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The Dissemination of Antimicrobial Resistance Determinants in the Environment Through Horizontal Gene Transfer.

By

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ABSTRACT

The extensive use of antibiotics led to the rise and dissemination of antibiotic-resistant bacteria. One of the most important factors facilitating the spread of resistance determinants is mobile genetic elements. Understanding the mechanisms associated with their circulation in the environment is essential to public health. The prevalence of antibiotic-resistant bacteria in surface water is a growing concern. We aimed at studying and defining the role of mobile genetic elements, particularly that of plasmids, in the propagation of resistance determinants in non-clinical environments. Water samples were collected from El Qa'a refugee camp and five major rivers in Lebanon. All collected samples were diluted and inoculated on MacConkey agar. The recovered isolates (n=91) were tested using the Kirby-Bauer disc diffusion assay. Multidrug-resistant (MDR) isolates were further characterized using whole-genome sequencing (WGS), pulse-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). *Escherichia coli* (36/91) and *Klebsiella pneumoniae* (11/91) were the most common among the recovered isolates. *In silico* plasmid analysis was performed and validated using PCR-based replicon typing (PBRT) to identify and confirm

incompatibility groups. Isolates from El Qa'a refugee camp were diverse and multidrug-resistant (MDR), with a multi-replicon *bla*$_{\text{NDM-5}}$ positive *E. coli* being recovered from the site. *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{TEM-1B}}$ were also detected in the majority of the MDR isolates. Different ST types were identified including five isolates from Saida, Zahle, Beirut and El Qa'a belonging to the highly virulent *E. coli* ST131 phylogroup B2 and serotype O25: H4b. In this study, we determined the role of antibiotic resistance determinants in the contamination of water supplies in Lebanon. Our results showed a common occurrence of bacterial contaminants in surface water including ESBL- and carbapenemase-producing *Enterobacteriaceae* and an increase in the risk of resistance genes dissemination with the rise in the human population, population mobility and widespread lack of wastewater treatment.

Keywords: Lebanon, Surface water, Population Mobility, *Escherichia coli*, *Klebsiella pneumoniae*, Seasonal Changes, β-lactamases, Whole Genome Sequencing

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LIST OF ABBREVIATIONS

AmpC:	Class C β-lactamase
AMR:	Antimicrobial resistance
ARGs:	antibiotic resistance genes
bp:	Base Pair
CARD:	Comprehensive antibiotic resistance database
CGE:	Center for Genomic Epidemiology
CI:	Chromosomal Integron
DNA:	Deoxyribonucleic acid
EC:	*E. coli*
ESBL:	Extended spectrum β-lactamase
gDNA:	genomic Deoxyribonucleic Acid
HGT:	Horizontal Gene Transfer
Inc:	Incompatibility
KP:	*K. pneumoniae*
MDR:	Mutlidrug resistant
MI:	Mobile Integron
MLST:	multi-locus sequence typing
PBRT:	PCR-Based ReplicoN Typing
PCR:	Polymerase Chain ReactioN
PFGE:	Pulse-field gel electrophoresis
PLACNET:	Plasmid Constellation Network
PMQR	Plasmid-mediated Quinolone Resistance

RAST:	Rapid Annotation using Subsystem Technology
rRNA:	Ribosomal Ribonucleic Acid
ST:	Sequence Type
UNHCR:	United Nations High Commissioner for Refugees
VRE:	Vancomycin-resistant *Enterococci*
WGS:	Whole Genome Sequencing

Chapter 1

Introduction

1.1 Overview of Antimicrobial Resistance in Surface Water

Antimicrobial resistance (AMR) is a worldwide growing threat (Matamoros et al., 2017). The spread of antibiotic resistance is reported mainly in clinical and/or veterinary settings but has also been detected in other environments including wastewater (Varela, Macedo, Nunes & Manaia, 2015). Wastewater, being nutrient rich and having the optimal temperature with a high bacterial load, is an optimal environment for microbial growth and transfer of genes between different species (Osińska, Korzeniewska, Harnisz & Niestępski, 2017; Zhang et al., 2013). With the lack of proper disposal and treatment of sewage, the risk of transmission of multidrug resistant (MDR) bacteria into the community is extremely high and poses a public health threat (Finley et al., 2013). Hospitals are also a major contributor to the development and transfer of resistance determinants with antimicrobial agents from different classes, partially or fully unmetabolized, being dumped in the sewage systems (Zhang et al, 2017). Once MDR bacteria persist in the environment, resistance determinants get passed to other organisms through multiple gene transfer mechanisms increasing as a result their prevalence in natural environments including water systems (Mohanta & Goel, 2014). Therefore, aquatic environments receiving antibiotic-resistant bacteria from municipal, industrial, hospital and agricultural waste may constitute a route for the dissemination of drug-resistant bacteria and antibiotic resistance genes (ARGs) (Egervärn et al., 2017; Sanganyado & Gwenzi, 2019).

1.2 Microbial contamination of water sources

Surface water sources in Lebanon are used as the main supply for agricultural activities, electricity generation, leisure and human consumption (Daou, Salloum, Legube, Kassouf & Ouaini, 2018). However, most water sources in Lebanon are contaminated with raw sewage and industrial waste (Faour-Klingbeil, Kuri, Fadlallah & Matar, 2016). In 1992, a study assessing water quality indicated that most of the water sources in Lebanon do not conform with the WHO standards for bacterial contamination. In 2007 the study of coastal rivers showed high levels of fecal coliforms confirming a significant raw sewer water input (Houri & El Jeblawi 2007). In 2018, Diab et al. further validated the contamination of different water sources in Lebanon including spring and well waters, which are directly consumed without further treatment, and estuaries which are used in watering crops and water animals.

The most common family of bacteria found in surface water is the *Enterobacteriaceae*; a large family of Gram-negative, non-spore forming rods. The family includes potential pathogens of several genera such as: *Escherichia*, *Enterobacter*, *Klebsiella*, *Proteus*, *Citrobacter*, *Serratia*, *Salmonella* and *Shigella* that can be transmitted through fecal material (Ye et al., 2017). In Lebanon, the presence of multidrug resistant *Enterobacteriaceae* in water has been previously documented including extended spectrum β-lactamase (ESBL) and carbapenemase producing isolates with *E. coli* being the main contaminant present and *K. pneumoniae* being commonly isolated (Diab et al, 2018). *E. coli*, which is part of the gut microbiota of both ill and healthy humans and warm-blooded animals, disseminates in natural environments either directly through feces or indirectly through treated wastewater, making it the main indicator of water, food, and

soil fecal contamination (Osińska, Korzeniewska, Harnisz & Niestępski, 2017). *E. coli* usually enters water systems through various routes including human activities, animal, hospital and municipal discharges (Singh, Singhal & Virdi, 2018). The presence of *E. coli* in surface water presents a major health risk for people who are dependent on such supplies for drinking especially that it survives for long terms in natural environments (Sharan, Chhibber, Attri & Reed, 2009). In Lebanon, *E. coli* was isolated from different aquatic environments including water collected from households (Korfali & Jurdi, 2007), marine water (Korfali & Jurdi, 2012), estuaries, wells and spring water (Diab et al., 2018). In a preliminarily study conducted recently and covering rivers from different parts in Lebanon and including El Qa'a refugee camp, ESBL-producing *E. coli* were recovered from all the studied sites highlighting the magnitude of the problem associated with the dissemination of drug-resistant Gram-negative bacteria (Tokajian et al., 2018).

K. pneumoniae, a Gram-negative, non-motile, encapsulated organism, a normal inhabitant of the gastrointestinal tract of healthy individuals, and an opportunistic pathogen also accounts for one-third of all Gram-negative infections worldwide (Navon-Venezia, Kondratyeva & Carattoli, 2017). Pathogenic *K. pneumoniae* were linked to various infections including bloodstream and urinary and respiratory tract infections (Candan & Aksöz, 2015). *K. pneumoniae* is ubiquitously found in environmental sources including soil, plants and surface water (Podschun, Pietsch, Holler & Ullmann, 2001). *K. pneumoniae* can move from the environment to humans through the consumption of contaminated water or plants or from humans to the environment via sewage and these strains can carry antimicrobial resistance genes and/or plasmids (Wyres and Holt, 2018).

Enterococci are also among the widespread environmental bacteria; they are Gram-positive organisms frequently isolated from water systems. *Enterococci* are part of the gastrointestinal normal flora of humans and animals and often used as an indicator of fecal contamination in water. *Enterococci* released into the environment directly or through sewage, were found to be MDR and linked to nosocomial infections (Sahlström, Rehbinder, Albihn, Aspan & Bengtsson, 2009). The detection of *Enterococci* in environmental water sources is of interest due to the natural tolerance of this organism to extreme conditions (low pH, high temperatures and high salt concentrations) (Loong et al., 2016).

1.3 Resistance transfer

The primary means for resistance determinants to spread across bacterial communities is by horizontal gene transfer (HGT) usually carried on mobile genetic elements such as plasmids, transposons and integrons (Osińska, Korzeniewska, Harnisz & Niestępski, 2017; Baquero, Martínez & Cantón, 2008). The transfer of resistance genes between environmental and clinical bacteria is common and could take place in aquatic environments (Taylor, Verner-Jeffreys & Baker-Austin, 2011).

1.3.1 Plasmids

Plasmids are extra-chromosomal circular DNA fragments that are present in most bacterial species and their sizes could vary from a few to more than several hundred kilobase pairs (Carattoli et al., 2005). Several plasmids carrying resistance genes were additionally found to carry factors mediating virulence such as: bacteriocins, siderophores, cytotoxins, and adhesins (Carattoli, 2011; Rahube, Viana, Koraimann & Yost, 2014). Hedges and Datta in 1971 proposed a scheme for plasmid classification based on their

stability during conjugation and referred to it as plasmid incompatibility. Plasmid incompatibility is defined as the inability of two plasmids to propagate stably in the same lineage (Carattoli, 2009). Plasmid families that are largely prevalent and widely associated with specific resistance genes include InFII, IncA/C, IncL/M and IncI1; these plasmids are detected in different countries and in bacteria from different origins making them a major health threat (Carattoli, 2009). IncF group of plasmids was the most frequently detected in *E. coli* recovered from water in Tanzania, these plasmids were associated with different resistance genes including *tet*(A), $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-15}}$. Prevalence of IncF plasmids from different water sources was reported also in Lebanon, with plasmids involved carrying the $bla_{\text{CMY-42}}$ and $bla_{\text{CTX-M-15}}$ resistant determinants (Diab et al., 2018).

