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Beta-Lactam Susceptibility Profiles of Bacteria Isolated from the Ozama River in Santo Domingo, Dominican Republic

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Abstract: The spread and contamination of antimicrobial-resistant bacteria in ambient waters is an emerging concern in urban, rural, medical, and industrial settings. A large amount of domestic, hospital, and industrial wastewater discharged directly into the rivers through the different channels can turn them into extensive reservoirs of antibiotic-resistant bacteria. In the present study, surface water samples from three collection sites were analyzed, according to different levels of anthropogenic impacts, along the Ozama River, one of the most important rivers in the Dominican metropolitan area, a source of water and food for human consumption. Seventy-six bacterial isolates were selected based on resistance to beta-lactams, using culture media previously enriched with cefotaxime and imipenem. Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF) subsequently identified them. The isolates covered 12 genera of bacteria; more than 30% were clinically relevant, and 43% had phenotypes classified as multidrug resistance. A total of 10 (44%) presented resistance. However, only seven presented resistance to 3 or more of the 14 groups of antibiotics, considered to be a multiresistant phenotype, which was sequenced using the high-throughput sequencing technique or New Generation (NGS). This study is part of the initiative to understand the profiles of the dangers of multidrug resistance in the metropolitan and rural areas of the Dominican Republic and its possible implications for human health.

Keywords: antimicrobial resistance; multi-drug resistance; Ozama River; Dominican Republic

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1. Introduction

New antibiotics have been developed over the last fifty years to ease the increasing need for effective antimicrobial prophylaxis and therapies [1–3]. The adoption of antibiotics to prevent and treat infections in the animal husbandry industry and their use at sub-therapeutic concentrations are growth promoters for agriculture [4–8]. As a result, they have propelled their abundance in the environment [9]. However, if performed irresponsibly, these practices lead to inadequate disposal of antibiotics and other pollutants directly to essential water sources. These deposits, especially in aquifer systems of developing countries, contribute to the rise of drug-resistant bacteria in aquatic environmental reservoirs such as rivers and lakes [10–12], increasing antibiotic resistance (AR) in clinically relevant bacteria [13]. For this reason, the emergence and spread of antibiotic-resistant bacteria (ARB) and antibiotic-resistant genes (ARG) has become a significant public health crisis worldwide [14].

As a result of the increase in antibiotic prescription and misuse, aquatic systems are now considered critical reservoirs of these genetic elements [5]. However, this is not only due to the misuse of antibiotics; recent studies have demonstrated that these same aquatic environments accumulate substantial amounts of contaminants (such as fecal matter, metals, disinfectants, and hormones) [15]. These same contaminants are involved in selecting resistant bacteria in the environment [16]. As a result, antibiotic-resistant bacteria reside in multiple aquatic environments, and on some occasions, they can enter the food chain through food and water from these environments [17]. Examples of these are *Klebsiella pneumonia*, *Enterococci* spp., *Pseudomonas* spp. which is consistently found as a multidrug-resistant bacteria across many studies and is considered to be extremely detrimental to patients [8]. Multidrug-resistant colonies of these bacteria are now commonly found in environmental waters, now as emergent contaminants themselves [18].

Consequently, ARBs are one of the greatest threats to public health in the 21st century, as described by the World Health Organization [19]. Therefore, international large-scale and local monitoring systems are urgently needed to assess their occurrence in the environment, primarily in water bodies that are essential sources for the survival of humanity. This study unveils the multidrug resistance prevalence and microbiome characteristics of the Ozama River in the Dominican Republic.

The Ozama River is one of the main drainage basins of the Dominican Republic, covering an approximate area of 2847.15 km^2, traversing rural and urban areas and finally discharging its waters into the Caribbean Sea. The river's area of influence has a population density of approximately 3.8 million people [20–22]. The city of Santo Domingo was founded on its banks in 1498 and has relied on the river for its socio-economic development, with its waters used for agriculture, fishing, recreation, and potabilization [23,24]. However, the growth in demand has resulted in increased pollution, with the river receiving sewage and waste from at least 54 waterways, 241 companies, and settlements [25,26]. The high levels of contamination result in the proliferation of water hyacinth and harmful bacteria, including *Escherichia coli*, *Klebsiella* spp., *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Proteus* spp., and *Salmonella* spp., among others [27].

This research aims to describe the antimicrobial susceptibility profiles of the bacterial communities present at three points with different anthropogenic impacts in the Ozama River. This study will deliver results through microbiology techniques and the sequencing of the genomes of the isolates that present higher levels of resistance. In addition, we present the use of the Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) methodology for the identification of bacteria in river samples, which has not been reported before in the Dominican Republic, this being high-value information for the public health community in the country for decision making when it comes to the treatment of bacterial infections [28].

2. Materials and Methods

2.1. Sampling and Physico-Chemical Tests

Our team sampled the rivers in June 2019. Our main goal was to sample the Ozama River (18º28′0.12″ N, 69º52′59.88″ W) in three distinct states: pristine (untouched by humans) (18.552950, −69.822415), which was Point C (Punto 3), moderate human intervention (near a settlement) (18.526683, −69.860283) which was Point A (Punto 1), and considerably intervened (near wastewater disposal conduits) (18.518502, −69.895071) which was Point B (Punto 2) (Figure 1). The sampling was conducted in a time-lapse of six hours per point, each two hours, one replica was sampled. These waters are not treated, all samples were taken directly from the river, and proper sewage and wastewater systems are yet to be implemented. During our sampling, we found animal residues due to an active abattoir; at the time, we could not conclude whether the abattoir was contributing to the river's state.

