0.0 (0)	5.3 (1)	0.0 (0)	0.0 (0)	0.0 (0)	1.8 (1)	7.1 (4)	8.9 (5)
DNA synthesis									
Ciprofloxacin (R:15, I:20)	5.3 (1)	0.0 (0)	5.3 (1)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	1.6 (1)	1.6 (1)
Nalidixic Acid**^^ (R:13, I:19)	10.5 (2)	0.0 (0)	10.5 (2)	0.0 (0)	0.0 (0)	0.0 (0)	41.1 (23)	1.8 (1)	42.9 (24)
Folic Acid synthesis									
SXT**^^ (R:10, I:15)	10.5 (2)	15.8 (3)	26.3 (5)	2.5 (4)	1.9 (3)	4.4 (7)	14.3 (8)	3.6 (2)	17.9 (10)
MDR**	15.8 (3)			0.01 (1)			37.5 (21)		
MAR index**	0.206			0.090			0.196		
AMR**	100 (19)			76.6 (121)			100 (56)		

MAR index: Median multiple antibiotic resistance index
MDR: % (no.) resistant to 3+ classes of antibiotics
AMR: % (no.) resistant to 1+ antibiotic
AMC: Amoxicillin-Clavulanic Acid
SXT: Sulfamethoxazole-Trimethoprim
Resistance measurements are in mm

*p < 0.05, resistant isolates
**p < 0.005, resistant isolates
^p < 0.05, resistant + intermediate isolates
^^p < 0.01, resistant + intermediate isolates
x: CLSI does not provide intermediate breakpoint for cefazolin or tigecycline
MAR from index amongst the sources was determined usting ANOVA each source compared to the beach

To aid in assessing the public health risk of environmental populations of *Aeromonas,* antibiotic resistance was determined from beach (n=158) and POC wastewater (n=56) isolates and compared to clinical isolates (n=19). Among the clinical isolates identified from various sources (Table 1), resistance was evident against amoxicillin/clavulanate (AMC), cefazolin, aztreonam, cefotaxime, gentamicin, tetracycline, ciprofloxacin, nalidixic acid, and SXT (Table 5). Differences in SXT, tetracycline, and gentamicin resistance were statistically different across the three sources (Table 5: *P*<0.01, R+I isolates; *P*<.005, resistant isolates). Antimicrobial resistance (AMR) was defined as resistance to at least one antibiotic, and 100% of clinical isolates exhibited AMR. Of the clinical isolates, 15.8% of them were multi-drug resistant, which was defined as resistant to three or more classes of antibiotics. POC wastewater exhibited the highest prevalence of MDR, followed by the clinic and the beaches (Table 5: 37.5%, 15.8%, 0.01%, respectively; *P*< 0.005).

Next, we examined the resistance profiles of environmental isolates from POC wastewater and beaches compared to the clinic. POC had a significantly higher prevalence of resistant populations compared to beaches. The AMR was very similar between the clinic and the POC wastewater (100%); however, both were significantly higher compared to the beaches (76.6%) (Table 5: $P<0.01$). Wastewater and clinical isolates exhibited increased resistance to AMC, nalidixic acid, tetracycline, and SXT compared to beach samples (Table 5), suggesting beaches pose a milder health risk compared to POC wastewater. The multiple antibiotic resistance (MAR) index, calculated as the total number of antibiotics to which the isolate is resistant divided by the total number of antibiotics tested (tigecycline was not used in this calculation due to the fact that only clinical strains were analyzed for this), was similar for the clinic and POC populations (Table 5; 0.206 and 0.196, respectively); however, they both were significantly higher compared to the beach (0.091) (Table 5; $P<.005$).

Of all antimicrobials tested, nalidixic acid resistance showed the strongest difference among the sources with significantly higher resistance levels in POC wastewater compared to both the beach and clinical isolates (Table 5: $P<0.005$ R and $P<0.01$ R+I). The beach showed no resistance, while the clinical isolates only had two isolates resistant to nalidixic acid. This suggests nalidixic acid resistance may be an indicator of WW origin (Table 5).

Comparison of antibiotic resistance genes among beaches and post-chlorinated wastewater isolates to clinical isolates.

Table 6. Comparison of antibiotic resistance genes among clinical, recreational beaches, and post-chlorinated resistant Aeromonas isolates

Clinical (n)	Beach (n)	POC Wastewater (n)
SXT resistance (3)	SXT resistance (7)	SXT resistance (10)
sul1 (0)	*sul1* (0)	*sul1* (5)
sul2 **(1)**	***sul2*** **(5)**	***sul2*** **(2)**
sul1/sul2 **(2)**	*sul1/sul2* (0)	***sul1/sul2*** **(1)**

TET resistance (1)	TET resistance (1)	TET resistance (5)
tetA (0)	*tetA* (0)	*tetA* (0)
tetB (0)	*tetB* (0)	*tetB* (0)
tetC (0)	*tetC* (0)	*tetC* (0)
tetD (0)	*tetD* (0)	*tetD* (0)
***tetE* (1)**	*tetE* (0)	***tetE* (5)**
3CEF/CARB (4)	3CEF/CARB (5)	3CEF/CARB (1)
KPC (0)	*KPC* (0)	*KPC* (0)
CTX-M1 subgr (0)	*CTX-M1* subgr (0)	*CTX-M1* subgr (0)
CTX-M2 subgr (0)	*CTX-M2* subgr (0)	*CTX-M2* subgr (0)
CTX-M9 subgr (0)	*CTX-M9* subgr (0)	*CTX-M9* subgr (0)
***TEM* (3)**	***TEM* (4)**	***TEM* (1)**
***OXA-1* (2)**	***OXA-1* (1)**	*OXA-1* (0)
Class 1 integron (19)	Class 1 integron (31)	Class 1 integron (56)
***Int1* (3)**	*Int1* (0)	***Int1* (5)**

3CEF: 3rd gen. cephalosporins; CARB: carbapenem

Antibiotic resistant gene profiles were analyzed among resistant populations to assess the clinical risk of environmental populations. SXT resistant isolates were examined for *sul1* and *sul2* genes. In the clinic, no isolates were found to have *sul1* alone, but one isolate had *sul2,* and two isolates had *sul1* and *sul2.* All sources exhibited the *sul2* gene alone and POC wastewater isolates also encoded the *sul1/sul2* genotype similar to clinical isolates. Additionally, POC wastewater was the sole source presenting the *sul1* gene only (Table 6).

Aeromonas populations are known to carry intrinsic beta-lactamases providing resistance to beta-lactams, such as AMC, and first generation cephalosporins, like cefazolin. Resistance to aztreonam, cefotaxime, and meropenem were analyzed for various ESBLs: *CTX-M* subgroups, *KPC, TEM,* and *OXA-1.* Clinical, beach, and POC wastewater all encoded *TEM,* while OXA-1 was only evident in beach and clinical populations (Table 6).

Isolates resistant to tetracycline were analyzed for *tetA, tetB, tetC, tetD,* and *tetE.* Resistance to tetracycline amongst the clinical and POC wastewater *Aeromonas* populations

was driven by the presence of *tetE,* while it is unknown what the driving factor of resistance is in the one resistant beach isolate (Table 6).