1.3.2 Integrons Insertion sequences and transposons

Integrons are mobile genetic elements associated with insertion sequences and broad-host range plasmids, contribute to the spread of resistant determinants through HGT (Khan, Knapp & Beattie, 2016; Xu et al., 2017). Insertion sequences and transposons are DNA fragments that can insert almost randomly into new locations within the same or into different organisms (Patridge, Kwong, Firth & Jensen, 2018). Transposons are found carrying ARGs. Due to their ability to move both intra- and inter-molecularly, they mediate the conjugative transfer of the carried ARGs into plasmids or chromosomes and at times in multiple copies (Mathur & Singh, 2005). Two categories of integrons were so far documented: chromosomal integrons (CIs) and mobile integrons (MIs) with the latter being non self-transmissible and usually found associated with other mobile genetic elements (Kaushik, Kumar, Kapoor & Gulati, 2019). On the other hand, five classes of MIs were detected (classes 1, 2, 3, 4 and 5) with class 1 being the most ubiquitous among

water-borne pathogens including *E. coli*, *Salmonella*, *Shigella*, *Vibrio*, *Campylobacter* and *Pseudomonas* (Rosser & Young, 1999). In *E. coli*, the occurrence of integrons from raw and treated wastewater was respectively reported to be 10% and 9.6%, with the class 1 and/or class 2 being found in the genomes of 11% of MDR *E. coli* isolates (Laroche, Pawlak, Berthe, Skurnik & Petit; 2009).

1.3.3 Bacteriophages

Bacteriophages contribute, and through horizontal gene transfer, to the relocation of genetic material between different organisms by the release of DNA fragments into the environment, which will be readily available for transformation (Bierbart et al, 2012). A wide variety of metabolic and functional genes have been identified in phage genomes including toxins, superantigens, intracellular survival/host cell attachment proteins and antimicrobial resistance genes (Calero-Cáreres, Ye & Balcázar, 2019). Bacteriophages recovered from environmental bacteria were found to carry resistance genes belonging to different drug classes including aminoglycosides, β-lactams, quinolones and tetracyclines. Phages recovered from river water collected in Spain carried bla_{KPC}, bla_{NDM}, bla_{TEM}, *vanA*, *qnrA* and *qnrB* and, while *aac*(6')-*lb-cr* encoding resistance to quinolones was also recovered from China (Yang et al., 2018; Marti, Variatza & Balcázar, 2014). The presence of phages in water settings could mediate the transmission of ARGs between distant bacterial cells as phages could survive for long periods of time and do not require direct cell-to-cell contact (Touchon, Moura de Sousa & Rocha, 2017).

1.4 Mechanisms of resistance in *Enterobacteriaceae*

1.4.1 Extended spectrum β-lactamase

The increase in the dissemination of resistance and specifically those associated with the extended spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* in the environment and through wastewater is a public health concern (Dropa et al., 2016; Ye et al., 2017). β-lactamases encoding genes are carried either on plasmids or chromosomal DNA and are detected predominantly in *E. coli* and *K. pneumoniae* as we well as *Pseudomonas aeruginosa*, *Acinetobacter baumanii* and *Shigella* spp. (Jury, Vancov, Stuetz & Khan, 2010; Tokajian, Eisen, Jospin, Farra & Coil, 2015). Genetic modifications in the β-lactamase encoding genes led to the development of ESBLs that can hydrolyze penicillin, first, second, third and a few of the fourth generation cephalosporins, and aztreonam (Jury, Vancov, Stuetz & Khan, 2010; Tokajian, Eisen, Jospin, Farra & Coil, 2015).

More than 300 subtypes of ESBLs were so far detected. The TEM, CTX (CTX-M group 1 and CTX-M group 9), OXA and SHV are the most common ones in *Enterobacteriaceae* (Korzeniewska, Korzeniewska & Harnisz, 2013), with CTX-M being the most commonly detected ESBL worldwide (Xu et al., 2017). The $bla_{\text{CTX-M-15}}$ gene is usually found on plasmids belonging to the IncF group associated with $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$ and *aac(6')-Ib-cr* resistance genes (Carattoli, 2009).

In Lebanon, the most commonly detected ESBL from water resources was the CTX-M-15 with *E. coli* being the most commonly recovered organism carrying the $bla_{\text{CTX-M-15}}$ (Diab et al., 2018). This was also recently confirmed with the ESBL-producing *E. coli*

recovered from river effluents and El Qa'a refugee camp mainly carrying the CTX-M-15 along with other β-lactams CTX-M-27, CTX-M-14, CTX-M-9, TEM-1, OXA-1 and SHV-12 (Tokajian et al., 2018)

1.4.2 AmpC β-lactamases

AmpC β-lactamase genes detected in pathogens constitute a persistent threat in clinical and environmental settings (Coertze & Bezuidenhout, 2018). AmpC β-lactamase family of genes are commonly carried on the chromosome in *Enterobacteriaceae*, and which at times would be overexpressed due to mutations in regulatory genes (Pfeifer, Cullik & Witte, 2010; Coertze & Bezuidenhout, 2018). Twenty-nine plasmid-borne AmpC β-lactamases were identified including: MOX-, CIT-, DHA-, ACC- and FOX- with CMY-2 and CIT-type enzymes being the most common worldwide and representing one of the main key players in resistance against the β-lactams (Perez- Perez & Hanson, 2002; Ye et al, 2017). Both chromosomal and plasmid encoded AmpC genes convey resistance to a wide variety of β-lactam antibiotics including cefoxitin and cefotetan, which are not inactivated by ESBL producing organisms (Amador, Fernandes, Prudêncio, Barreto & Duarte, 2014). In addition to their presence in hospital settings, AmpC producers were detected in the environment as well as in food, domestic and wild animals, and healthy humans (Ye et al, 2017).

1.4.3 Carbapenemases

Carbapenems are part of the β-lactam group of drugs that are used as a last resort for treatment of MDR Gram-negative bacilli (Djahmi et al, 2014). The global dissemination of carbapenemases, which confer resistance to carbapenems, has been a

major concern worldwide with more than 700,000 deaths caused by infections yearly linked to carbapenem-resistant *Enterobacteriaceae* (O'Neill, 2016). In *Enterobacteriaceae,* three classes of carbapenemases are common: the Ambler class A (such as KPC), Ambler class B known as metallo β-lactamases (such as IMP, VIM and NDM) and Ambler class D (such as OXA-48) and its variants (Poirel, Potron, Ash & Nordmann, 2012). Reports indicate that animals, food products and the environment constitute a reservoir for carbapenemase producing organisms (Wyres and Hold, 2018; Lepuschitz et al., 2019). Carbapenamases have been sporadically reported in rivers in Germany (KPC-2 and NDM-1), Switzerland (VIM) and the United States (IMP-2) (Aubron, Poirel & Nordmann, 2005; Zurfluh, Hachler, Nuesch-Inderbinen & Stephan, 2013). In Lebanon, carbapenemase producing *Enterobacteriaceae* were recovered from water samples including estuaries with $bla_{\text{OXA-48}}$ being the most commonly identified gene from *E. coli* and *K. pneumoniae* (Diab et al., 2018).

1.4.4 Quinolone and fluoroquinolone resistance

Quinolone resistance in *Enterobacteriaceae* is usually the result of chromosomal mutations leading to alterations in target enzymes. Plasmid-mediated quinolone resistance (PMQR) can also occur by the acquisition of *qnr, qepA* and *aac(6')-Ib-cr* genes (Carattoli, 2009). Very often, the spread of MDR plasmids among *Enterobacteriaceae* was found to be associated with plasmids carrying quinolone resistant genes, ESBLs and/or aminoglycoside resistance determinants (Paterson, 2006). PMQR was detected in hospital, animal and environmental isolates with aquatic environments being a reservoir for these genes including the *qnrS1* and *qnrS2*. Both *qnrS1* and *qnrS2* were previously isolated from a Seine river in Paris and *qnr* as well as *qepA* and *aac-(6')-Ib-cr* from

different rivers in China (Yan et zal., 2017; Cattoir, Poirel, Aubert, Soussy & Nordmann, 2008). Finally, quinolone-resistant *E. coli* and *K. pneumoniae* were also recovered from hospital effluent, outflow sewage and surface waters in Brazil (Conte et al., 2017).

1.5 Vancomycin resistance in *Enterococci*

Vancomycin resistant *Enterococci* (VRE) were detected in sewage, stools of healthy farm animals and surface water (Schwartz et al, 2003). VRE are known to cause difficult to treat hospital-associated infections because of their multidrug resistance (Schwartz et al, 2013). Nine types of glycopeptide resistance determinants, particularly vancomycin resistance, are so far detected in enterococci (*vanA, vanB, vanC, vanD, vanE, vanG, vanL, vanM and vanN*) (Sabouni et al., 2016). *vanA* and *vanB* are considered the most commonly detected ones conferring high-level of resistance to vancomycin (Iversen, Kuhn, Franklin & Mollby, 2002). Recently, A study from Lebanon evaluating the levels of contamination in natural water sources, intestinal *Enterococcus* were found to be the most commonly in rivers, well and lake water resources and all exhibited resistance to at least two types of antibiotics (Mokh, El Khatib, Koubar, Daher & Al Iskandarani, 2017).

1.6 Population Mobility and Surface Water Quality

The emergence of resistance could be linked to population mobility, which is one of the factors associated with the globalization of public health threats (MacPherson et al., 2009). Lebanon with a surface area of 10,452 km^2 has an approximate population of 4,467 million with more than 220,000 Palestinian and around 40,000 Iraqi refugees as well as an additional 100,000 workers from different nationalities (Cherri, González & Delgado, 2016). The country received additionally more than 1.5 million Syrian refugees starting 2012 and till the end of 2018 with around 74% lacking legal status (Human rights watch).

Refugees are dispersed in four major areas: Akkar (36.3%), Bekaa (26.2%), Beirut (25.6%) and South Lebanon (11.9%) (Cherri, González & Delgado, 2016; UNHCR, 2017). The infrastructure and public sanitation have suffered a lot in Lebanon after the war in 1975 and the influx of refugees who are living in areas lacking public sanitation and proper access to care facilities is exposing them to significant health risks (Daoud et al., 2018). The UNHCR revealed that 39% of the Syrian refugee families have no access to private sanitation facilities, 13% are deficient in personal needed hygiene items, 68% live below the poverty line, and 40% cannot access appropriate toilets (UNHCR, 2017). Additionally, Amel Association showed that 47% of the Syrian refugees suffered from skin diseases (lice, staphylococcal skin infection, and leishmaniasis), 27% had digestive system problems, and 19% had respiratory diseases (Cherri, González & Delgado, 2016).