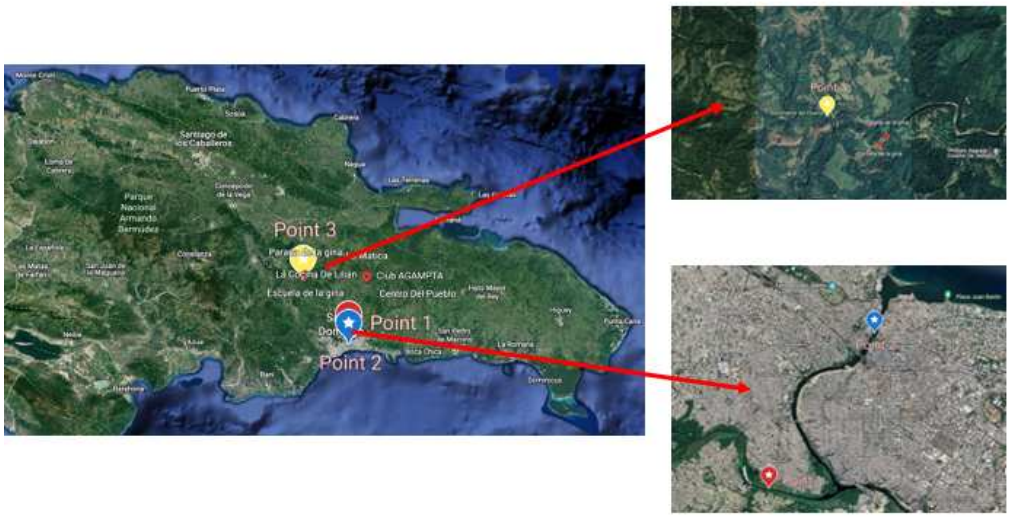

Figure 1. Distribution of collection points in the Ozama River with a 20 km bar distance.

For the first state, we sampled the river source in "Loma Siete Cabezas, Sierra de Yamasá", which covers 148 km and empties into the Caribbean Sea. Our team moved to these in-river locations using a small motorboat. Once well-positioned, we proceeded to take samples. Water was collected in the middle of the river (approximately fifty meters from the banks) with adequately sterilized polypropylene bottles. We collected one liter of water for each replicate and made triplicates (technical repetitions) for microbiological and physico-chemical analyses. We repeated this process for every point we sampled. We chose a community upstream of the city as the moderately intervened point and a wastewater drainage output for the most contaminated. The bottles were kept in an icebox and transported to the microbiology and physical-chemistry laboratories within six hours of collection.

We measured pH in situ with a multi-parameter probe (Chek-Mite pH-20; cat.: Z543047); Chemical Oxygen Demand (COD), Biochemical Oxygen Demand (BOD), Dissolved oxygen, turbidity, total phosphorous, and total nitrogen were the chemico-physical parameters determined, following the procedures described in the book "Standard Methods for the Examination of Water and Wastewater" [29]. We used UV spectrophotometry to determine the turbidity, phosphorus, nitrogen, and chemical oxygen demand (COD), while Biochemical oxygen demand (BOD) was determined through barometry (BOD Track II; cat.: 2952400). We also measured total coliforms in all of our samples. All these analyses and results were provided by the Environmental Chemistry Laboratory of Instituto Tecnológico de Santo Domingo (INTEC).

2.2. Bacteria Isolation

Aliquots of 1, 10, and 50 mL were filtered from the samples using the standardized membrane filtration method using a 0.22 µm diameter cellulose filter (Millipore). The membranes were placed on 2 MacConkey agar media (Oxoid, UK), one with 4 g/mL of imipenem and the other with 8 g/mL of cefotaxime, incubated at 37 °C for 24–48 h, following the recommendation of [10]. The individual colonies were purified on the same media and stored in 25% glycerol at −70 °C. Then, the individual colonies from this process were isolated in chromogenic culture media (ChromoAgar) and stored in 25% glycerol at −70 °C.

2.3. Bruker BioTyper Bacterial Identification

About 0.1 mg of each culture was inoculated into a kit sample carrier (MPS 96 target polished steel), then the samples were coated with 1 µL of matrix solution consisting

of cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trichloroacetic acid and allowed to dry at 25 °C for 15 min. The identification of the isolated strains was performed with the MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) technique (29), with the BioTyperR©3.1 software (Bruker Daltonics, Germany) equipped with the MBT 6903 MPS library (2019), using the MALDI BioTyper Standard Processing Method and the MALDI Biotyper MSP Standard Identification Method adjusted by the manufacturer.

The MALDI-TOF Mass Spectrometry technique was chosen for the identification of the isolates because of its cost-effectiveness relationship. It is generally used in the clinical area, but it has been demonstrated to be effective when working with environmental data as well [30]. In this method, bacterial proteins are ionized and their spectral patterns are collected and compared to databases for identification. The only limitation for this technique, currently, is the need to keep updating databases for increasing sensitivity and accuracy [31], yet there have been studies comparing MALDI-TOF to 16S sequencing and having over 90% similitude [30].

2.4. Antibiotic Susceptibility Test

The selected strains were replicated in chromogenic culture (ChromoAgar) and incubated at 37 °C for 24 h. The susceptibility test was performed based on the minimum inhibitory concentration (MIC) and then classified according to the recommendations of the Institute of Clinical and Laboratory Standards [30].