All of the isolates were also analyzed for a class 1 integron for the integrase gene. This gene is associated with multi-drug resistance because it allows bacteria to acquire ARGs from exogenous sources, such as plasmids and transposons, and adjust how the genes are expressed (72). The POC wastewater mirrored the clinic with the presence of *Int1,* which was not observed in the beach isolates.

Species identification of *Aeromonas* isolates in each source.

Figure 2

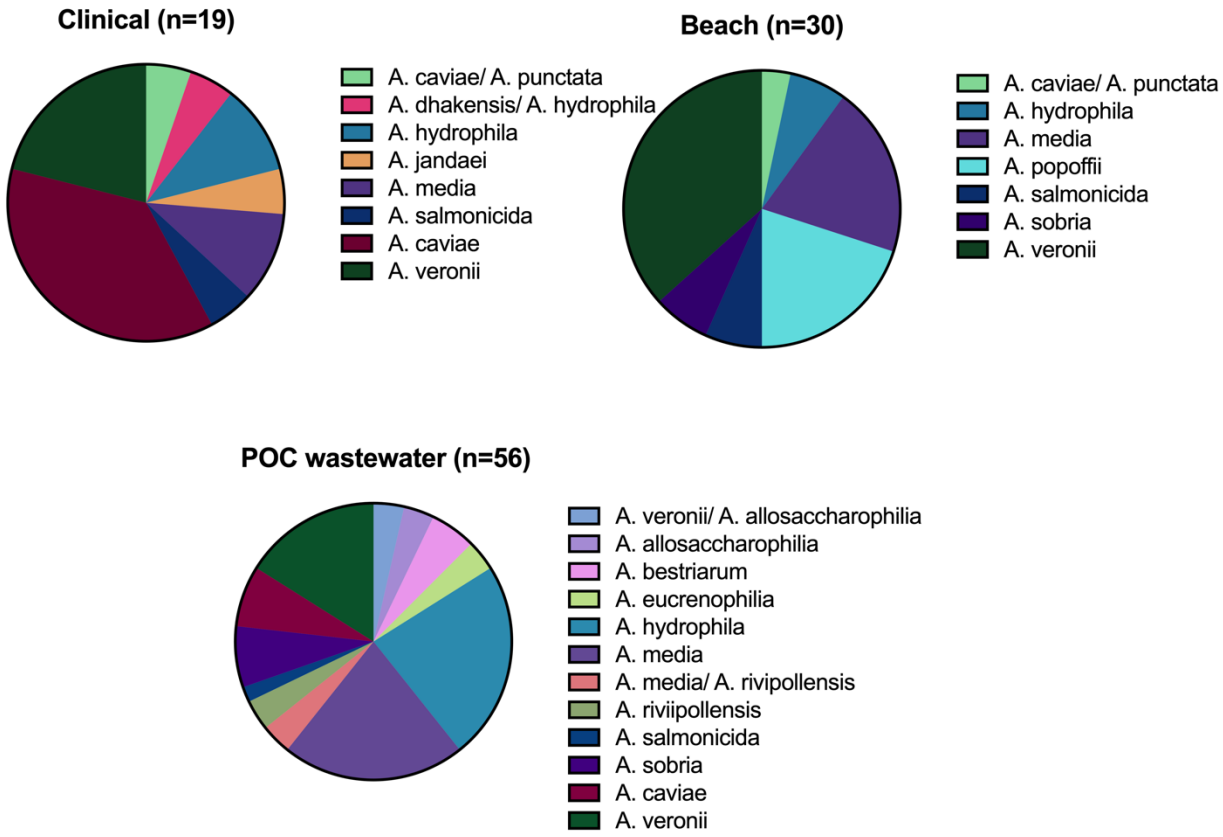


Figure 2. Prevalence of *Aeromonas* species among each source. All clinical isolates (n=19) and POC (n=56) isolates, as well as beach isolates demonstrating AMR to non-intrinsic beta lactam combined with a random subpopulation of the beach isolates (n=30) were identified to

species utilizing the *gyrB* housekeeping gene. The pie charts represent the prevalence of *Aeromonas* species found in each source.

Of the species analyzed, all were determined with greater than 98% similarity against known species using BLAST. Amongst our clinical population, we found *A. dhakensis/A. hydrophila, A. caviae,* and *A. veronii* as the most common species (79%), which agrees with previous findings (24, 79). However, we also found species that are not typically clinical pathogens including *A. jandaei* (5.3%)*, A. media* (10.5%)*,* and *A. salmonicida* (5.3%)*.*

Of the environmental sources, the species diversity of *Aeromonas* populations was found to be the highest in POC wastewater with 10 different species though *A. hydrophila* (23%)*, A. media* (21%)*,* and *A. veronii* (16%) predominated. Of the clinically predominant species, the recreational beaches primarily contained *A. veronii* (37%) and *A. hydrophila* (7%), with *A. media* (20%) and *A. popoffii* (20%) present as well. Together, *A. veronii* and *A. hydrophila* were present in all sources, with *A. hydrophila* and *A. caviae* more common among POC wastewater and clinical isolates. In general, the environmental sources contained similar species commonly found within the clinic, further reinforcing the potential health risks of POC wastewater and recreational beaches.