A previous study conducted by Tokajian et al. 2018, evaluating the effects of population influx on the prevalence of ESBL-producing *E. coli* isolated from river effluents in Lebanon, revealed high level contamination by drug resistant organisms as well as the introduction of new resistant patterns into the water systems. Accordingly, this work is a continuation of what was previously done to include larger number of samples and to cover different seasonal patterns.

Objectives:

1- Investigation and characterization of the bacterial contaminants present in surface water in Lebanon, and the comparison between different resistance patterns for samples collected from sites close to refugee camps to that collected from other surface water sources;
2- Genetic characterization of multidrug resistant organisms including resistance determinants, virulence genes and phylogeny using whole-genome sequencing;
3- Study of plasmid content, incompatibility groups, resistance determinates linked to plasmids and other mobile elements (insertion sequences, phase, and transposons) and their role in the spread of such factors in the environment;
4- Typing and clonal relatedness of resistant isolates;
5- Investigation of the effect of temperature changes on bacterial density, type, and antimicrobial resistance.

Chapter 2

Materials and Methods

2.1 Study settings

Water samples were collected from Al Qa'a refugee camp and four other major river effluents across Lebanon with possible sewage contamination including: Nahr Abou Ali (Tripoli), Nahr Al-Awali (Saida), Al- Berdawni river (Zahle) and Beirut (Beirut) (Figure 1).

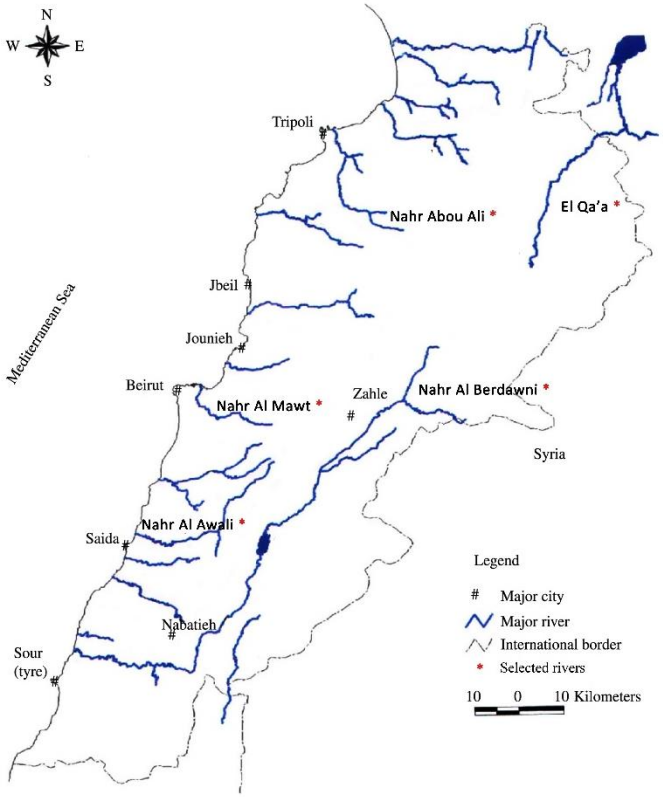


Figure 1: Geographical distribution of the chosen surface water sources included in this study; the red asterisk (*) indicates the rivers from which isolates were collected.

2.2 Bacterial isolates

Water samples (5L) were collected in clean containers, diluted 1/10 with sterile saline and inoculated on Blood and MacConkey agar. A total of 91 isolates were recovered from the water samples and isolates were named based on the organism ID. Table 1 shows the identification details of each isolate (name, location, season and organism).

Table 1: Distribution of the based on the source and time of collection.

S17= summer 2017, W17= winter 2017, W18= winter 2018

Organism	Labeling	Season	Area	Organism	Labelings	Season	Area
E. coli	EC1	S17	El Qa'a	*A. baumanii*	AB1	W17	Tripoli
	EC2 - EC5	W17	El Qa'a	*Aeromonas* spp.	AE1 - AE7	W17	El Qa'a
	EC6 - EC8	S17	Beirut	*Citrobacter freundii*	CI1	W17	Zahle
	EC9 - EC12	W17	Saida		CI2	S17	Al Qa'a
	EC13 - EC15	S17	Zahle		CI3	W17	Beirut
	EC16 - EC23	W18	El Qa'a	*Citrobacter gilleni*	CI4	W17	Zahle
	EC24 - EC26	W18	Beirut	Delftia spp.	DE1	W17	Beirut
	EC27 - EC29	W18	Saida	*Hafnia*	HA1	W17	Zahle
	EC30 - EC32	S17	Tripoli	*Kluyvera ascorbata*	KL1	W17	Zahle
	EC33 - EC36	W18	Zahle	*P. aeuroginosa*	PA1	W17	Beirut
K. pneumoniae	KP1 - KP2	S17	Tripoli		PA2	W17	Tripoli
	KP3	S17	Beirut	*P. otitidis*	PO1	S17	Saida
	KP4	W17	Beirut	Providencia spp.	PR1	S17	Tripoli
	KP5	S17	Saida		PR2	W17	Al Qa'a
	KP6 - KP9	W17	Tripoli	Raoultella spp.	RA1 - RA3	W17	Zahle
	KP10	S17	Zahle	*Salmonella enterica*	SA1	S17	Zahle
Enterococcus spp.	EN1	S17	El Qa'a	*Serratia macrescens*	SM1	S17	Tripoli
	EN2- EN5	W17	El Qa'a	Shewanella spp.	SH1	W17	Beirut
	EN6	S17	Beirut	*Enterobacter* spp.	EB1	W17	Beirut
	EN7	W17	Beirut		EB2 - EB3	W17	Tripoli
	EN8	S17	Saida		EB4	W17	Zahle
	EN9	S17	Tripoli		EB5	S17	El Qa'a
	EN10 - EN12	W17	Tripoli		EB6 - EB7	S17	Beirut

2.3 Antimicrobial Testing

All Gram-negative isolates were tested for resistance using the Kirby-Bauer disk diffusion method against 29 different antimicrobial agents using representatives of ten different classes (Amoxicillin, Amoxicillin/Clavulanic Acid, Ticarcillin, Piperacillin/Tazobactam, Imipenem, Ertapenem, Meropenem, Aztreonam, Cefalotin, Cefuroxime, Cefoxitin, Cefotaxime, Ceftriaxone, Ceftazidime, Cefepime, Cefixime, Tobramycin, Gentamycin, Amikacin, Ofloxacin, Ciprofloxacin, Norfloxacin, Levofloxacin, Tetracycline, Tigecycline, Trimethoprim/Sulfamethoxazole, Nitrofurantoin, Fosfomycin (*E. coli* urine) and Colistin).

Gram-positive isolates were tested against 11 different antimicrobial agents (Amoxicillin, Kanamycin high, Streptomycin high, Gentamycin high, Vancomycin, Teicoplanin, Levofloxacin, Tetracycline, Tigecycline, Trimethoprim/Sulfamethoxazole, and Erythromycin).

All results were interpreted according to the Clinical Laboratory Standards Institute guidelines (CLSI, 2018).

2.4 DNA extraction

DNA extraction was performed using the NucleoSpin® Tissue DNA extraction kit (Macherey- Nagel, Germany) following the manufacturer's instructions. Recovered DNA was quantified using Nanodrop technology and stored at -20℃.

2.5 Species Identification

The 16S rRNA was amplified using primers SSU-bact-27F (5'-AGAGTTTGATCMTGGCTGAG- 3') and SSU-bact-519R (5'-GWATTACCGCGGCKGCTG- 3') as previously described by Schmidt, DeLong & Pace,

1991. The resulting PCR products were sequenced using the Genetic analyzer 3500 (Applied Biosystems™) and the sequences were blasted against the NCBI 16S rRNA database.

E. coli and *K. pneumoniae*, the two most commonly isolated organisms, were chosen for further characterization.

2.6 PFGE fingerprinting

Pulse-field gel electrophoresis (PFGE) was used to type and determine the genetic relatedness of recovered isolates identified as either *E. coli* or *K. pneumoniae*. The restriction enzyme *Xba*I (ThermoScientific, Waltham, MA, USA) was used. 1% SeaKem agarose gel and *Salmonella enterica* susbp. enterica serovar Braenderup (ATCC BAA664TM) was used as the universal laboratory standard and isolates were processed according to the standard PulseNet protocol (http:/www.pulsenetinternational.org).

Electrophoresis was performed using Bio-Rad laboratories CHEF DR-III system (Bio-Rad Laboratories, Bio-Rad Laboratories Inc., Hercules, CA, USA). Gels were stained with Ethidium Bromide.

BioNumerics software version 7.6.1 (Applied Maths, Belgium) was used to analyze the PFGE profiles and pulsotypes were clustered through dice correlation coefficients with 1.5% optimization and 1.5% tolerance.

2.7 Multi-locus sequence typing (MLST)

2.7.1 *K. pneumoniae*

MLST was performed as described on the Institute Pasteur MLST database targeting seven housekeeping genes (*rpoB, gapA, mdh, pgi, phoE, infB* and *tonB*) and using primers with universal sequencing tails. Genes were sequenced using universal

primer pair (Diancourt, Passet, Verhoef, Grimont, & Brisse, 2005). STs were assigned using the Pasteur Institute database (www.pasteur.fr/mlst).

2.7.2 *E. coli*

The allelic profiles of *E. coli* isolates were also reported for seven housekeeping genes: *adk, fumC, gyrB, icd, mdh, purA* and *recA* using primers with universal sequencing tails. Genes were sequenced using the same primers and STs were assigned using the MLST Warwick database (www.enterobase.warwick.ac.uk).

The Achtman multilocus sequence typing (MLST) was performed on all whole-genome sequenced isolates using MLST 2.0 database available on the Center for Genomic Epidemiology (CGE) (www.genomicepidemiology.org) (Larsen et al., 2012)

2.8 Whole-genome sequencing

Based on the antimicrobial susceptibility test results, 26 MDR isolates were selected for further analysis. Isolates were defined as being MDR following Magiorakos et al. 2012; isolates non-susceptible to at least one agent in three or more antimicrobial categories are considered as being MDR.

Library preparation was performed using the Illumina Nextera XT DNA Library preparation kit (Illumina, San Diego, CA, USA). In summary, genomic DNA (gDNA) was used as input for library preparation. The gDNA was subjected to end-repair, A-tailing, ligation of adaptors including sample-specific barcodes as per the manufacturer's recommendation. Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) was used to quantify the resulting library which was sequenced on an Illumina MiSeq (Illumina, San Diego, CA, USA) with paired-end 500 cycles protocol to read a length of 250 bp. Genome were assembled *de novo* with SPAdes v3.13 with applying read error correction (Nurk et

al., 2013). The assembled draft genomes were annotated using RAST (http://rast.nmpdr.org/rast.cgi) (Aziz et al, 2008).