BD Phoenix ID broth and AST broth were used for sample preparation in conjunction with the BD PhoenixTM AP kit, where the inoculum was standardized from 0.25 to 0.5 according to the required McFarland. The broths with the samples were placed on a BD PhoenixTM NMIC-406 panel for gram negatives, and the automated identification and susceptibility test systems BD PhoenixTM 100 and BD PhoenixTM M50 with the data management system BD EpiCenterTM, were the susceptibility test was carried out at a time interval of 12 to 13 h. Isolates were treated with the following antibiotics: ampicillin, amikacin, amoxicillin-clavulanic acid, ceftazidime, cefuroxime, ciprofloxacin, gentamicin, imipenem, trimethoprim/sulfamethoxazole, meropenem, and ertapenem.

2.5. Data Analysis

The physicochemical parameters were compared with the Quality Standard for Surface Waters and Coastal Zones established by the Ministry of Environment and Natural Resources of Dominican Republic [20] to determine possible relationships between the levels of contamination and the bacteria found. The data were processed through component analysis to identify similarities between sampling sites.

2.6. Genomic DNA Extraction from Isolates

Genomic DNA was extracted from colonies incubated in TSB for 24 h at 35 °C. One aliquot of 4 mL of culture was centrifuged at 8000× *g* for 2 min. The cell pellet was subjected to the QIAamp DNA extraction kit (Qiagen, Germany) with the adaptations indicated next: the bacterial pellet was suspended in 420 µL of the modified lysis buffer (20 µL proteinase K, 200 µL of TSB, and 100 µL of Qiagen's ATL buffer), and incubated for 10 min at 56 °C. The addition of 50 µL of absolute ethanol followed by 3 min incubation at room temperature concluded the adaptations; from this point in the process, the protocol continued according to the manufacturer's recommendations. DNA obtained was suspended in 50 µL of Qiagen's TE buffer. The integrity of the extracted DNA was evaluated in 1% agarose gels stained with SYBR Green and ran at 100 V for 60 min.

2.7. Genome Sequencing, Assembly, and Analysis

For the construction of sequencing libraries, (I) the genomic DNA was randomly fragmented by sonication; (II) DNA fragments were ended polished, A-tailed, and ligated with the full-length adapters of Illumina sequencing, and followed by further PCR amplification with P5 and indexed P7 oligos; and (III) the PCR products as the final construction of the

libraries were purified with AMPure XP system (Beckman Coulter Inc., Indianapolis, IN, USA). Sequencing library size distribution quality control was performed with an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) and quantified by real-time PCR (to meet the criteria of 3 nM). Whole genomes were sequenced using Illumina NovaSeq 600 using the PE 150 strategy at the America Novogene Bioinformatics Technology Co., Ltd., Sacramento, California, United State

Genomes were assembled using the Assembly HiSeq Pipeline, a SnakeMake pipeline to assemble sequencing data produced by Illumina [10]. The pipeline integrates different quality control tools such as FastQC [32] to analyze and visualize read quality, AdapterRemoval v2 [33] for removing sequencing adapters, and KmerStream [34] for computing k-mer distribution. For the genome graph construction, two leading assemblers were used: Edena V3 [35] and Spades 3.9.1 [36]; CD-HIT [37] and Unicycler [38] were used to optimize and integrate the assemblies previously produced. Whole-genome annotation was performed with RAST [39] and Prokka [40]. To predict and reconstruct individual plasmid sequences in the genome assemblies, we used MOB-recon [41]. Finally, QUAST [42] computed assembly quality metrics, and each genome phylogenetic affiliation was confirmed through JSpeciesWS web tools [43] using the contigs generated by the assemblies. All genome shotgun projects have been deposited to DDBJ/ENA/GenBank.

The genomes were uploaded to the RAST [39] annotation server to identify the subsystem of each genome, obtaining information on genes related to different functions, including virulence, pathogenicity, plasmids, and antibiotic resistance. Additionally, pathogenicity and virulence analyses were conducted for each genome through the PathogenFinder tool and the VirulenceFinder tool in the Center for Genomic Epidemiology. In addition, a plasmid identification was performed through the PlasmidFinder tool, also in the Center for Genomic Epidemiology. Finally, the identification of the resistomes through the CARD database Resistance Gene Identifier tool was performed [44]. There were a total of seven different species to describe.

Bioinformatic analyses of the multi-resistant genomes started with Resistance Genes Identifier (RGI) with the CARD protein database [44] and ResFinder-4.0 [45] to predict the resistance genes. Plasmid detection was conducted through the MOB-suite [41] and PlasmidFinder-2.1 [46]. For the pathogenicity classification of each of the strains, PathogenFinder-1.1 [47] was utilized. VirulenceFinder-2.0 [48] was used to determine the virulence factors of each genome. The serotypes of the *E. coli* genomes were determined using SerotypeFinder-2.0 [49], and the number of mobile elements was determined by MobileElementFinder [50].

All genomes were properly deposited on NCBI's genome database with Accession number: JAGJVC000000000, JAGJVD000000000, JAGJVE000000000, JAGJVF000000000, JAOPFR000000000, JAOPFS000000000, SAMN18612605.

3. Results

3.1. Physical, Chemical, and Biological Parameters of Water

We performed a series of analyses described by the Environmental Standard for Surface and Coastal Zones Water Quality [51] for acceptable physical, chemical, and biological limits. For this study, we classified surface waters based on the current or potential use of its waters: Class A, these waters are suitable for vegetable irrigation, recreational uses with direct contact (e.g., swimming), and human and animal consumption without previous treatment; and class B waters, which could serve as a public drinking water supply, irrigation of crops, industrial uses, and livestock maintenance, given adequate treatment [51]. Our research team sampled the Ozama River in June 2019; this predates the COVID-19 pandemic, which will give us a baseline for our subsequent surveillance studies. Due to our sampling nature, we did not assess seasonal microbiome profile variation. However, it is fundamental to note the consistent temperature in these regions, as they usually fluctuate between 30 °C and 35 °C throughout the year. During our sampling, we did not record any rains.