Figure 3

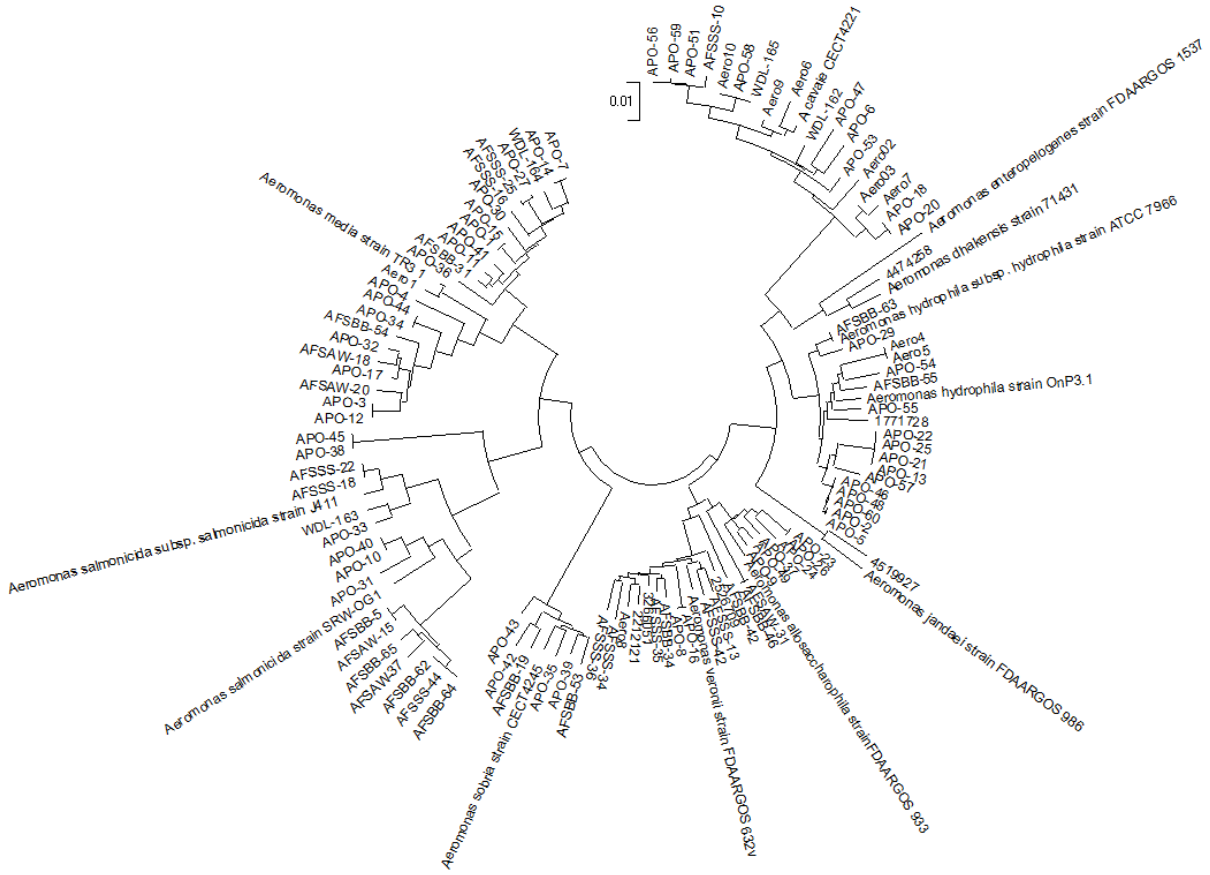


Figure 3. Phylogenetic tree of *gyrB* nucleotide sequence among *Aeromonas* isolates. For all isolates, the *gyrB* sequence was analyzed against reference strains to help determine the species.

The *gyrB* gene sequences of the clinical, beach, and POC wastewater isolates were aligned with reference strains to help differentiate between species when two were found in BLAST. Branch lengths represent genetic distances, with shorter branches indicating more closely related nucleotide sequences.

Table 7. MAR index per species per source

	Clinical (n=19)	Beach (n=31)	POC Wastewater (n=56)	Total (n=106)
A. allosaccharophila			0.182 (2)	0.182 (2)
A. bestiarum			0.121 (3)	0.121 (3)
A. caviae	0.182 (7)		0.192 (9)	0.187 (16)
A. caviae/ A. punctata	0.182 (1)	0.182 (1)		0.182 (2)
A. dhakensis/ A. hydrophila	0.182 (1)			0.182 (1)
A. eucrenophila			0.091 (2)	0.091 (2)
A. hydrophila	0.409 (2)	0.273 (2)	0.238 (13)	0.307(17)
A. jandaei	0.182 (1)			0.182 (1)
A. media	0.09 (2)	0.182 (6)	0.22 (12)	0.164 (20)
A. media/ A. rivipollensis			0.227 (2)	0.227 (2)
A. popoffii		0.091 (6)		0.091 (6)
A. rivipollensis			0.182 (2)	0.182 (2)
A. salmonicida	0.182 (1)	0.091 (2)	0.091 (1)	0.121 (4)
A. sobria		0 (2)	0.136 (4)	0.068 (6)
A. veronii	0.159 (4)	0.053 (12)	0.182 (4)	0.131 (20)
A. veronii/ A. allosaccharophila			0.227 (2)	0.227 (2)

To assess if specific *Aeromonas* species are more common to acquire AMR, the MAR index was determined per species and per source. To obtain a value for a specific species, the MAR index for all species in that source were averaged together. *A. hydrophila* had the highest overall MAR index, 0.307, followed by *A. caviae/A. puncata* and *A. media/A. rivipollensis,* at 0.197 for both (Table 7). Considering the strong association of *A. hydrophila* with clinical infections and common presence in the environment, the high MAR index brings further public health concern.

Characterizing nalidixic acid resistant populations in POC wastewater compared to clinical and beach isolates.

Table 8. Nalidixic acid resistance among clinical, recreational beaches, and post-chlorinated wastewater isolates

	Percentage of isolates with Nalidixic Acid resistance or intermediate resistance		
	Clinical *(N)*	Beach *(N)*	POC Wastewater *(N)*
A. allosaccharophila	0.0 (0)	0.0 (0)	100.0 (2)
A. bestiarum	0.0 (0)	0.0 (0)	33.3 (3)
A. caviae	14.3 (7)	0.0 (0)	0.0 (9)
A. caviae/ A. punctata	0.0 (1)	0.0 (1)	0.0 (0)
A. dhakensis/ A. hydrophila	0.0 (1)	0.0 (0)	0.0 (0)
A. eucrenophila	0.0 (0)	0.0 (0)	0.0 (2)
A. hydrophila	50.0 (2)	0.0 (2)	38.5 (13)
A. jandaei	0.0 (1)	0.0 (0)	0.0 (0)
A. media	0.0 (2)	0.0 (6)	58.3 (12)
A. media/ A. rivipollensis	0.0 (0)	0.0 (0)	50.0 (2)
A. popoffii	0.0 (0)	0.0 (6)	0.0 (0)
A. rivipollensis	0.0 (0)	0.0 (0)	0.0 (2)
A. salmonicida	0.0 (1)	0.0 (2)	0.0 (1)
A. sobria	0.0 (0)	0.0 (2)	50.0 (4)
A. veronii	0.0 (4)	0.0 (11)	100.0 (4)
A. veronii/ A. allosaccharophila	0.0 (0)	0.0 (0)	100.0 (2)
Total	**10.5 (19)**	**0.0 (30)**	**42.9 (56)**

(N) represents the number of isolates in each category

To further investigate the unique abundance of nalidixic acid, a quinolone antibiotic, resistance within wastewater, we identified the species of all nalidixic acid resistant populations. There were only two clinical isolates resistant to nalidixic acid, an *A. hydrophila* and an *A. caviae* (Table 8). *A. veronii/A. allosaccharophilia* were all resistant to nalidixic acid among POC wastewater, while all *A. caviae/A. puncata* (n=9) were susceptible (Table 8). *A. media* and *A. hydrophila* were the most prevalent species among nalidixic acid resistant wastewater populations, demonstrating 50% and 38.5% resistance, respectively (Table 8). The observed resistance in *A. hydrophila* strains from wastewater (38.5%) could suggest a potential origin of infections associated with wastewater, contrasting with the absence of nalidixic acid resistance observed in beach samples (though only two isolates were present). This indicates a possible association between nalidixic acid resistance and POC wastewater sources.

ii. Specific Aim 2

Comparison of *Aeromonas* virulence profiles between clinical, beach, and POC wastewater populations.