2.9 Plasmid Identification

Plasmid identification for all MDR *Enterobacteriaceae* was performed using the DIATHEVA PCR-Based Replicon Typing (PBRT) kit (Diatheva, Fano, Italy). Eight multiplex PCRs were performed for the amplification of 28 replicons: HI1, HI2, I1, I2, X1, X2, L/M, N, FIA, FIB, FIC, FII, FIIS, FIIK, W, Y, P, A/C, T, K, U, R, B/O, HIB-M and FIB-M representative of major plasmid incompatibility groups and replicase genes that are typically found on resistance plasmids circulating among *Enterobacteriaceae*. All PCR reactions were performed according to the manufacturer's instructions and visualized on a 2.5% agarose gel stained with ethidium bromide.

2.10 Phylogenetic typing- *E. coli*

The phylogenetic origin of all *E. coli* isolates was determined following the protocol proposed by Clermont O. et al 2013. *E. coli* isolates were classified as belonging to one of seven phylogenetic groups: A, B1, B2, C, D, E and F. Four marker genes were used for the detection of phylogenetic groups: *yja* gene, *chuA* gene, TspE4C2 fragment and *arpA* gene. Results were further validated on GoSeqit (GoSeqit.com) using the pipeline developed to determine the phylogroup. The predicted phylotype was also determined using the same pipeline (Joensen et al, 2015).

Table 2: primer sequences and sizes of PCR products used in phylogenetic grouping of *E. coli*

Target	Primer ID	Primer Sequences	PCR product (bp)
ChuA	chuA.1b	5′-ATGGTACCGGACGAACCAAC-3’	288
	chuA.2	5′-TGCCGCCAGTACCAAAGACA-3′	
yjaA	yjaA.1b	5′-CAAACGTGAAGTGTCAGGAG-3′	211
	yjaA.2b	5′-AATGCGTTCCTCAACCTGTG-3′	
TspE4.C	TspE4C2.1	5′-CACTATTCGTAAGGTCATCC-3′	152
	TspE4C2.2b	5′-AGTTTATCGCTGCGGGTCGC-3′	
arpA	AceK.f	5′-AACGCTATTCGCCAGCTTGC-3′	400
	ArpA1.r	5′-TCTCCCCATACCGTACGCTA-3	

2.11 Capsule typing- *K. pneumoniae*

wzi gene typing was performed to determine the *K. pneumoniae* capsular type, using wzi-F (GTGCCGCGAGCGCTTTCTATCTTGGTATTCC) and wzi-R (GAGAGCCACTGGTTCCAGAA[C or T]TT[C or G]ACCGC) primers as previously described (Brisse et al, 2013). K-types were assigned using the Pasteur Institute database (http://bigsdb.pasteur.fr/klebsiella).

2.12 Whole- genome based *in silico* typing and virulence and resistance profiling

In silico Plasmid and pMLST typing were performed on all whole-genome sequenced isolates using PlasmidFinder 1.3 and pMLST 2.0 respectively using the Center for Genomic Epidemiology (CGE) tools (Carattoli et al., 2014). Virulence genes were identified using VirulenceFinder 1.5 (Joensen et al, 2014). ResFinder v2.1 and the Comprehensive Antibiotic Resistance Database (CARD) were used to identify resistance genes (Zankari et al, 2012; Jia et al, 2017). Phage Search tool (PHASTER) was used for Phage identification (Arndt et al, 2016). IS-finder was used to identify insertion sequences

(ISs) and IS-families (Siguier, Perochon, Lestrade, Mahillon & Chandler, 2006). PLACNETw was used to separate from raw reads chromosomal from accessory and plasmid genomes (Vielva, de Toro, Lanza & de la Cruz, 2017). Genome alignments and comparison were performed using BioNumerics software version 7.6.1 (Applied Maths Belgium).

2.13 Pan-genome and recombination analysis

To further study the diversity of *E. coli*, genomes were annotated using Prokka (version 1.13) with a similarity cutoff e-value 10^{-6} and minimum contig size of 200 bp (Seemann, 2014). Annotated GFF3 files were piped into Roary (version 3.12). Choosing a minimum blastp identity of 95 and core gene prevalence in all (>99%) of the isolates (Page et al., 2015)

2.14 Conjugation

The $bla_{\text{NDM-5}}$ positive *E. coli* EC23 recovered from El Qa'a refugee camp was chosen for further characterization and a conjugation assay was performed to assess the transferability of the $bla_{\text{NDM-5.}}$ The donor (EC23) and recipient (Azide resistant *E. coli* J53) strains were grown overnight in Luria-Bertani (LB) broth at 37℃ and shaking speed of 180 rpm. Donor was grown in the presence of 10 µg/mL of meropenem, while the recipient in 150 µg/mL of sodium azide. Donor and recipient cells were mixed at a 1:1 ratio and 100 µL of the transconjugants were plated on LB agar supplemented with meropenem and sodium azide. PBRT was performed on colonies recovered in the presence of meropenem and sodium azide.

Chapter 3

Results

3.1 Identification of isolates

A total of 91 isolates were recovered in this study period (2017 and 2018 covering winter and summer) from four major areas: Tripoli (19/91, 20.9%), Saida (10/91, 10.9%), Zahle (17/91, 18.7%) and Beirut (17/91, 18.7%), in addition to the El Qa'a refugee camp (28/91, 30.8%).

Among the recovered organisms, the most common were *E. coli* (36/91; 39.6%), *Enterococcus* spp. (12/91; 13.2%) and *K. pneumoniae* (11/91; 12.1%). The remaining 35.1% were distributed between *S. marcescens*, *Salmonella*, *A. baumanii*, Delftia spp., *Hafnia*, *Kluyvera ascrobata*, *Shewanella* spp., *Providencia* spp., *P. aeuroginosa*, *Raoultella* spp., *Citrobacter* spp., *Enterobacter* spp. and *Aeromonas* spp. (Figure 2)

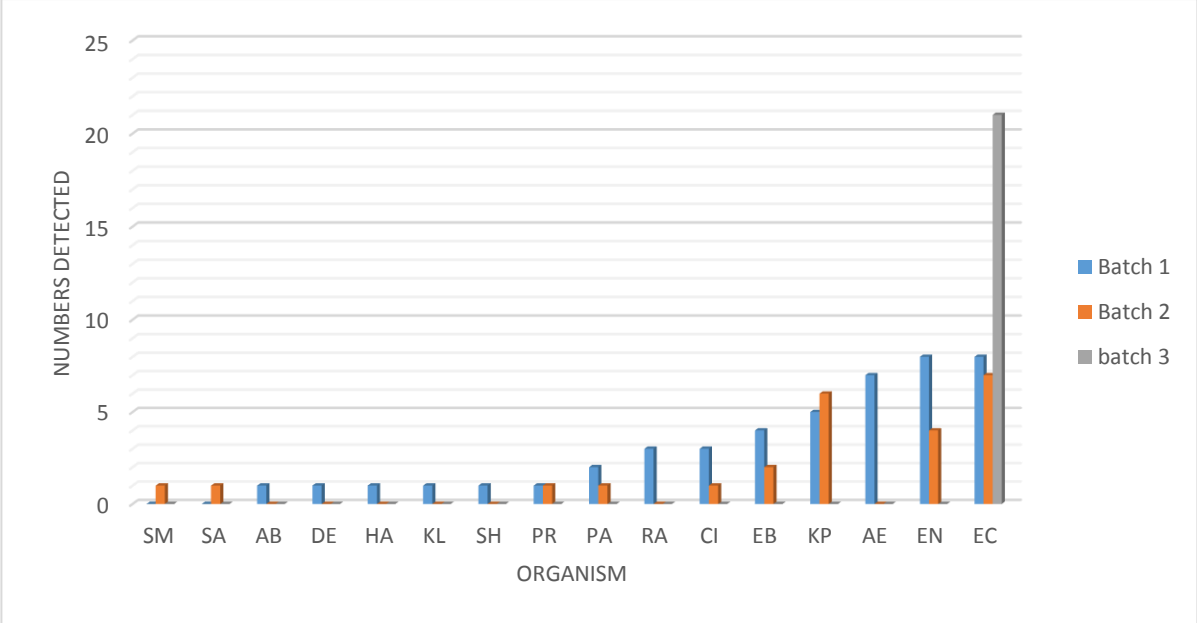


Figure 2: Distribution of organisms isolated from river effluents (Zahle, Beirut, Tripoli and Saida) and El Qa'a refugee camp in Lebanon.