Our assays consisted of nine measurements, one microbiological and eight physicochemical, based on the Environmental Standard for Surface and Coastal Zones Water Quality. We described these assays in Table 1.

We concluded that sampling points A and B could be classified as Class B Water Quality based on our results. We also provided evidence suggesting point C corresponds to a Class A Water Quality. However, we also encountered evidence for all three points suggesting contamination: our assays revealed that Point C had considerably altered (25% higher) chemical oxygen demand (COD) than recommended standards and higher than our two other sampling sites. We also evidence higher biological oxygen demands (BOD) for these points, as they were over two orders of magnitude above recommended values for one sample. Our total coliform concentration is also at least twice the recommended values for most samples.

Table 1. Physicochemical and microbiological results of water sampled from Ozama River.

Point	Parameter	Mean	Standard Deviation	Permitted Value	Units
A	Temperature	33.3	2.49	NA	°C
	pH	6.75	0.24	6.5–9.0	None
	BOD	17.04	6.88	5.0	mg/L
	COD	30.66	12.36	300.0	mg/L
	TP	1.0	0	0.025	mg/L
	TN	2.4	1.2	30.0	mg/L
	fDO	6.98	0.19	>5	mg/L
	Turbidity	2.39	0.024	Und	NFU
	Coliforms	7700	711.8	1000	CFU/100 mL
	Water Class	NORDOM B			
B	Temperature	31.67	0.94	NA	°C
	pH	8.14	0.87	6.5–9.0	None
	BOD	67.81	3.87	5.0	mg/L
	COD	125.0	4.32	300.0	mg/L
	TP	1.0	0	0.025	mg/L
	TN	2.7	0	30.0	mg/L
	DO	6.79	0.074	5	mg/L
	Turbidity	6.69	0.84	Und	NFU
	Coliforms	7166	1027.4	1000	CFU/100 mL
	Water Class	NORDOM B			
C	Temperature	29.3	0.47	NA	°C
	pH	7.52	0.021	6.5–8.5	None
	BOD	163.0	53.78	2.0	mg/L
	COD	163.0	53.78	150.0	mg/L
	TP	1.0	0	0.025	mg/L
	TN	1.2	0	20.0	mg/L
	DO	6.79	0.074	6.4	mg/L
	Turbidity	1.07	0.24	Und	NFU
	Coliforms	2100	535.41	1000	CFU/100 mL
	Water Class	NORDOM A			

Abbreviations used. BOD: Biochemical oxygen demand; COD: Chemical Oxygen Demand; TP: Total Phosphorous, TN: Total Nitrogen, DO: Dissolved Oxygen.

3.2. Composition and Distribution of the Isolates

Our isolation methodology consisted of an initial antibiotic-based selection and culturing in differentiation media. First, we performed the selection phase with MacConkey agar supplemented with antibiotics: cefotaxime (CTX) and imipenem (IMP), to screen for resistance. This methodology yielded seventy-six isolates. Next, our team performed colony isolation using chromogenic agar, where five colonies were taken from each sample. Using this method, we recovered forty isolates from media initially selecting CTX resistance and thirty-six isolates from IMP media. Finally, we isolated most of our specimens from Point B, totaling twenty-eight colonies, while Point A and Point C combined yielded twenty-four isolates.

The most frequent genus in isolates was *Stenotrophomonas* spp. (46%), the predominant species was *Stenotrophomonas maltophilia* (n = 33), followed by *Pseudomonas* spp. (23%), with *Pseudomonas protegens* being the most abundant species (n = 4), the Acinetobacter genus (22%) with the highest presence of *Acinetobacter baumannii* (n = 7), then the *Klebsiella* genus (5%), with the *Klebsiella pneumoniae* (n = 4), and Escherichia genus (4%) with *Escherichia coli* (n = 3), representing the smallest number of isolates found (Figure 2).

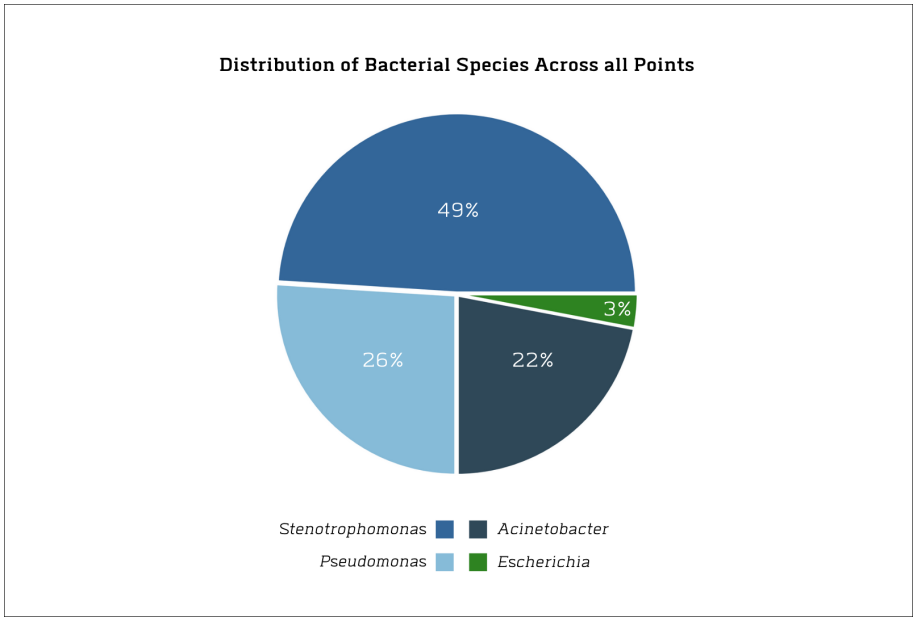

Figure 2. Distribution of the most frequent bacterial genera found in all points of the Ozama River.