Figure 4

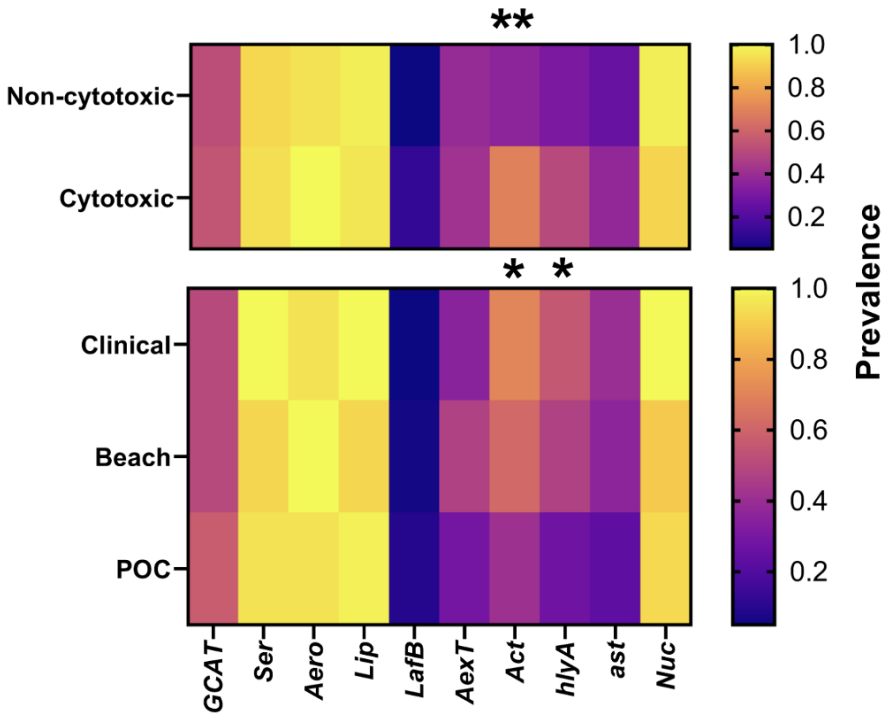


Figure 4. The association of virulence genes with cytotoxicity and within each source. The speciated isolates, clinical (n=19), beach (n=30), and POC (n=56), were analyzed for ten different virulence factors and cytotoxicity against HeLa cells. The heat map represents the prevalence of each virulence factor within the source and cytotoxic phenotype. *P<0.05; **P<0.005

Virulence factors of clinical isolates were compared to environmental isolates to determine the potential of these isolates to cause disease. *Act* and *hlyA* were significantly more prevalent in clinical populations compared to the environment, though some isolates in all sources still encoded both virulence factors. *Act* was also significantly more prevalent among isolates secreting cytotoxic compounds compared to that of non-cytotoxic. Almost all isolates amongst the sources contained serine protease (*ser*), aerolysin (*aero*), lipase (*lip*), and nuclease (*nuc*) genes, while very few isolates contained lateral flagella (*lafB)* (Figure 4). Overall, there were minimal differences seen among most virulence factors between all three sources and cytotoxic potential suggesting they are all potentially pathogenic.

Total virulence factors for most prevalent species:

Figure 5

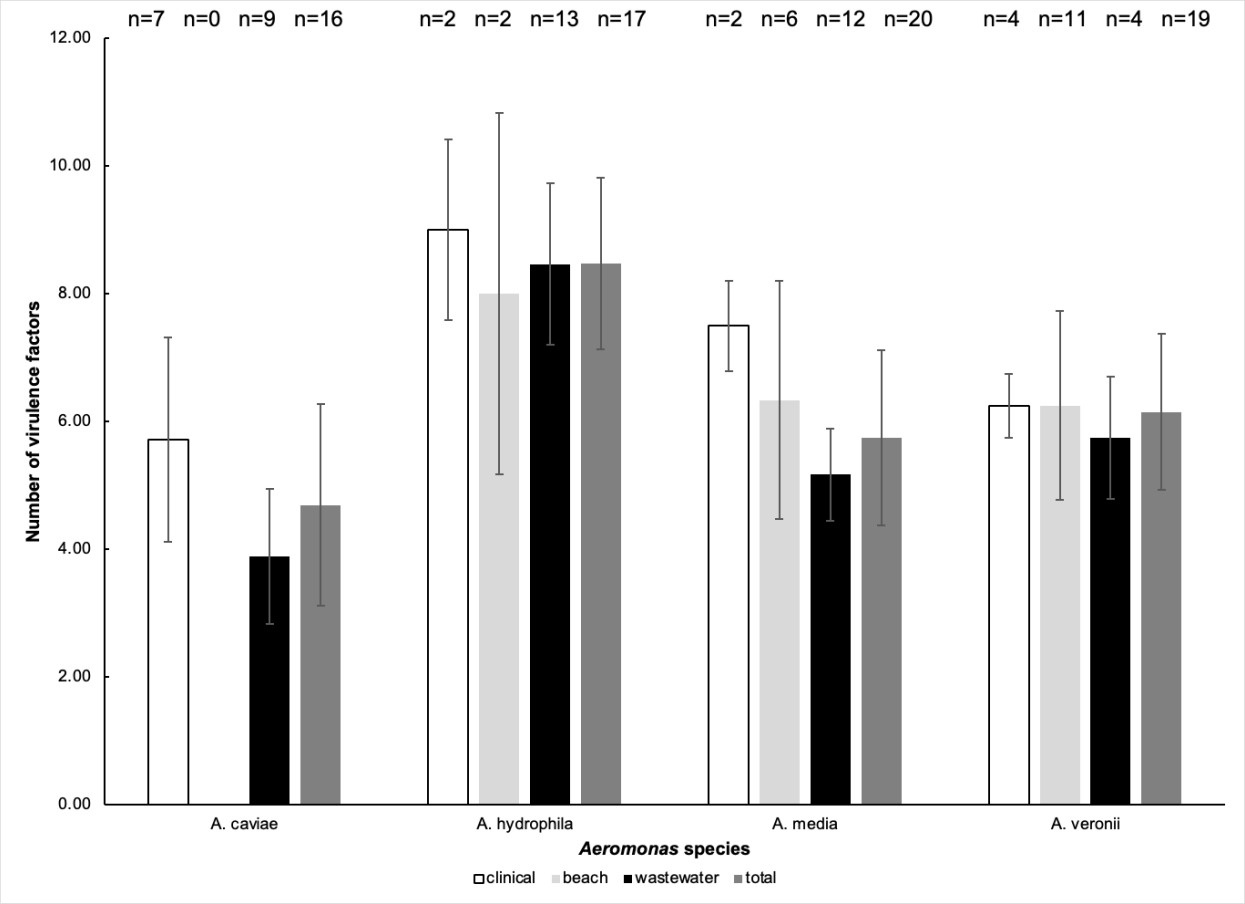


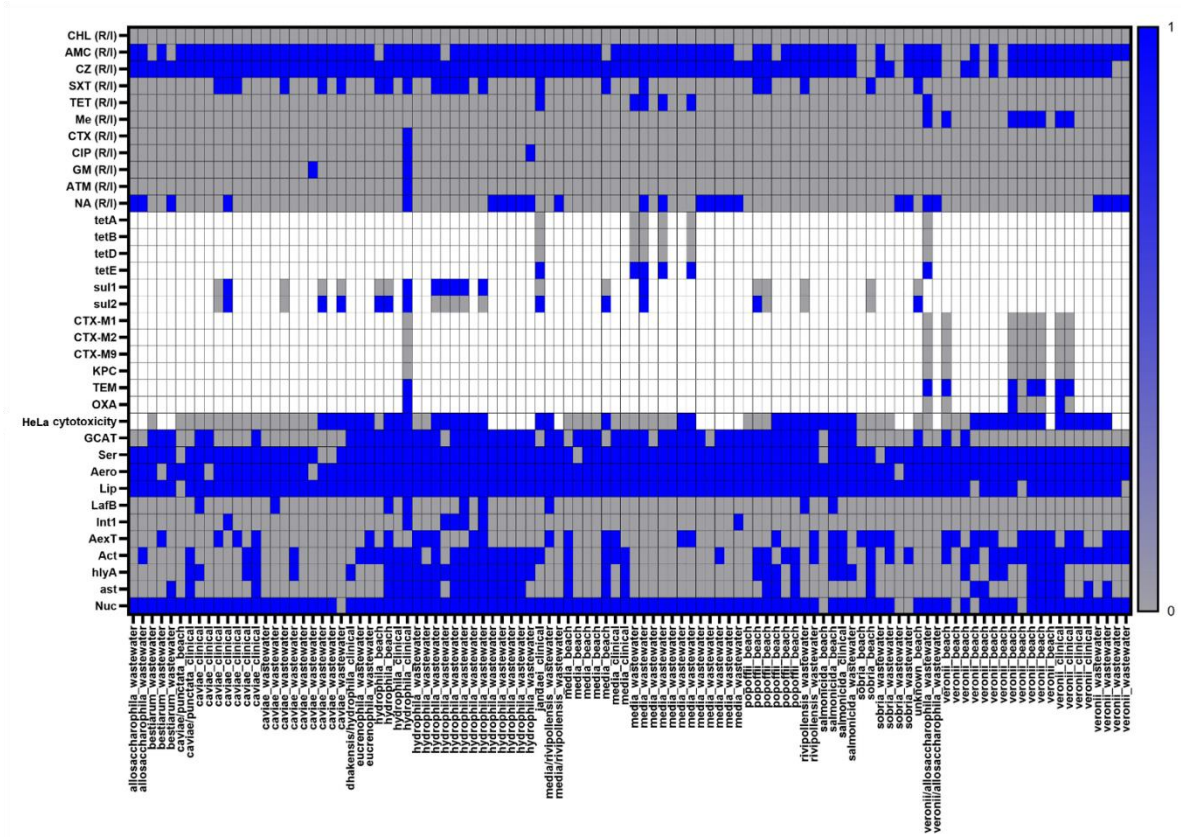
Figure 5. Prevalence of virulence factors among the most common species. The most common species among all sources were determined and the number of virulence factors identified for each species. The bar graphs represent the average number ± standard deviation of virulence factors per species in each source and the combination of all 3 sources. Above each bar, the number of isolates is denoted next to the *n*.

The total number of virulence factors in the four most prevalent species across all sources were analyzed to better understand their pathogenic potential. The predominating species were *A. caviae, A. hydrophila, A. media,* and *A. veronii.* The total virulence genes for each species were analyzed and minimal differences were seen amongst each source and the total (Figure 5). *Aeromonas hydrophila* demonstrated the highest number of virulence factors of

all the species analyzed with the clinic having the highest at 9 and the average of all sources with 8.5 (Figure 5). Interestingly, *A. media* had the second highest number of virulence factors for the clinic (7.5) and beach (6.3), which was unexpected as it is not a typical human pathogen. *A. veronii* had approximately six virulence factors per source, which was the third highest of the four species for the clinic and beach and second highest for POC wastewater. Of note, there were no *A. caviae* on the beaches, but *A. caviae* did appear to have the least amount of virulence factors of the species analyzed (4.7). Therefore, the overall order of total virulence factors among these species was *A. hydrophila* > *A. media* > *A. veronii* > *A. caviae.* There is also a trend with the clinical isolates having more virulence factors compared to the environmental isolates, highlighting their potential relevance in pathogenicity.

Figure 6

a.


CHL (R/I)
AMC (R/I)
CZ (R/I)
SXT (R/I)
TET (R/I)
Me (R/I)
CTX (R/I)
CIP (R/I)
GM (R/I)
ATM (R/I)
NA (R/I)
tetA
tetB
tetD
tetE
sul1
sul2
CTX-M1
CTX-M2
CTX-M9
KPC
TEM
OXA
HeLa cytotoxicity
GCAT
Ser
Aero
Lip
LafB
Int1
AexT
Act
hlyA
ast
Nuc
1
0

b.