Batch 1= winter 2017, batch 2= summer 2017 and batch 3= winter 2018

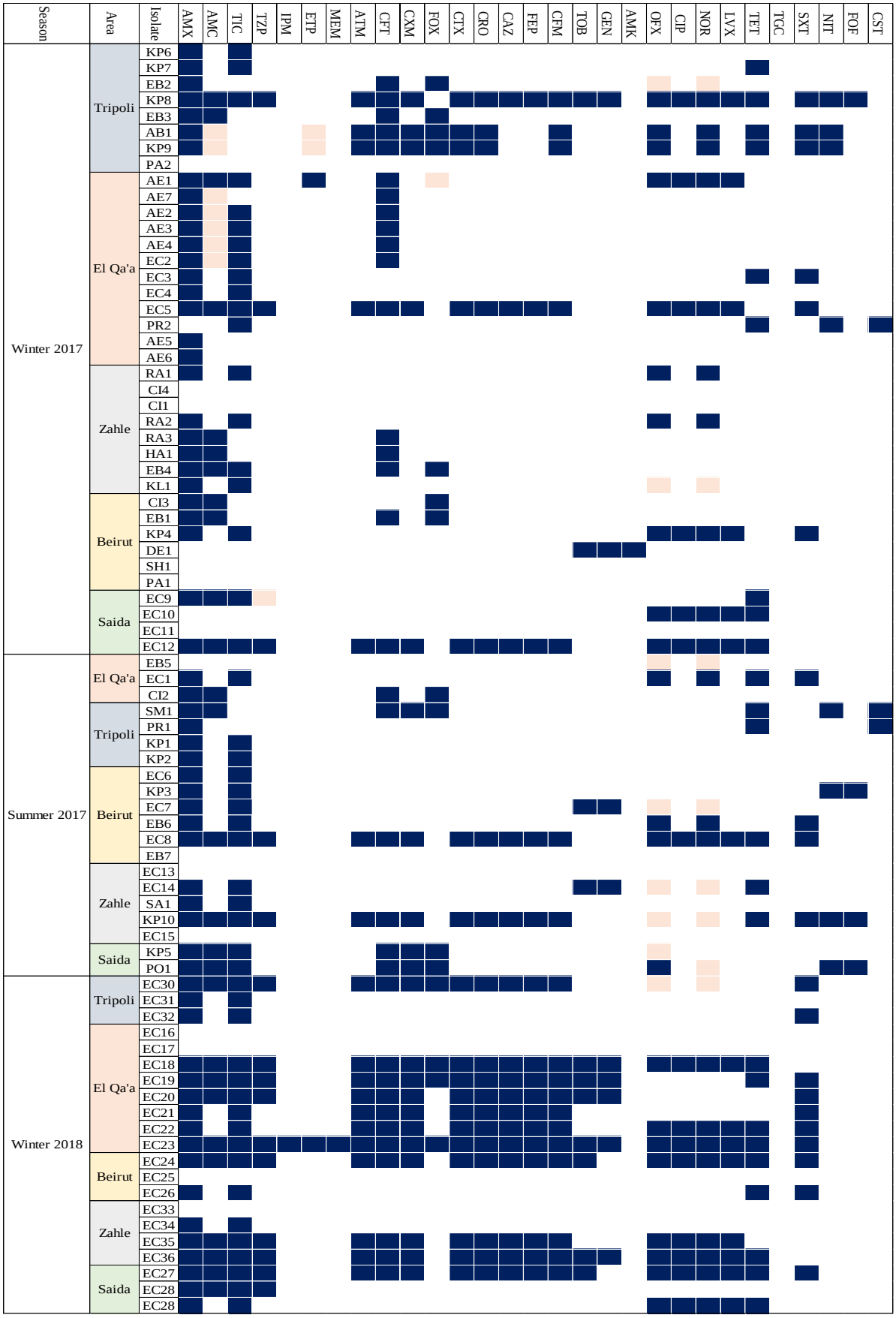
SM= *Serratia marcescens*, SA= *Salmonella* spp., AB= *Acinetobacter baumanii*, DE= *Delftia* spp., HA= *Hafnia*, KL= *Kluyvera ascorbata*, SH= *Shewanella* spp., PR= *Providencia* spp., RA= *Raoultella* spp., CI= *Citrobacter* spp., EB= *Enterobacter* spp., KP= *Klebsiella pneumoniae*, AE= *Aeromonas* spp., EN= *Enterococcus* spp., EC= *E. coli.*

3.2 Antibiotic Resistance Profiles

3.2.1 Resistance in the Gram-negative isolates

Of all isolated organisms 86.8% (79/91) were Gram-negative. The disk diffusion assay revealed that 77% (62/79) were resistant to amoxicillin, 62% (49/79) to ticarcillin, 43% (34/79) to cefalotin and 34.2% (27/79) to amoxicillin/clavulanic acid. All isolates were sensitive to tigecycline. *E. coli* EC23 was the only isolate resistant to ertapenem, meropenem and imipenem (Table 3).

Table 3: Antibiotic susceptibility results for all recovered Gram-negative organisms from the river effluents and El Qa'a refugee camp in Lebanon using 29 different antimicrobial agents covering ten different classes.


Season
Area
Isolate
AMX
AMC
TIC
TZP
IPM
ETP
MEM
ATM
CFT
CXM
FOX
CTX
CRO
CAZ
FEP
CFM
TOB
GEN
AMK
OFX
CIP
NOR
LVX
TET
TGC
SXT
NIT
FOF
CST
Winter 2017
Tripoli
KP6
KP7
EB2
KP8
EB3
AB1
KP9
PA2
El Qa'a
AE1
AE7
AE2
AE3
AE4
EC2
EC3
EC4
EC5
PR2
AE5
AE6
Zahle
RA1
CI4
CI1
RA2
RA3
HA1
EB4
KL1
Beirut
CI3
EB1
KP4
DE1
SH1
PA1
Saida
EC9
EC10
EC11
EC12
Summer 2017
El Qa'a
EB5
EC1
CI2
Tripoli
SM1
PR1
KP1
KP2
Beirut
EC6
KP3
EC7
EB6
EC8
EB7
Zahle
EC13
EC14
SA1
KP10
EC15
Saida
KP5
PO1
Winter 2018
Tripoli
EC30
EC31
EC32
El Qa'a
EC16
EC17
EC18
EC19
EC20
EC21
EC22
EC23
Beirut
EC24
EC25
EC26
Zahle
EC33
EC34
EC35
EC36
Saida
EC27
EC28
EC28

AMX= Amoxicillin, AMC= Amoxicillin/ Clavulanic Acid, TIC= Ticarcillin, TZP= Piperacillin/Tazobactam, IPM= Imipenem, ETP= Ertapenem, MEM= Meropenem , ATM= Aztreonam, CFT= Cefalotin, CXM= Cefuroxime, FOX= Cefoxitin, CTX= Cefotaxime, CRO= Ceftriaxone, CAZ= Ceftazidime, FEP= Cefepime, CFM= Cefixime, TOB= Tobramycin , GEN= Gentamicin , AMK= Amikacin , OFX= Ofloxacin, CIP= Ciprofloxacin, NOR= Norfloxacin, LVX= Levofloxacin, TET= Tetracycline, TGC= Tigecycline, SXT= Trimethoprim/Sulfamethoxazole, NIT= Nitrofurantoin, FOF= Fosfomycin (*E. coli* urine) and CST= Colistin)

3.2.2 Resistance in the Gram-positive isolates

Gram-positive organisms represented 13.2% (12/91) of the recovered isolates in this study. The majority of the Gram-positive isolates (83.3%; 10/12) were resistant to trimethoprim/sulfamethoxazole and 25% (3/12) to erythromycin. All isolates were sensitive to amoxicillin, vancomycin, teicoplanin and tigecycline (Table 4).

Table 4: Antibiotic susceptibility results for all recovered Gram-positive organisms from the river effluents and El Qa'a refugee camp in Lebanon using 12 different antimicrobial agents covering eight different classes.

Isolation site	Organism	AMX	KAN High	STR High	GEN	VAN	TEC	LEV	TET	TGC	SXT	ERY
Tripoli	*Enterococcus* spp.											
	Enterococcus spp.											
	Enterococcus spp.											
El Qa'a	*Enterococcus* spp.											
	Enterococcus spp.											
	Enterococcus spp.											
	Enterococcus spp.											
Beirut	*Enterococcus* spp.											
El Qa'a	*Enterococcus* spp.											
Tripoli	*Enterococcus* spp.											
Beirut	*Enterococcus* spp.											
Saida	*Enterococcus* spp.											

AMX= Amoxicillin, KAN high= Kanamycin high, STR high= Streptomycin high, GEN high= Gentamicin high, VAN= Vancomycin, TEC= Teicoplanin, LEV= Levofloxacin, TET= Tetracycline, TGC= Tigecycline, SXT= Trimethoprim/Sulfamethoxazole, ERY= Erythromycin.

3.3 Whole-genome sequencing

Isolates defined as MDR based on the definition of Magiorakos et al., 2011 were chosen for further characterization using whole-genome sequencing. *E. coli* (17/26, 65.4%) and *K. pneumoniae* (4/26, 15.4%) were the two most recovered MDR isolates in this study (Table 5). In 2017, a total of 46 isolates was collected within the winter batch compared to isolates from the summer batch, out of which eight and seven were classified as being MDR from winter and summer batches, respectively. MDR *E. coli* were equally

distributed between the different batches (three from each season), while MDR *K. pneumoniae* was more prevalent in the winter (three in winter and one in the summer) (Table 5).

Table 5: Detailed overview on the MDR isolates that we chose for further characterization using whole-genome sequencing including the: season, year, site of isolation and NCBI accession numbers.

EC= *Escherichia coli*, KP= *Klebsiella pneumoniae*, PR1= *Providencia rettgeri*, PR2= *Providencia alcalifacians*, SM= *Serratia marcescens*, and PO= *Pseudomonas otitidis*

Season	Year	Site	Name	Organism	Accession Number
Winter	2017	El Qa'a	EC5	*E. coli*	PXIF00000000
Winter	2017	Tripoli	KP8	*K. pneumoniae*	PXIG00000000
Winter	2017	Tripoli	AB1	*A. baumanii*	PXHY00000000
Winter	2017	Tripoli	KP9	*K. pneumoniae*	PXHZ00000000
Winter	2017	Saida	EC12	*E. coli*	PXIA00000000
Winter	2017	Beirut	KP4	*K. pneumoniae*	SDUE00000000
Winter	2017	El Qa'a	PR2	*P. rettgeri*	Pending release by NCBI
Winter	2017	Saida	EC10	*E. coli*	SDUD00000000
Summer	2017	El Qa'a	EC1	*E. coli*	SDUF00000000
Summer	2017	Tripoli	SM1	*S. marcescens*	PXIB00000000
Summer	2017	Tripoli	PR1	*P. alcalifacians*	PXIC00000000
Summer	2017	Beirut	EC7	*E. coli*	SDUG00000000
Summer	2017	Beirut	EC8	*E.coli*	PXID00000000
Summer	2017	Zahle	KP10	*K. pneumoniae*	PXIE00000000
Summer	2017	Saida	PO1	*P. otitidis*	PXJI00000000
Winter	2018	El Qa'a	EC18	*E.coli*	SDPR00000000
Winter	2018	El Qa'a	EC19	*E.coli*	SDPS00000000
Winter	2018	El Qa'a	EC20	*E.coli*	VAUG01000000
Winter	2018	El Qa'a	EC21	*E.coli*	SDSF00000000
Winter	2018	El Qa'a	EC22	*E.coli*	SDSG00000000
Winter	2018	El Qa'a	EC23	*E.coli*	SDSH00000000
Winter	2018	Tripoli	EC30	*E.coli*	SGIV00000000
Winter	2018	Saida	EC27	*E.coli*	SGIX00000000
Winter	2018	Beirut	EC24	*E. coli*	SGIW00000000.1
Winter	2018	Zahle	EC35	*E. coli*	Pending release by NCBI
Winter	2018	Zahle	EC36	*E. coli*	Pending release by NCBI

3.4 Resistance determinants

The antibiotic susceptibility testing results were further validated *in silico* using ResFinder v1.2, and using which we detected 56 different genes each conferring resistance to one of the nine tested categories of antimicrobial agents (β-lactams, fluoroquinolones, fosfomycin, sulphonamides, trimethoprim, tetracycline, phenicol, macrolides and aminoglycosides).

Twelve different variants of genes linked to β-lactam resistance were detected with the $bla_{\text{TEM-1B}}$ (54%, 14/26) and $bla_{\text{CTX-M-15}}$ (46%, 13/26) being the most common among the detected ESBLs and mainly being recovered from the El Qa'a refugee camp (5/14 and 4/13 respectively). Only one gene conferring resistance to carbapenems, namely $bla_{\text{NDM-5}}$, was detected in *E. coli* EC23 and which was isolated from the El Qa'a refugee camp during winter 2018.