The region that presented the most variety was the one corresponding to the River Delta (Point B) with five genera, in which the most abundant were the *Stenotrophomonas* (47%) and the *Pseudomonas* (21%), standing out for the presence of the *Klebsiella* (14%). The Cañada de la Rubia region (Point A) presented four genera, of which the most abundant genera were *Stenotrophomonas* (50%) and *Pseudomonas* (25%). The River Source (Point C) presented only three genera corresponding to *Stenotrophomonas* (42%), *Pseudomonas* (25%), and *Acinetobacter* (33%), this last genus was the most abundant at this point compared with its presence in Points 1 and 2 (Figure 3).

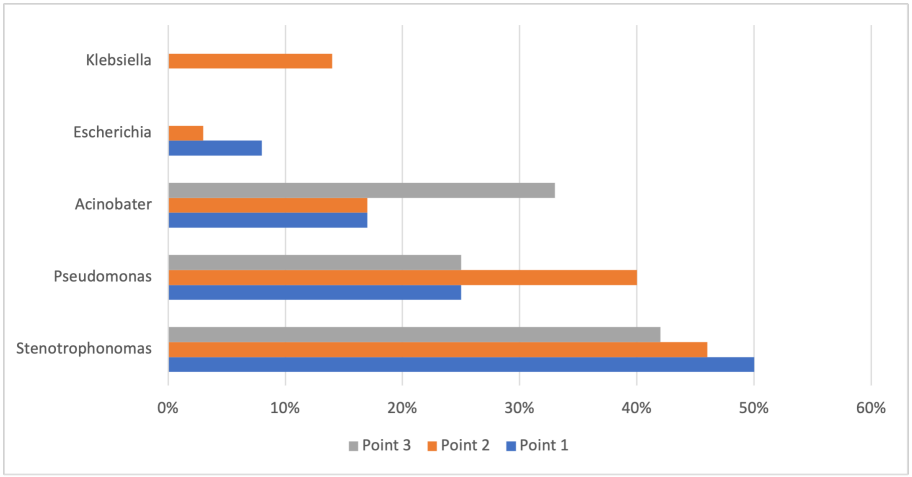

Figure 3. Distribution of the bacteria genera present in each of the monitored points in the Ozama river basin.

3.3. Antibiotic Resistance

A total of ten (44%) of the twenty-three isolates presented resistance. However, only seven presented resistance to 3 or more of the 14 groups of antibiotics, considering themselves to be a multi-resistant phenotype (Table 2). Klebsiella (five isolates) and Escherichia (one isolate) presented substantial phenotypic variety; we measured resistance to seven antibiotics, especially cephalosporins. Additionally, these isolates tested positive for broad-spectrum beta-lactamase genetic markers, among other antibiotic-resistance genes. We found similar resistance diversity to those presented in previous studies based on antibiotic resistance assays performed with ciprofloxacin, gentamicin, and trimethoprim/sulfamethoxazole [9].

Table 2. Resistance phenotype of the different isolates of clinical interest obtained from the Ozama river.

ID	Species	Resistance Phenotype
DC1	*Acinetobacter baumannii*	No resistance
DC2	*Escherichia coli*	AM, CZ, FEP, CRO, SXT
DC5	*Acinetobacter baumannii*	No resistance
DC8	*Escherichia coli*	AM, SAM, CZ, FEP, CRO, CIP, SXT
DC9	*Acinetobacter baumannii*	No resistance
DC10	*Acinetobacter pittii*	No resistance
EC1	*Klebsiella pneumoniae*	SAM, CZ, FEP, CRO, CIP, GM, SXT
EC4	*Klebsiella pneumoniae*	SAM, CZ, FEP, CRO, CIP, GM, SXT
EC6	*Escherichia coli*	SAM, CZ, FEP, CRO, CIP, GM, SXT
EC7	*Klebsiella pneumoniae*	SAM, CZ, FEP, CRO, CIP, GM, SXT
EC11	*Acinetobacter baumannii*	No resistance
EC13	*Acinetobacter baumannii*	No resistance
EC14	*Acinetobacter baumannii*	FEP, IPM
EC15	*Acinetobacter baumannii*	MEM
EC16	*Acinetobacter baumannii*	No resistance
EC16.2	*Klebsiella pneumoniae*	SAM, FEP, CRO
FC1	*Acinetobacter pittii*	No resistance
FC4	*Acinetobacter baumannii*	No resistance
FC5	*Acinetobacter baumannii*	No resistance
FC6	*Acinetobacter baumannii*	No resistance
FC7	*Acinetobacter baumannii*	FEP, MEM
FC8	*Acinetobacter baumannii*	No resistance
FC12	*Acinetobacter baumannii*	No resistance

Abbreviation: Ampicillin, AM; Ampicillin/Sulbactam, SAM; Cefazolin, CZ; Cefepime, FEP; Ceftriaxone, CRO; Ciprofloxacin, CIP; Gentamicin, GM; Imipenem, IPM; Meropenem, MEM; Trimethoprim-Sulfamethoxazole, SXT.