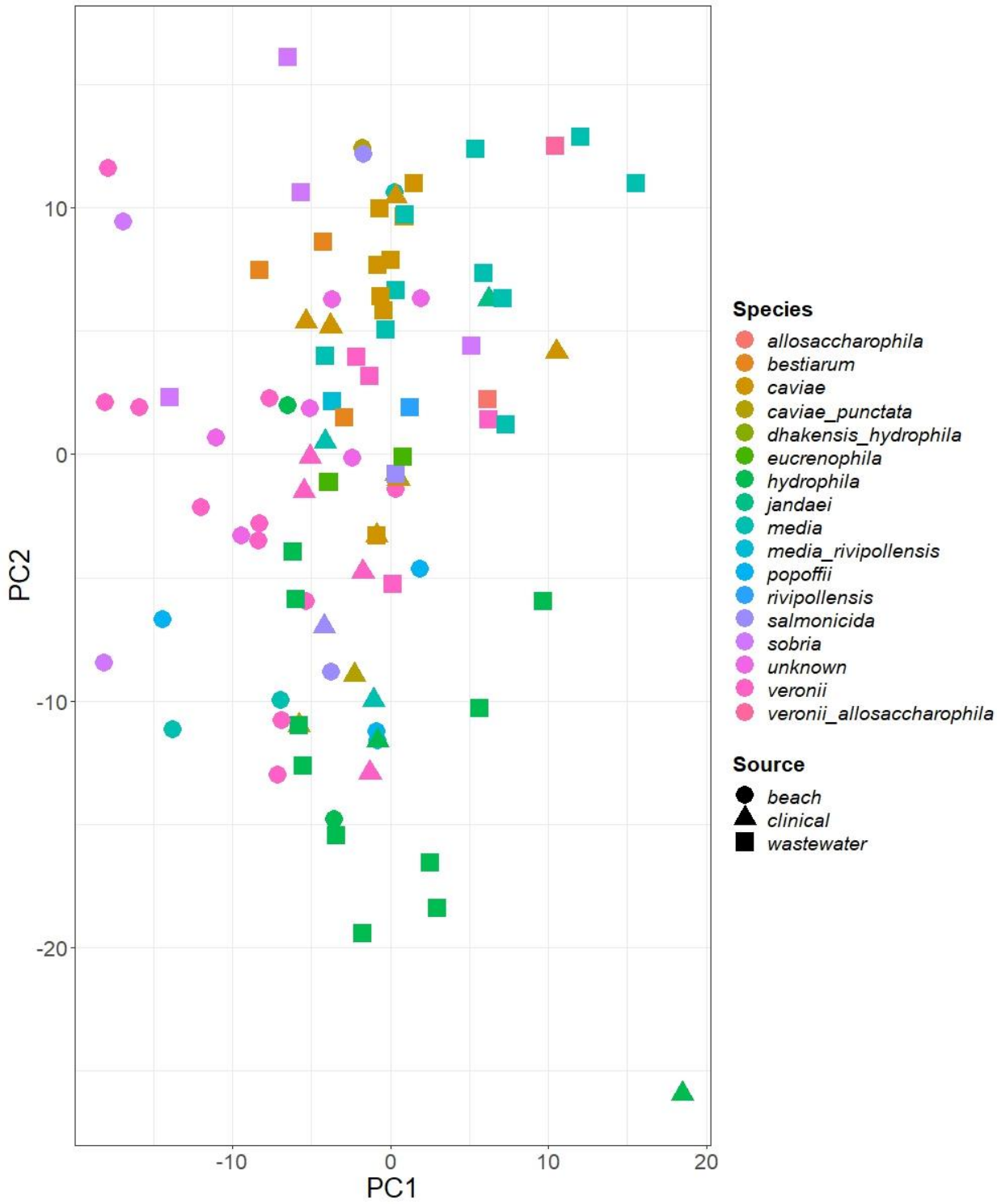


Figure 6. Principal component analysis of antimicrobial resistance, virulence factors, and species among all samples. To construct the plots, phenotypic antimicrobial resistance, corresponding ARGs, virulence genes, cytotoxicity, and species were utilized. If an isolate was not tested for an ARG due to the lack of resistance, this was also reflected in the plots. A. Binary heatmap of each isolate and their associated antibiotic resistance profile (phenotype and

genotype) and virulence factors. Blue boxes are positive, grey is negative, and white was not tested. B. Each point represents an individual isolate within a source. Location of the point was determined by the antimicrobial resistance and virulence factors encoded by each isolate. The color of each point corresponds with the species and shape with the source.

To determine and visualize similarity between the sources, a binary heatmap and a principal component analysis (PCA) were performed. The heatmap contains all isolates along with their phenotypic antimicrobial resistance, corresponding ARGs, virulence genes, and cytotoxicity, enabling a direct comparison between species and sources of *Aeromonas* isolates (Figure 6A). In the heatmap, blue boxes represent positive ARGs and virulence factors, while grey boxes are negatives, and white boxes denote untested isolates. Minimal differences are evident between isolates from various sources and species. The heatmap even shows an *A. caviae* clinical and wastewater isolate with the same virulence genes, *nuc, hylA, act, lip, aero, ser,* and AMR pattern (Figurer 4A). The logistic PCA plot shows each isolate as an individual point, corresponding to the binary heatmap, with point colors indicating the different species (Figure 6B). Therefore, each point accounts for the phenotype and genotype for antibiotic resistance and virulence. If the isolate was not analyzed for specific ARGs due to a lack of resistance, the presence or absence was not included in its PCA. In the logistic PCA plot, the closer the distance between the points being analyzed, the more similar the isolates. Therefore, isolates of the same color in close proximity of different origins supports the similarity of bacterial populations between sources. Overall, the PCA provided 64.2% of the explained variation, further confirming the overlap between environmental and clinical sources (Figure 6B), supporting that the environment could serve as a source of *Aeromonas* infections due to minimal differences.

Chapter 4: Discussion

In this study, we analyzed antibiotic resistance and virulence among presumptive *Aeromonas* isolates from clinical, beach, and post-chlorinated wastewater. The genus

Aeromonas has the potential to serve as an indicator species from a One Health perspective due to its ubiquity in a variety of environments. Specifically, we compared known pathogenic *Aeromonas* clinical isolates to those from recreational beaches and POC wastewater. To our knowledge, no previous studies have characterized *Aeromonas* populations from recreational beaches. Our findings identified overlapping similarities between antimicrobial resistance and virulence gene profiles in environmental and clinical *Aeromonas* isolates. Among all sources, the predominant species were *A. hydrophila/A. dhakensis, A. caviae,* or *A. veronii.* Considering these species are among the most commonly associated with human infections, it stresses the potential risk of infection from the environment. Additionally, we found nearly 50% of post-chlorinated wastewater isolates were resistant to nalidixic acid in post-chlorinated wastewater, whereby complete susceptibility was evident among beach populations and only 2 clinical isolates resistant, suggesting nalidixic acid resistance can serve as an indicator phenotype for wastewater origin. Meanwhile, POC wastewater also had 100% AMR and the highest MDR, with *A. hydrophila* exhibiting the highest MAR index. We also observed if these resistant isolates had the capability to cause disease. Overall, minimal differences were observed in virulence phenotypes and genotypes between isolates from the clinic, beach, and post-chlorinated wastewater among 10 different virulence factors. However, virulence genes *act* and *hlyA* were more associated with clinical isolates and cytotoxicity. Together, these findings support the potential of environmental isolates to cause human disease.

Treated urban wastewater that is returned to the environment in the city of Milwaukee, as in many cities around the world, contains AMR opportunistic pathogens. Previous studies have established that although WWTPs reduce the number of bacteria during the treatment process (16), opportunistic AMR bacteria persist in wastewater even after disinfection, questioning its safety and use even in agricultural irrigation (57). Our study further confirms this as we found the highest level of antimicrobial resistance and multi-drug resistance in our treated wastewater. Beattie *et al*. analyzed hospital sewage, combined influent, pre-chlorinated effluent,

and post-chlorinated effluent wastewater in Milwaukee, Wisconsin and found that *Aeromonas,* among other bacteria, predominated in hospital sewage and combined influent (16). Following wastewater treatment, *Aeromonas* continued to predominate among culturable proteobacteria, comprising approximately 20% of the relative abundance of amplicon sequence variants (16). More concerning, among ceftazidime resistant populations *Aeromonas* accounted for approximately 5% of the total bacterial population (16). While we did not observe this ceftazidime resistance pattern in our isolates, this could be attributed to our analysis focusing on random isolates, whereby this study sequenced all isolates grown on TSA with ceftazidime. This indicates a potential health risk if deposited on beaches and in recreational waters. The persistence of *Aeromonas* in POC wastewater underscores the need for further research into the potential health risk and transmission of wastewater bacteria to human populations.