Fourteen different aminoglycoside resistance genes were also detected. The most common were *aph(3")-lb* (8/26; 33%) and *aph(6)-Id* (7/26; 29%). Phenicol, macrolide, tetracycline, sulphonamide, Fosfomycin and quinolone resistance determinants were also among the detected resistance determinants (Figure 3).

Season
Isolate
Location
Winter 2017
Summer 2017
Winter 2018
EC5 El Qa'a
KP8 Tripoli
AB1 Tripoli
KP9 Tripoli
EC12 Saida
KP4 Beirut
PR1 El Qa'a
EC10 Saida
EC1 El Qa'a
SM1 Tripoli
PR2 Tripoli
EC7 Beirut
EC8 Beirut
KP10 Zahle
PO1 Saida
EC18 El Qa'a
EC19 El Qa'a
EC20 El Qa'a
EC21 El Qa'a
EC22 El Qa'a
EC23 El Qa'a
EC30 Tripoli
EC27 Saida
EC24 Beirut
EC35 Zahle
EC36 Zahle
Plasmids detected
IncI1α, IncX3, IncFIB(K), IncFII(pCoo)
IncFII, IncFIB(K), IncR
IncFIA, IncFIB, IncFII
IncFIA, IncFIB(K), IncFII(K),IncR
IncFIA, IncFII
IncFIB(K)
IncFIB, IncFII
IncB/O/K/Z, IncFIA, IncFIB, IncFII
IncB/O/K/Z, IncFIB(K), IncFIB, IncFII(K), IncQ1
IncI1α, IncFIA, IncFIB, IncI1γ, IncU, IncFII, IncY
IncFIA, IncFIB, IncFII
IncFIB KN
IncFIA, IncFIB, IncFIIS, IncU, IncFII
IncI1α, IncFIA, IncFIB, IncFIIS, IncU, IncFII
IncFIA, IncI1γ, IncFII, IncY
IncFIB, IncFII, IncFII(pCoo)
IncFIA, IncFIB, IncFII
IncFIA, IncFIB, IncFII
IncFIA, IncFII
IncFIA, IncFII
A
aac(6')Ib-cr
aadA16
aadA5
aadA2
aadA1
strB
strA
aph(6)-Id
aph(3")-Ib
aac(6')-Ic
aph(3')-Ia
aac(3)-iIa
aac(3)-iId
ant(2")-Ia
O
oqxA
oqxB
qnrB1
qnrB4
qnrB6
qnrB66
qnrD
qnrS1
qnrS2
H
mph(A)
mdf(A)
T
tet(A)
tet(B)
tet41
P
catA1
catA2
catB3
catB4
cmlA1
floR
F
FosA
D
dfrA1
dfrA7
dfrA12
dfrA14
dfrA17
dfrA27
dfrA30
S
sul1
sul2
B
blaTEM-1B
blaCTX-M-15
blaOXA-1
blaSHV-1
blaSHV-11
blaCMY-42
blaADC-25
blaOXA-132
blaSRT-2
blaPOM-1
blaDHA-1
blaNDM-5

Figure 3: Types and distribution of resistance determinants and plasmid typing results. *in silico* analysis was done using ResFinder v1.2, while plasmid Inc group determination was through PBRT-based PCR assays combined with *in silico* analysis using PlasmidFinder 1.3.

The used antimicrobial categories were labeled as follows: B, β-lactam resistance genes; S, sulfonamide resistance genes; D, trimethoprim resistance genes; F, fosfomycin resistance genes; P, chloramphenicol resistance genes, T, tetracycline resistance genes; H, macrolide resistance genes; O, quinolone resistance genes and A, aminoglycoside resistance genes.

3.5 Virulence factors

Virulence factors encoding genes were also studied in all MDR isolates. *gad*, a glutamate decarboxylase associated with *E. coli* survival under highly acidic conditions, was the most prevalent (82.3%, 14/17). *iss* gene was the second most common (52.9%, 9/17), encoding for an outer membrane protein and linked to serum resistance. The enteroaggregative *E. coli* heat-labile enterotoxin 1 (EAST1) encoding gene *astA* was found in *E. coli* EC22 from El Qa'a and EC24 from Beirut both belonging to ST131. In *K. pneumoniae* fimbriae encoding genes (*mrk*ABCDFHIJ) were detected only in KP8 and KP9. KP10 recovered from Zahle carried, in addition to the fimbriae encoding genes, yersiniabactin and siderophores encoding genes (*ybt* and *irp*). *K. pneumoniae* KP10 had the highest number of VFs, while none was detected in KP4 (Table 6).

Table 6: Virulence factors detected using VirulenceFinder 1.5 and their respective functions among the MDR isolates recovered from the river effluents and the El Qa'a refugee camp in Lebanon.

EC= *E. coli*, KP= *K. pneumoniae*

Season	Year	Site	Name	Virulence genes
Summer	2017	El Qa'a	EC1	increased acid resistance (*gad*), adhesin (*ipfA*)
Winter	2017	Saida	EC10	aggregative adherence (*aafC*), increased acid resistance (*gad*), adhesin (*iha*), increased serum resistance (*iss*), non-fimbrial adhesionh (*nfaE*), secreted autotranspoter toxin (*sat*)
Winter	2017	Saida	EC12	enteroaggregative immunoglobulin repeat protein (*air*), expression of invasion genes (*eilA*), increased acid resistance (*gad*), adhesin (*iha*), secreted autotranspoter toxin (*sat*), toxin (*senB*)
Winter	2018	El Qa'a	EC18	increased acid resistance (*gad*), long polar fimbriae (*iphA*)
Winter	2018	El Qa'a	EC19	iron uptake (*iroN*), increased serum resistance (*iss*), microcin transporter protein (*mchF*), protease (*tsh*)
Winter	2018	El Qa'a	EC20	increased acid resistance (*gad*), increased serum resistance (*iss*)
Winter	2018	El Qa'a	EC21	increased acid resistance (*gad*), iron uptake (*iroN, ireA*), increased serum resistance (*iss*), microcin transporter protein (*mchF*, *mchB*, *mchC*, *mcmA*), toxins (*senB*, *pic*, *cnf1*), autotransporter (*vat*)
Winter	2018	El Qa'a	EC22	Toxins (*astA*, *senB*), increased acid resistance (*gad*), adhesin (*iha*), increased serum resistance (*iss*), secreted autotranspoter toxin (*sat*)
Winter	2018	El Qa'a	EC23	capsular gene (*capU*), increased acid resistance (*gad*)
Winter	2018	Beirut	EC24	toxins (*astA*, *senB*), increased acid resistance (*gad*), adhesin (*iha*), increased serum resistance (*iss*), secreted autotranspoter toxin (*sat*)
Winter	2018	Saida	EC27	increased acid resistance (*gad*), increased serum resistance (*iss*), long polar fimbriae (*iphA*), toxin (*senB*)
Winter	2018	Tripoli	EC30	enteroaggregative immunoglobulin repeat protein (*air*), capsular gene (*capU*), expression of invasion genes (*eilA*), increased acid resistance (*gad*), adhesin (*iha*), increased serum resistance (*iss*), long polar fimbriae (*iphA*), secreted autotranspoter toxin (*sat*), toxin (*senB*)
Winter	2018	Zahle	EC35	adherence (*aafC*), increased acid resistance (*gad*), adhesin (*iha*), increased serum resistance (*iss*), non-fimbrial adhesionh (*nfaE*), secreted autotranspoter toxin (*sat*)
Winter	2018	Zahle	EC36	increased acid resistance (*gad*), adhesin (*iha*), increased serum resistance (*iss*), secreted autotranspoter toxin (*sat*)
Winter	2017	El Qa'a	EC5	increased acid resistance (*gad*), adhesin (*iha*, *aatA*), long polar fimbriae (*iphA*), microcin transporter protein (*mchF*,*mchB*, *mchC*), secreted autotranspoter toxin (*sat*)
Summer	2017	Beirut	EC7	adherence (*aafC*), increased acid resistance (*gad*), adhesin (*iha*), non-fimbrial adhesionh (*nfaE*), secreted autotranspoter toxin (*sat*), toxin (*senB*)
Summer	2017	Beirut	EC8	enteroaggregative immunoglobulin repeat protein (*air*), expression of invasion genes (*eilA*), increased acid resistance (*gad*)
Summer	2017	Zahle	KP10	siderophore encoding gene (*irp25*) ,fimbriae encoding gene (*mrkA3*, *mrkB3*, *mrkD1*, *mrkF8*, *mrkH10*, *mrkI7*, *mrkJ19*), yersiniabactin encoding gene (*ybtA1*, *ybtA39*, *ybtE5*, *ybtP5*, *ybtQ6*, *ybtS5*, *ybtT5*, *ybtU9*, *ybtX11*, *fyuA11*)
Winter	2017	Tripoli	KP8	fimbriae encoding gene (*mrkA2*, *mrkB2*, *mrkF4*, *mrkI1*)
Winter	2017	Tripoli	KP9	fimbriae encoding gene (*mrkF44*, *mrkH1*, *mrkI17*)

3.6 Plasmid Incompatibility profiling

PBRT and PlasmidFinder 1.3 successfully identified 15 different Inc groups. Inc groups that were not identified using the PBRT kit such as IncY, were detected through *in silico* analysis. The most commonly identified Inc groups were IncFII (21%), IncFIA (18%) and IncFIB (18%) and many others as shown in Figure 3. The highest number of detected Inc groups was seven in *E. coli* EC18 (IncI1α, IncFIA, IncFIB, IncI1γ, IncU, IncFII and IncY).

3.7 *E. coli* MLST typing

All recovered *E. coli* isolates from the river effluents and El Qa'a refugee camp were subjected to PFGE and MLST typing to determine the genetic relatedness. Results obtained didn't show any significant pattern. It is noteworthy that all *E. coli* ST131, which were recovered from different sites (Beirut, Zahle and Saida), clustered together expect for EC24 isolated from Beirut river effluent. *E. coli* EC3 isolated from the El Qa'a refugee camp was untypeable using *Xba*I restriction digestion and was as a result excluded.

Based on Clermont et al. (2013) classification, there are seven *E. coli* phylgroups: A, B1, B2, C, D, E and F. All 36 *E. coli* isolates were analyzed and based on the marker gene combinations they were assigned to their corresponding phylogroups. Accordingly, Phylogroup A (10/36; 27.8%), B2 (13/36; 36%), and D (6/36; 17%) were the most common in this study (Figure 5).