The phenotypes described in our study indicate that the highest level of resistance to multiple antibiotics is at Point 2, corresponding to the region of the River Delta. The abundance of resistance to ampicillin/sulbactam, cefazolin, ceftriaxone, gentamicin, and trimethoprim/sulfamethoxazole was more than two times greater than in Point 1; therefore, there exists the possibility of positive selection for these phenotypes. Our results show no significant resistance patterns to most antibiotics, except for cefepime and meropenem (Figure 4).

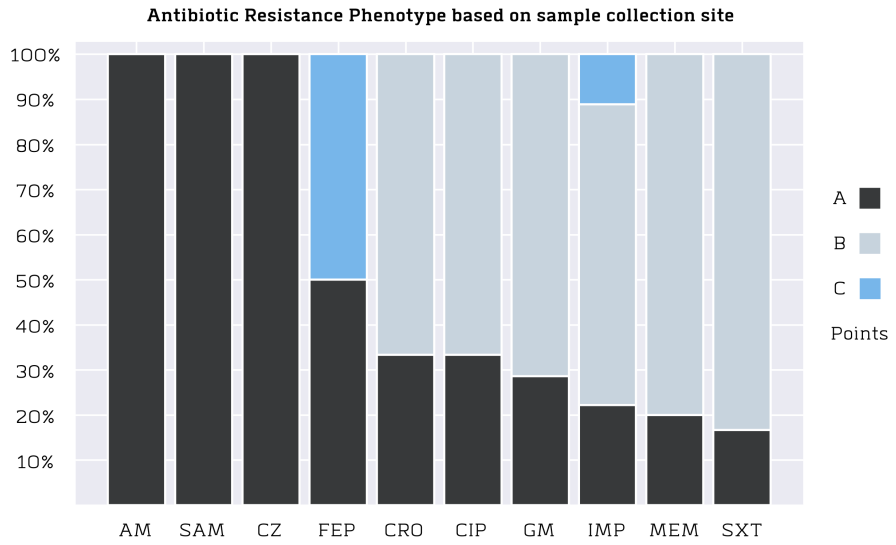

Figure 4. Distribution of resistant isolates at different sampling points. See Table 2 for abbreviations.

3.4. Analysis of Multi-Resistance Genomes

In order to describe resistomes found in these waters, we proceeded with whole genome sequencing of our isolates. We sequenced and assembled seven genomes. Our pipeline consisted of the following steps: quality control, data pruning, and de Bruijn graph assembly. Our team built an in-house assembly pipeline for this analysis. We named our genomes DC2, DC8, DC10, EC4, EC7, FC5, and FC7, as displayed in our Table 3.

3.4.1. *Escherichia coli*

Two *E. coli* isolates were sequenced (DC2 and DC8). Some of the computed parameters were the genome size, GC% content, and total predicted coding sequences (CDS). The isolates presented an average genome size of 4.6 Mb with a GC% content of 51%, and between 4637 and 4664 CDS, as seen in Table 3.

Table 3. Major genomic characteristics of genomes obtained from Ozama river.

Sample ID	Species	Genome Size (pb)	GC (%)	CDS	N50	Plasmids
DC8	*Escherichia coli*	4,710,092	50.7	4664	148,992	IncY, IncFIB
DC2	*Escherichia coli*	4,657,304	51.1	4637	56,323	IncY
DC10	*Acinetobacter pitii*	3,907,124	38.6	3705	245,032	-
EC4	*Klebsiella pneumoniae*	5,527,705	57.2	5457	201,357	IncFBI(K), IncFII(K), Col440II
EC7	*Klebsiella pneumoniae*	5,424,764	57.3	5346	320,811	IncFBI(K), IncFII(K0), Col440II
FC5	*Acinetobacter baumanii*	3,809,530	38.9	3622	324,095	-
FC7	*Acinetobacter baumanii*	3,841,875	38.9	3660	323,883	-

A total of 107 CDSs were related to virulence, disease, and defense. PlasmidFinder1.1 found IncY plasmids sequence, and VirulenceFinder2.0 results presented a significant amount of virulence factors, including terC, traT, neuC, capU, ipfA, and iss. Among the Antibiotic Resistance Genes (ARGs) found in the sequences with perfect hits were msbA, acrB, acrA, mdtH, QnrS1, marA, tolC, mdtG, dfrA12, aadA2, sul1, baerR, and H-NS. They presented resistance to antibiotic classes such as penem, sulfonamide, fluoroquinolone,

and carbapenem. The ARG mechanisms found were antibiotic efflux, antibiotic target alteration, antibiotic inactivation, antibiotic target protection, antibiotic target replacement, and reduced antibiotic permeability, as seen in Table 2. Serotype classification for these genomes was O104, O8, O9, O9a, H11, and H30. According to PathogenFinder1.1, these isolates have a probability of 94% of being human pathogens.

3.4.2. *Pseudomonas putida*

One Pseudomonas was sequenced. The isolate presented a genome size of 5.5 Mb with a GC% content of 63.4%, and a total of 5234 CDS as seen in Table 3. Approximately 1% of the genes were related to virulence, disease, and defense. In addition, there were found resistances to fluoroquinolone and tetracycline in the resistome. Some of the identified genes were adeF and Acinetobacter baumannii abaQ. PathogenFinder1.1 calculated that this isolate has a 17.4% probability of being a human pathogen. This genome did not present any mobile genetic element (MGE).