The contamination of the environment, including recreational beaches, by wastewater poses a potential public health concern. Lake Michigan beaches in Chicago were analyzed and thought to be free of discharge from WWTPs due to the flow of the Chicago River but found evidence of fecal human contamination (80), suggesting wastewater contamination. Studies have also shown that wastewater can influence the bacterial communities in beach sand and water, making them more similar to those found in wastewater, with human activities also being a contributor (81, 82). Recreational swimming areas downstream of wastewater treatment plants increase the potential of exposure to ESBL-producing *E. coli* (83). One study found that *E. coli* concentration in swimming water was related to high concentrations in beach sand (84), including up ato 86,500 times higher levels than nearby recreational swimming water (84). Of note, there are other factors impacting contamination of beach sand like gull feces, which can contain *Aeromonas populations* (85). Additionally, *Aeromonas* has been found in recreational beach sand (86), but it has not yet been characterized for antibiotic resistance. Our study identified MDR was not common in beach sand; however, the high concentration of bacteria in sand makes it challenging to accurately assess the prevalence of MDR because the entire

beach cannot be analyzed. While we did not observe an increase in resistance in the beach, the bacterial population on the beaches may still be influenced by the wastewater discharge. However, due to lakes being larger or lake currents, these wastewater populations may be quickly diluted, making it more challenging to detect these populations on the beach. The detection of resistance within the small population analyzed on the beach suggests more research is needed to further assess resistance, especially to last resort antibiotics.

Understanding the distribution and antibiotic resistance profiles of *Aeromonas* across the clinic is crucial for assessing the potential risks posed by diverse environmental sources, such as wastewater contamination at recreational beaches. A study performed in China analyzed clinical *Aeromonas* strains for 7 years, finding high resistance to extended-spectrum cephalosporins, ciprofloxacin, and trimethoprim-sulfamethoxazole (87). This was reflected in our study for trimethoprim-sulfamethoxazole resistance, but not extended-spectrum cephalosporins and ciprofloxacin.

Comparative analysis of antibiotic resistance profiles among clinical, beach, and post chlorinated wastewater sources revealed some common resistance patterns across all three sources. AMR and MDR were higher in the clinic and wastewater compared to the beach, which is supported by previous research. A study analyzing multiple urban wastewater treatment plants found that antimicrobial resistance of the wastewater mimicked that of the clinic (88). Other studies have further confirmed this higher level of resistance in wastewater compared to other environmental sources like river water, surface water, ground water, and drinking water (61, 89). A study in Poland analyzed *Aeromonas* in river water upstream and downstream of a WWTP, finding MDR populations in 73.3% of treated wastewater samples and 81.8% of downstream river water, but 0% detected in upstream river water (90). This indicates that wastewater entering the environment can impact the bacterial population, elevating resistant populations. Although our study did not observe a similar resistance pattern change, we analyzed beaches bordering a lake, north and south of the WWTP. We did see antibiotic

resistance on the beach, some of which overlapped with resistance in POC wastewater populations. Significant differences existed between tetracycline, nalidixic acid, and SXT amongst the sources, consistent with previous studies (61). Meanwhile, a study of post chlorinated wastewater in South Africa found stronger levels of resistance against important antibiotics like chloramphenicol, gentamicin, and cefotaxime whereby our study found most of the isolates susceptible (91). This could be due to socioeconomic differences between the countries, which has been inversely associated with antimicrobial resistance (Jones, et al 2024 meta-analysis). However, this also further stresses the importance that each wastewater treatment plant should be analyzed for resistance patterns due to variations in treatment, origin of wastewater, and physical parameters such as pH and organic compounds that can have an effect on resistance (10). It has been previously shown that there is bacterial wastewater contamination on recreational beaches, outweighing the contamination from birds (92). Since there were similar resistance and virulence patterns across the sources, this study supports beaches as a potential source of an *Aeromonas* infection due to the close similarity of environmental populations to clinical *Aeromonas* isolates.

Identification of wastewater origin provides a challenge, and our group has consistently seen elevated nalidixic acid in wastewater compared to other sources, suggesting it could serve as an indicator of wastewater origin. In this study, nalidixic acid resistance had the highest percentage in the POC wastewater, but there was no resistance on the beach. Of interest, both POC wastewater and clinical isolates contained nalidixic acid resistance. Numerous studies have found increased nalidixic acid resistance in post-chlorinated wastewater compared to their receiving reservoirs (61, 89). For example, a study in Portugal compared wastewater *Aeromonas* isolates to that of surface water and found that the wastewater isolates were five times more likely to be resistant to nalidixic acid at 90.6% compared to surface water (17.6%) *Aeromonas* (89). Other studies have found low levels, less than 10% of resistance, in clinical and environmental strains (93). Considering that resistance to nalidixic acid is caused by point

mutations that arise in *gyrA,* the higher levels of resistance observed in wastewater could be due to increased mutation rates due to wastewater stressors (94), thus serving as a breeding ground for the acquisition of nalidixic acid resistance. In our study, we found almost all *A. veronii* were resistant to nalidixic acid, suggesting there may be a more complex story of nalidixic acid resistance. Interestingly, another study found clinical data supporting *A. veronii* predominately having resistance to nalidixic acid compared to other species (95). Together, this also suggests that wastewater may have resident *Aeromonas* bacterial populations resistant to nalidixic acid, though higher in some species.

Shared antibiotic resistant genes are evident among clinical, recreational beach, and POC wastewater, underscoring the interconnectedness. In our study, clinically relevant ARGs overlapped with the ARGs in both the beach and POC wastewater. An analysis of ARGs in clinical, tap water, and food in China found that subpopulations of all sources encoded CTX-M, *sul1*, *sul2*, and *mcr-3* (96). This is similar to our study where *sul2* and *sul1* were present, as well as *tetE.*

Aeromonas have been found to have a variety of ARGs, including ESBLs of high clinical importance (97), many of which have been acquired from other Enterobacteriaceae suggesting interspecies horizontal gene transfer. However, in our study, we did not find that many resistant strains against extended beta-lactam drugs, like third and fourth generation cephalosporins. Therefore, we did not find as many ESBL genes, except *TEM* and *OXA*. By spiking the media with these antibiotics, it would allow us to assess if these populations exist in the environment. While this genus of bacteria has been known to carry a variety of ESBLs, including recently identified SFO-1 (98), we only analyzed a few and found OXA and TEM. The presence of these ESBLs were common in a study of *Aeromonas* in treated wastewater , whereby 42.2% of the isolates contained *OXA*, 11.1% had *TEM*, and 2% *CTX* (90).

Class 1 integrons are genetic elements that play a role in multi-drug resistance by facilitating the acquisition and expression of ARGs from exogenous sources (72). These

integrons can encode the *int1* gene that encodes an integrase enzyme allowing for the capture and excision of exogenous gene cassettes, helping facilitate the spread of antibiotic resistance genes (99). In clinical *K. pneumoniae,* of the integron positive isolates, 96% of them had the *int1* gene, demonstrating the high clinical relevance (100). In our study, *int1* was found to be in the clinic and the majority of treated wastewater isolates, consistent with previous findings (90). This prevalence highlights the environmental reservoir of clinically relevant ARGs

Resistance to tetracycline is common among *Aeromonas* isolates due to a variety of genes. One study found tetracycline resistance with the genes *tetA, tetE,* and *tetD* among *Aeromonas* species found at a fish farm (101). This may be due to oxytetracycline being used in aquaculture to decrease disease incidence (102). These resistant bacteria from aquaculture can then be introduced to the environment and contribute to a broader pool of ARGs within the environment. Meanwhile, the study in China analyzing clinical, tap water, and food isolates found no isolates having *tetA, tetB,* or *tetE* in the clinical sample with phenotypic tetracycline resistance (96). This differed from our study where we found *tetE* resistance. Meanwhile, another study of POC wastewater and river water found *tetA* and *tetE* prevalent, while *tetB* and *tetC* were not detected (61). Our study aligned with this considering the absence of *tetB* and *tetC* and the presence of *tetE* but differed with the lack of *tetA* found. The most common tetracycline gene for treated wastewater in one study was *tetE* and tetO (90). Our study did not test for *tetO,* but the presence of *tetE* was the only tetracycline gene found, which is consistent with this work. Future work should include assessing for *tetO* presence among *Aeromonas* populations.