The Achtman method (https://enterobase.warwick.ac.uk/species/ecoli) was used to determine the MLST type, based on which ST131 (16.7%, 6/36) and ST69 (11.1%, 4/36) were the most common, with ST405, ST394 and ST410 being also among the detected

STs. Four of the isolates, however, did not match any allelic profile and as such were assigned under new STs (Figures 5 and 6).

ST131 isolates (*E. coli* EC10, EC22, EC24, EC35 and EC36) belonged to O25b:H4 serotype as determined *in silico* using GoSeqit (GoSeqit), except for EC7 having instead the O16:H5b serotype. Other serotypes were also detected including O102:H6 (ST405; *E. coli* EC8 and EC12), while EC5 (ST2349), EC19 (ST746), and EC27 (ST410) had untypeable O type gene.

3.8 Pan-genome analysis of *E. coli*

Pan-genome analysis of all the MDR *E. coli* revealed the presence of a total of 10315 unique protein coding sequences. Genes identified as part of the core genome were present in all the isolates and constituted 3154 protein coding sequences. Moreover, genes present in 15-95% of isolates were designated as being shell genes and constituted 2451 protein coding sequences, while those present in <15% constituted 4710 protein coding sequences.

Maximum likelihood tree based on the pan-genome analysis of the coding regions in *E. coli* showed the clustering of EC22, EC24, EC36, EC10 and EC35; all typed as ST131, phylogroup B2 and serotype O25:H4b. EC7 (ST131, phylogroup B2, serotype O16:H5b) on the other hand, clustered separately (Figure 6).

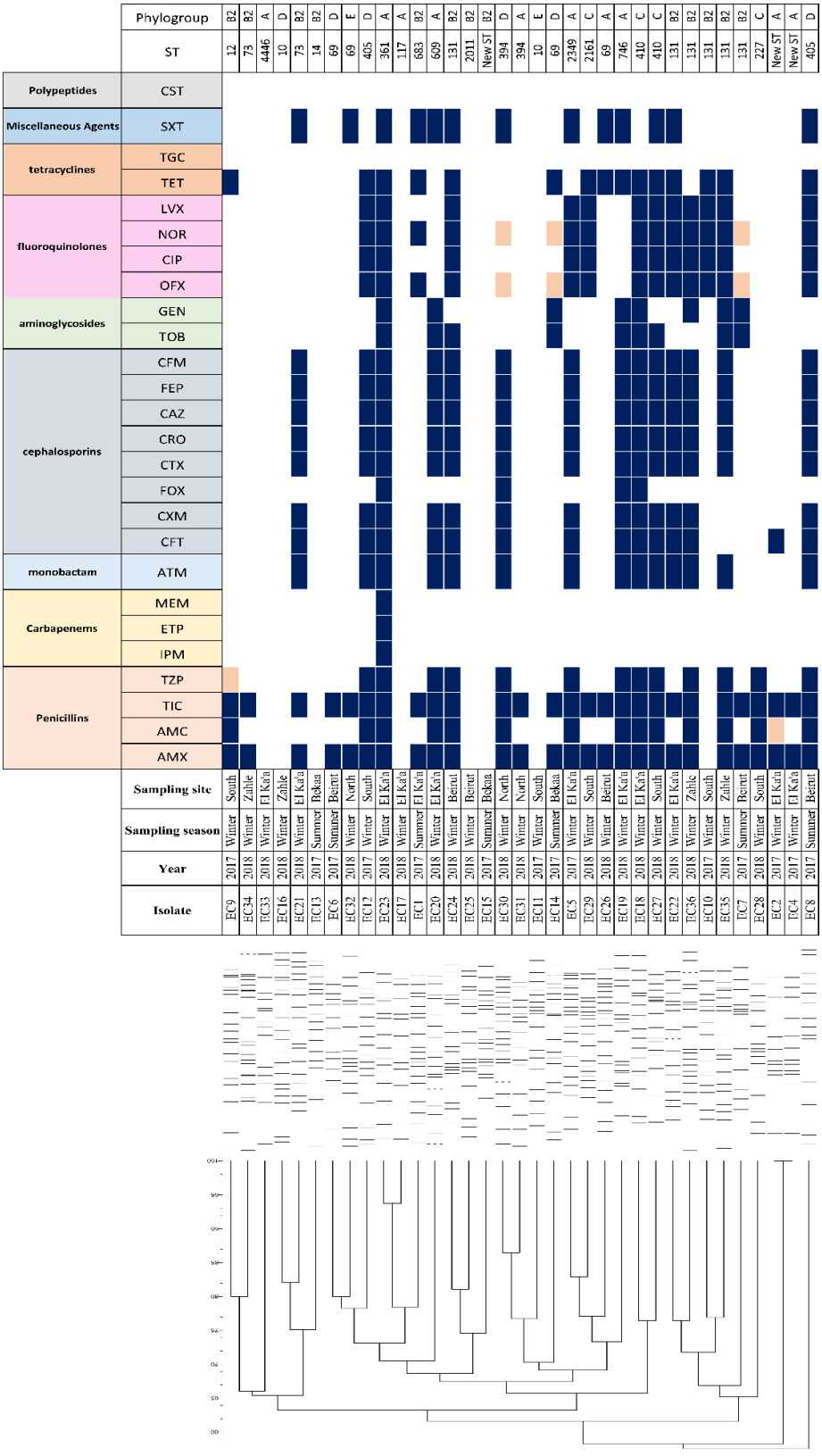

Phylogroup
ST
Polypeptides
CST
Miscellaneous Agents
SXT
tetracyclines
TGC
TET
fluoroquinolones
LVX
NOR
CIP
OFX
aminoglycosides
GEN
TOB
cephalosporins
CFM
FEP
CAZ
CRO
CTX
FOX
CXM
CFT
monobactam
ATM
Carbapenems
MEM
ETP
IPM
Penicillins
TZP
TIC
AMC
AMX
Sampling site
Sampling season
Year
Isolate

Figure 4: Detailed characterization of MDR *E. coli*. The dendrogram was generated using Bionumerics software 7.6.2 showing the relatedness of the isolates based on their banding patterns generated by *Xba*I restriction digestion.

Dark blue= Resistant, white= sensitive and light orange= intermediate resistant.
AMX= Amoxicillin, AMC= Amoxicillin/ Clavulanic Acid, TIC= Ticarcillin, TZP= Piperacillin/Tazobactam, IPM= Imipenem, ETP= Ertapenem, MEM= Meropenem , ATM= Aztreonam, CFT= Cefalotin, CXM= Cefuroxime, FOX= Cefoxitin, CTX= Cefotaxime, CRO= Ceftriaxone, CAZ= Ceftazidime, FEP= Cefepime, CFM= Cefixime, TOB= Tobramycin , GEN= Gentamicin, OFX= Ofloxacin, CIP= Ciprofloxacin, NOR= Norfloxacin, LVX= Levofloxacin, TET= Tetracycline, TGC= Tigecycline, SXT= Trimethoprim/Sulfamethoxazole and CST= Colistin, EC= *E. coli*, ST= sequence type.

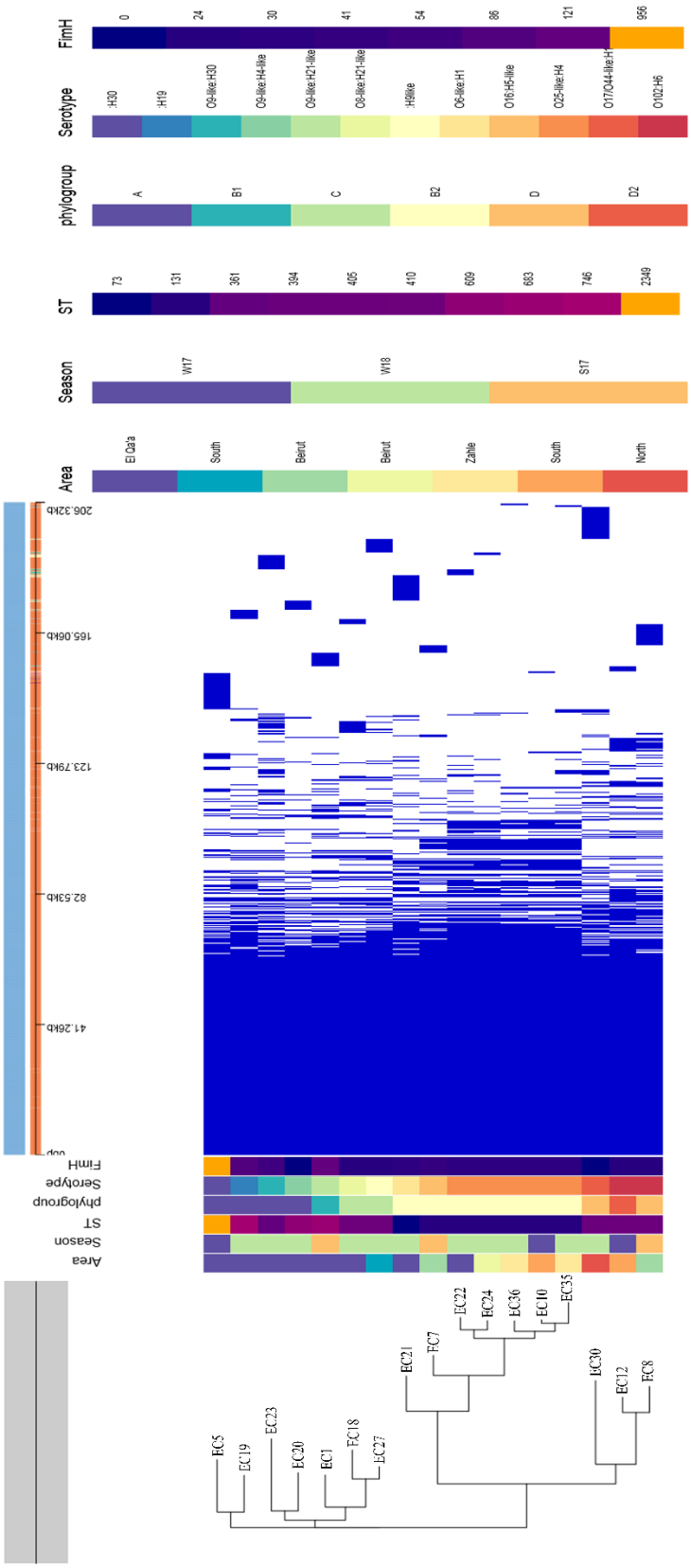

FimH
Serotype
phylogroup
ST
Season
Area
El Qa'a
South
Beirut
Beirut
Zahle
South
North
W17
W18
S17
206.32kb
165.06kb
123.79kb
82.53kb
41.26kb
EC5
EC19
EC23
EC20
EC1
EC18
EC27
EC21
EC7
EC22
EC24
EC36
EC10
EC35
EC30
EC12
EC8

Figure 5: Pan-genome of all sequenced *E. coli* constructed using Roary and showing the similarity matrix, area and season of isolation, ST type and phylogroup in addition to the maximum likelihood tree based on the core and accessory genomes.