3.4.3. *Acinetobacter pittii*

Only one isolate was obtained. This isolate contained a 3.9 Mb size with a 38.6% of GC content and 3705 CDS, where 43 were related to virulence, disease, and defense. According to the Resistance Genes Identifier, only nine strict hits were identified, these were ADC-15, OXA-421, adeF, abaQ, parC, AmvA, AbaF, and LpsB. Resistance to fluoroquinolone, tetracyclin, cephalosporin, macrolide, carbapenem, penam, aminocoumarin, Fosfomycin, and acridine dye was found. The resistance mechanism detected included antibiotic efflux, antibiotic inactivation, reduced permeability to antibiotics, and antibiotic target alteration. According to PathogenFinder1.1, this isolate had a 92.5% probability of being a human pathogen. No MGE were found in this genome.

3.4.4. *Klebsiella pneumoniae*

We sequenced two *K. pneumoniae* isolates. These genomes had an average size of 5.47 Mb with an average of 57.25% of GC content. Additionally, the CDS ranged between 5346 and 5457, where 136 were related to virulence, disease, and defense. There were nine perfect hits and 30 strict hits in the resistome identifier. Among the perfect hits, there were resistances to fluoroquinolone, glycylcycline, tetracyclin, carbapenem, diaminopyrimidine, nitrofuran, aminocoumarin, cephalosporin, penam, sulfonamide, penem, monobactam, rifamycin, and macrolide. Among the identified genes were oqxA, LptD, KpnF, SHV-28, sul2, TEM-1, CTX-M-15, QnrB1, and OXA-1. The resistance mechanism included antibiotic efflux, antibiotic inactivation, antibiotic target replacement, antibiotic target protection, antibiotic target alteration, and reduced antibiotic permeability. Among the mobile genetic elements, there were Inc and Col plasmids sequences. IncFIB(K), IncFII(K), and Col440II were found. According to PathogenFinder2.0, these genomes had approximately 89.4% probability of being a human pathogen. Finally, there were virulence factors identified by VirulenceFinder2.0 that included iutA, traT, fyuA, and irp2.

3.4.5. *Acinetobacter baumanii*

Two A. baumanii isolates were obtained. These genomes had an average size of 3.8 Mb with an average GC content of 65%. The CDS were between 3660 and 5346, where approximately 115 were related to virulence, disease, and defense. According to the Resistance Genes Identifier, there were four perfect hits and 12 strict hits. Among the perfect hits there were adeL, abeS, adeK, and adel. These genes included resistances to fluoroquinolone, tetracycline, macrolide, aminocoumarin, lincosamide, carbapenem, cephalosporin, rifamycin, diaminopyrimidine, phenicol, and penem. The resistance mechanisms of these genomes included antibiotic efflux, antibiotic inactivation, antibiotic target alteration, and reduced permeability to the antibiotic. According to PathogenFinder1.1, these isolates had approximately 88.5% probability of being a human pathogen. These isolates had no mobile genetic elements identified by PlasmidFinder2.0.

4. Discussion

The prolonged interactions between the Ozama River, its communities, and surrounding industries evolved into a harmful reservoir for antibiotic resistance in the Dominican Republic. Although this interaction was crucial to the development of the city of Santo Domingo, prolonged anthropogenic influences from the surrounding population are now evident through contamination.The abnormally high anthropogenic environmental impact causes the proliferation of water hyacinth, an invasive aquatic plant that develops in highly contaminated water basins [52]. Most of the contamination comes from organic matter, which causes the excessive proliferation of bacteria in these waters. Our research allowed us to describe multi-resistant bacteria in these aquatic habitats; nevertheless, their growth mechanisms, origin and prevalence remain to be studied. We demonstrated the quality of this river as a wastewater dump for its residents. Therefore, the ever-increasing population surrounding the perimeter of the river has become a public health and sanitary challenge, as delivering treated water properly to these settlements has not been possible.

The Ozama river has many tributaries; the Cabon River, researched by [53], where the microbiological tests found concentrations of fecal coliforms above the maximum allowed by the Dominican Standard Surface Waters Norm [51]. The Ozama river's *Enterobacteriaceae* count was estimated to be 70–70,000 CFU/100 mL in 44% of the samples obtained from the river; in the other 56%, the values were untouchable due to high microbial prevalence. This study also detected *Streptococcus* spp., *Salmonella* spp., *Shigella* spp., *Klebsiella* spp., *Alteromonas* spp., *Enterobacter* spp., and *Pseudomonas* spp. in the Ozama river samples.

Perturbation in these environments can result in a higher prevalence of antibiotic-resistant bacteria and resistance genes [9]; these modifications may pose a significant danger to human and environmental health. Our study explored and described the antimicrobial resistance composition in three contrasting areas of the Ozama River. Our primary focus was antibiotic-resistant Gram-negative bacteria of clinical relevance.

Comparing to a study that evaluated antibiotic resistance in numerous rivers from the United States, a country with a higher antibiotic use control, we could see some comparable resistance profiles [54]. Considering the Dominican Republic has a 33rd part of the USA population and we only evaluated one river, this can be alarming for the public health system and a much more strict control of antibiotic use should be evaluated.

4.1. Physico-Chemical and Microbiological Analyses

Our research team performed physico-chemical analyses as the Standard Methods for the Examination of Waters and Wastewater dictated. Our results indicate that Biological Oxygen Demand values remain within acceptable limits in all points, these levels of BOD are uncommon for a densely populated area with a poor-to-nonexistent sewage system. Furthermore, as these settlements do not have access to a proper sewage system, dumping all of their wastewater directly into the river as a result, we expected lower levels of BOD. It is of importance to make note of the presence of an active abattoir on their banks.