It has been established that the clinically relevant *Aeromonas* species are *A. hydrophila, A. veronii, A. caviae,* and *A. dhakensis* (25). Upon determining the species, 79% of our clinical isolates constituted these species. A study analyzing extra-intestinal and intestinal infections found approximately 92% of their *Aeromonas* isolates were of these four species (103). Concerning is that in our study, the beach had *A. veronii* (36.7%) as the predominating species,

and POC wastewater had *A. hydrophila* (23.2%). Our findings are in agreement with others showing high concentrations of *A. veronii (*51.5%) and *A. hydrophila* (18.2%) among POC wastewater (61), supporting environmental sources can serve as rich reservoirs of clinically relevant *Aeromonas* species.

We did find other species associated with clinical infections such as *A. media, A. salmonicida, and A. jandeai.* A meta-analysis of clinical infections found *A. media* in feces, wounds, blood, and urine, consisting of about 2% of the species analyzed (24). Traditionally a common pathogen among fish and cold-blooded vertebrates, *A. salmonicida* was isolated from a patient in India for one of the first documented cases in 2014 (104). The presence of *A. media* and *A. salmonicida* in both beach and POC wastewater isolates in our study supports a potential source of infection. Although still rare, *A. jandeai* has been found in multiple clinical cases including in stool, blood, and wounds (105). Therefore, while these species may be uncommon in the clinic, they have been recognized to cause human infection and commonly found in environmental sources.

To better predict the public health risk of environmental populations, *Aeromonas* isolates from all sources were tested for virulence factors. While there is no known specific virulence factor profile to correlate directly with pathogenicity of *Aeromonas,* we assessed some of the more common virulence factors including *GCAT, ser, aero, lip, lafB, nuc, AexT, act, hlyA, ast.* Minimal differences in virulence factor profiles existed between environmental and clinical populations suggesting they are all potentially pathogen(45, 106)It is important to consider that not all clinical isolates were cytotoxic, which may be due to them being commensals, this is especially true considering the large number from feces. (45, 106). It is important to consider that not all clinical isolates were cytotoxic, which may be due to them being commensals, this is especially true considering the large number from feces. However, a couple virulence factors, *act* and *hlyA*, were more common amongst the clinical compared to

recreational beaches and the POC wastewater, while *act* was found to be more prominent among cytotoxic populations.

The cytotoxic enterotoxin*, act,* is a well characterized virulence factor in *Aeromonas.* When injected intravenously in mice, *Aeromonas* carrying the *act* gene had a 50% increase in lethal dose compared to that of *Aeromonas* not carrying this gene (107). Additionally, *act* has been found to be a cytotoxic enterotoxin, which causes cell damage (45). Our findings of *act* among clinical and environmental isolates suggest the potential of environmental strains to cause disease. However, the absence of this gene amongst 31.6% of clinical strains adds to the complexity of this gene with virulence.

Cytotoxicity and hemolysis have both been linked with hemolysin A, or *hlyA,* in *Aeromonas.* Previous studies have shown *hlyA* contributes to the destruction of host cells. In combination with aerolysin, mutants lacking these two virulence factors did not exhibit cytotoxicity to buffalo green monkey kidney cells or hemolysis on horse blood agar (76). However, inactivation of aerolysin or *hlyA* alone did not completely eliminate hemolytic and cytotoxic effects (76).This highlights the potential role of *hylA* in pathogenicity of *Aeromonas* and its contribution to virulence.

To identify similarities of isolates from different sources,a PCA was conducted, assessing antimicrobial resistance profiles, virulence profiles, and species for each isolate. There were multiple overlapping isolates from various sources, including one clinical and wastewater isolate of the same species. Additionally, there were similar species overlapping from the clinic and wastewater, the beach and wastewater, and the clinic and beach. Throughout the PCA plot, there is minimal segregation of isolates between sources. This overlap of isolates from different sources further supports the interconnectedness of the three sources. Therefore, the recreational beaches and POC wastewater could potentially serve as sources of future *Aeromonas* clinical infections.

Chapter 5: Future directions and conclusions

The strong similarity of species, antimicrobial resistance and virulence profiles between the clinic with the environment suggest their interconnectedness. Future studies may involve obtaining more clinical isolates to have a greater understanding of the virulence genes and cytotoxicity of the isolates, as well as identifying antibiotic resistance trends. Increasing the sample size of the clinical isolates will allow for a more comprehensive understanding of the genes required to potentially cause disease and the clinically relevant ARG mechanisms. These could be compared amongst various sources to determine an interconnectedness. Some future studies in the lab will try to further dissect this interconnectedness by analyzing other sources of *Aeromonas*, such as food.

The Skwor lab is beginning to analyze *Aeromonas* from food samples. Previous studies have found *Aeromonas* in a variety of foods including raw lamb and chicken meat, fish, shrimp, fruits, and vegetables (108). Some of these food samples were colonized by antibiotic resistant strains of *Aeromonas*. A previous study in China analyzing food samples including fish, shrimp, clams, pigs, chickens, cows, and sheep from multiple supermarkets found *tetE, tetA, aac(6')-Ib, mcr-3, qnrS,* and *TEM* (109). Therefore, by investigating food samples, we can assess the potential impact of *Aeromonas* on food safety due to clinically relevant ARGs.

In conclusion, this study identified key findings regarding *Aeromonas* clinical and environmental isolates. Our major finding was that *Aeromonas* isolates from recreational beaches and POC wastewater in Milwaukee, WI, USA could be a reservoir of human pathogens because we saw minimal differences between antibiotic resistance and virulence profiles compared to clinical isolates. This is also supported by the fact that similar species were evident among all the sources, with the predominating population in each source being a common, clinically relevant species.

Notably, increased nalidixic acid resistance was observed in POC wastewater, particularly associated with *A. veronii*. Our findings that nalidixic acid resistance predominates amongst wastewater *Aeromonas* populations suggest this resistance phenotype could be indicative of wastewater origin if identified in the clinic. More research diving into the origins of this population in wastewater is important, as well as analyzing *Aeromonas* populations from other One Health sectors.

Therefore, the findings in this study underscore the interconnectedness of the environment with the clinical *Aeromonas* populations and the potential health risks associated with environmental sources.

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