Many restoration projects have taken place on the Ozama River. Among them is OceanCleanup's Interceptor 004 initiative [55], a very ambitious project to remove debris from the river. However, a proper follow-up to this project could examine the source of these strains, and if resulting in antibiotic abuse, which is most likely, there would be evidence to implement more strict policies around antibiotics and their use. Nowadays, all families can buy antibiotics directly from any pharmacy without a prescription. The frictionless acquisition of antibiotics by uneducated patients exacerbated our nation's position against antibiotic resistance during the pandemic, as these individuals attended to their viral infections and preventive needs with antibiotics. The deleterious impact of this popular practice has been stressed by many studies in recent years [56–58].

Our isolates and their peculiar distribution are not that surprising, as these are the same bacteria commonly found in infected patients at hospitals around the city, especially in low-income districts and neighborhoods. Infections by *Acinetobacter, Escherichia*,

and *Pseudomonas* are among the most common in clinical settings, and their resistance phenotypes can only suggest a continuous interaction with antibiotics.

Of all the isolates, those particularly dangerous in hospitals, nursing homes, and for patients whose care requires devices such as ventilators and intravenous catheters [59] (*Klebsiella*, *Escherichia*, and *Acinetobacter*) have undergone sensitivity tests. The genus *Stenotrophomonas* was not considered for testing because it is intrinsically multi-resistant despite being an opportunistic human pathogen [60]. Considering the distribution of our isolates, we compared their distribution to the National Laboratory of Public Health's, we found that both *Klebsiella pneumoniae* and *Escherichia coli* are presented as the most common pathogens.

A previous study [9] examined the dissemination of resistance to beta-lactams in the bacterial communities present in the Isabela River, the main tributary of the Ozama River. This tributary is also highly impacted by anthropogenic action. This study found that the most common genera in the Ozama's main tributary were *Acinetobacter* spp. (44.6%) and *Escherichia* spp. (18%). We also identified twenty clinically relevant bacteria isolated in urban areas of the tributary; these isolates presented the following genes: KPC-3, OXA-1, OXA-72, OXA-132, CTX-M-55, CTX-M-15, and TEM-1. All of these genes are directly responsible for antibiotic resistance to beta-lactams.

4.2. Genomic and Resistome Analyses

Our genomic analyses allowed us to identify our isolates through phylogenetics and discover the virulence factors, antibiotic-resistance genes, and essential features they harbor. These analyses mainly consisted of reconstructing each individual's genome to extract coding regions and compare them to known antibiotic resistance genes through genome alignment and annotation.

Our pipeline effectively identified regions of interest in our samples, ranging from ARG-harboring plasmids to pathogenic toxins and other antibiotic-resistance genomic features. Our main finding through this methodology is the extraordinary amount of pathogenic bacteria harboring antibiotic-resistance genes.

Isolate DC8, an *Escherichia coli* found in waters close to many residential zones, harbors resistance genes against beta-lactamases, fluoroquinolone, and cephalosporins. This is also the case for isolates EC1, EC4, EC6, and EC7, all recovered from the Ozama river's delta.

We also detected plasmids as part of our pipeline: DC8 harbored plasmids IncY and IncFIB. IncY commonly carries the antibiotic resistance gene CTX-M-15, a serine beta-lactamase, more specifically, a cefotaximase. This ARG is one of the most common causes of resistant infections worldwide. The other plasmid, IncFIB, is commonly associated with gene blaIMP, which transfers resistance to last-resort carbapenem antibiotics.

As we described, it is crucial to properly attend to these genes, plasmids, and other genomic features in the urban waters of Santo Domingo. Furthermore, inadequate wastewater disposal into this river can and ultimately will cause severe deterioration to the population and settlements that depend on this water resource. Therefore, it is paramount that we, the researchers, notify appropriate authorities of our results and be of assistance in generating new policies regarding monitoring, assessment, and continuous care of our environment and water supply.

5. Conclusions

In this study, we identified genera such as *Klebsiella*, the isolate that presented the most multiresistant phenotypes in the Ozama River samples. On the other hand, the *Acinetobacter* genus is the most abundant in terms of bacteria of clinical importance, but it is the one with the least resistant phenotype.

Our analyzes conclude that the Delta region of the river (Point 2) is the one that presents the greatest variety of bacterial genera and is the region with the greatest resistance phenotype, as well as abundance and multiresistant bacteria.

Continuing studies of this type are essential to understand the different profiles of resistance to antibiotics that are present in aquifers since approximately two billion people obtain their water from low-quality reservoirs throughout the world. These reservoirs consist of water contaminated with anthropogenic contaminants such as antibiotics, disinfectants, and other substances used to treat water in low-income societies and unregulated industries. Furthermore, the exposure of these antibiotics to drinking water reservoirs promotes the increase of clinically relevant bacteria with antibiotic resistance capabilities, becoming a public health crisis. Therefore, treating patients with infectious diseases and providing prophylactic measures against conditions associated with infection (such as post-surgical recovery) has become increasingly difficult. Based on this evidence framework, it is of considerable importance to analyze the bacterial profiles of rivers that are victims of anthropogenic pollution since these investigations on antibiotic resistance will, of course, benefit the lives of humans.

As for the limitations of this study, it is important to mention that the accuracy of the MALDI-TOF results relies on how robust the spectral database is, as well as the water flood changing everyday, which makes it hard to replicate the results.

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Abbreviations

The following abbreviations are used in this manuscript:

ARB	Antibiotic-resistant bacteria
ARG	Antibiotic-resistant genes
ChromoAgar	Chromogenic culture
MIC	Minimum inhibitory concentration
RGI	Resistance Genes Identifier
COD	Chemical oxygen demand
BOD5	Biological oxygen demands
TP	Total phosphorous

TN	Total Nitrogen
DO	Dissolved Oxygen
CTX	Cefotaxime
IMP	Imipenem

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