3.9 *K. pneumoniae* MLST Typing

All recovered *K. pneumoniae* isolates were typed using PFGE and MLST to study genetic relatedness. No specific pattern was observed except for KP9 and KP6 which were recovered during winter 2017 from Tripoli. The two isolates belonged to ST76 and were linked to K-type 31. KP9 and KP6 additionally, shared a 96% similarity despite the difference observed in their susceptibility patterns. KP9 showed resistance to aztreonam, cefalotin, cefuroxime, cefoxitin, cefotaxime, ceftriaxone, cefixime, ofloxacin, tetracycline, trimethoprim/sulfamethoxazole, and nitrofurantoin and intermediate resistance to ertapenem and amoxicillin/clavulanic acid. KP6 on the other hand, was sensitive to all the above-mentioned antibiotics.

No significant seasonal differences were observed in the number of isolates recovered, with highest being recovered from samples collected from Tripoli (60%, 6/10) and none from El Qa'a refugee camp (Figure 7).

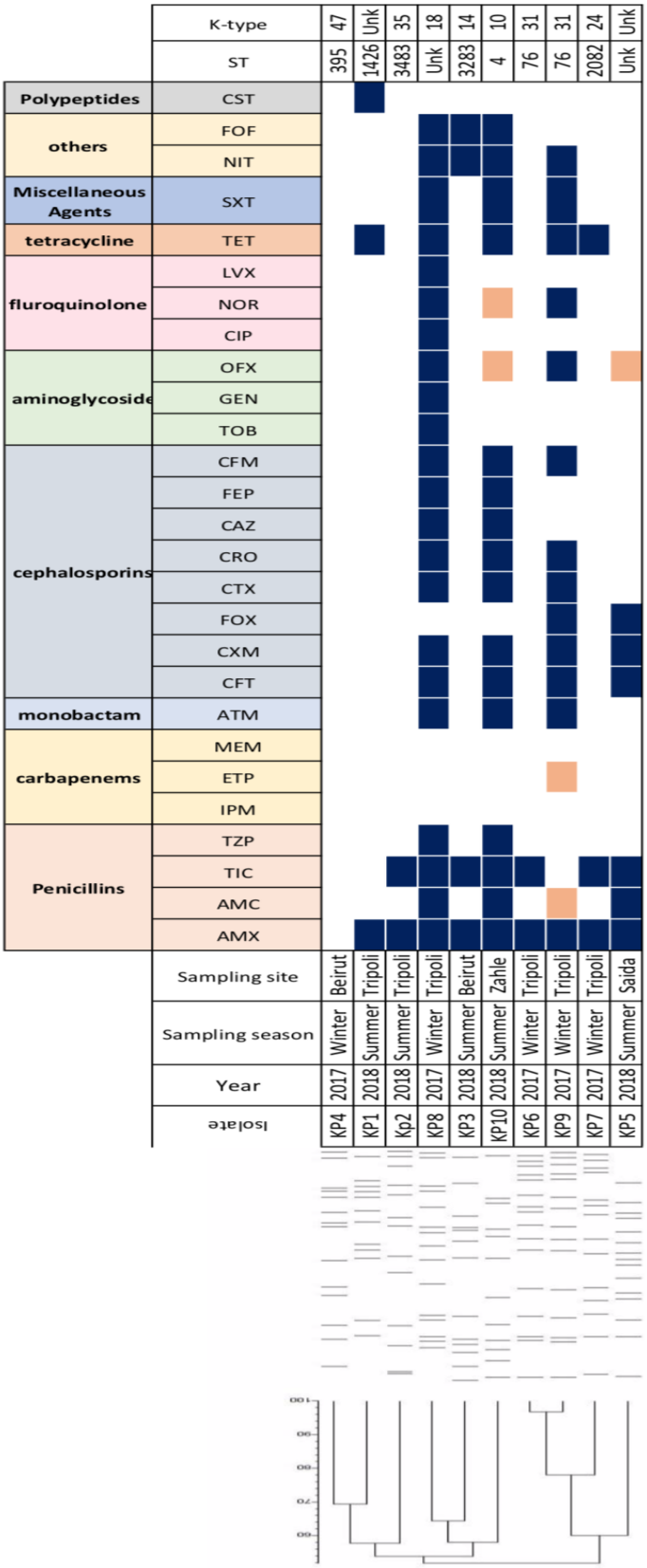

K-type
47
Unk
35
18
14
10
31
31
24
Unk
ST
395
1426
3483
Unk
3283
4
76
76
2082
Unk
Polypeptides
CST
others
FOF
NIT
Miscellaneous Agents
SXT
tetracycline
TET
fluroquinolone
LVX
NOR
CIP
aminoglycoside
OFX
GEN
TOB
cephalosporins
CFM
FEP
CAZ
CRO
CTX
FOX
CXM
CFT
monobactam
ATM
carbapenems
MEM
ETP
IPM
Penicillins
TZP
TIC
AMC
AMX
Sampling site
Beirut
Tripoli
Tripoli
Tripoli
Beirut
Zahle
Tripoli
Tripoli
Tripoli
Saida
Sampling season
Winter
Summer
Summer
Winter
Summer
Summer
Winter
Winter
Winter
Summer
Year
2017
2018
2018
2017
2018
2018
2017
2017
2017
2018
Isolate
KP4
KP1
Kp2
KP8
KP3
KP10
KP6
KP9
KP7
KP5

Figure 6: Detailed characterization of all *K. pneumoniae* isolates. The dendrogram was generated using Bionumerics software 7.6.2 and showing the relatedness between the isolates based on their banding patterns generated by *Xba*I restriction digestion.

Dark blue= Resistant, white= sensitive and light orange= intermediate resistant.
AMX= Amoxicillin, AMC= Amoxicillin/ Clavulanic Acid, TIC= Ticarcillin, TZP= Piperacillin/Tazobactam, IPM= Imipenem, ETP= Ertapenem, MEM= Meropenem , ATM= Aztreonam, CFT= Cefalotin, CXM= Cefuroxime, FOX= Cefoxitin, CTX= Cefotaxime, CRO= Ceftriaxone, CAZ= Ceftazidime, FEP= Cefepime, CFM= Cefixime, TOB= Tobramycin , GEN= Gentamicin, OFX= Ofloxacin, CIP= Ciprofloxacin, NOR= Norfloxacin, LVX= Levofloxacin, TET= Tetracycline, TGC= Tigecycline, SXT= Trimethoprim/Sulfamethoxazole and CST= Colistin, KP= *K. pneumoniae*, ST= sequence type

3.10 Conjugation-*bla*$_{\text{NDM-5}}$

The only carbapenem resistant isolate recovered from the El Qa'a refugee camp was *E. coli* EC23, carrying a *bla*$_{\text{NDM-5}}$, was selected for further characterization. The plasmid carrying *bla*$_{\text{NDM-5}}$ was successfully subjected to conjugative transfer from the donor to the recipient *E. coli* J53 and rendering it as a result to become resistant to meropenem. PBRT revealed that the plasmid had multi-replicons namely IncFIA, IncFII and IncI1γ. PLACNETw further showed that EC23 had one big-multi-replicon plasmid (IncFIA, IncFII and IncI1γ) and two other smaller ones (figure 8).

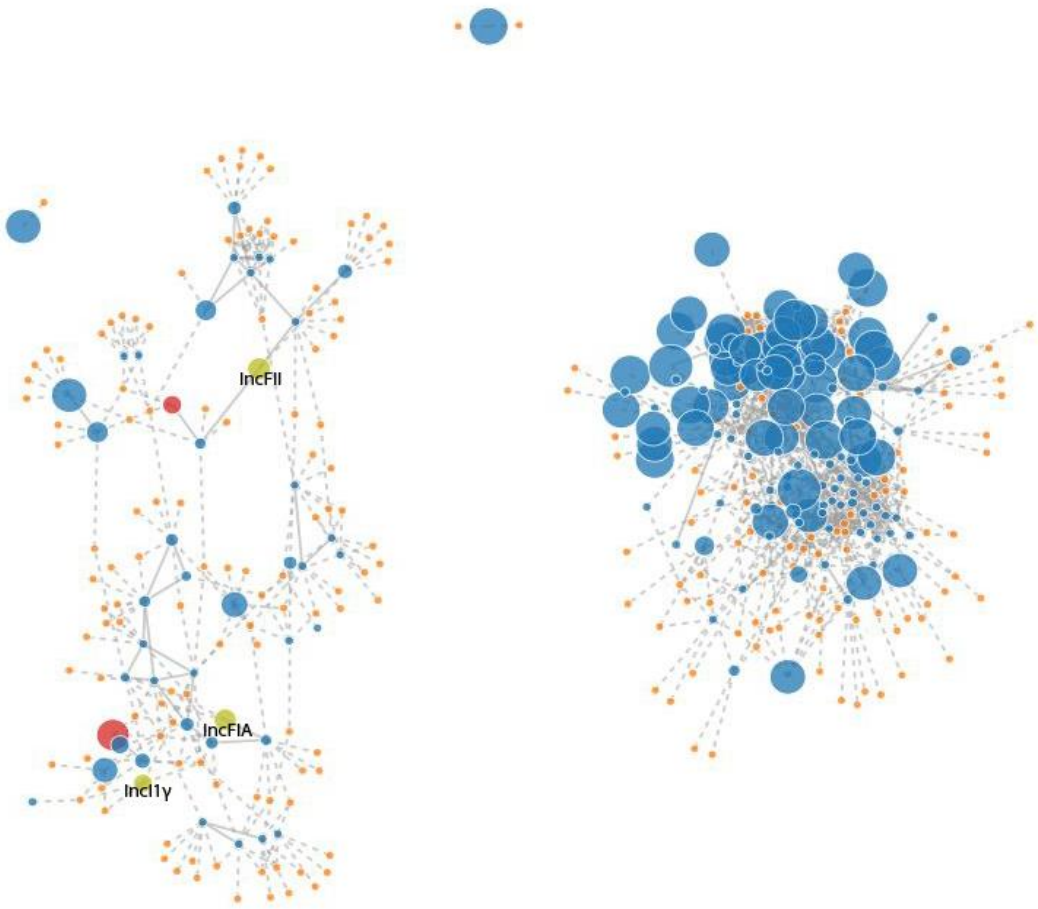


Figure 7: A schematic representation of *E. coli* EC23 genome using PLACNETw. EC23 had a multi-replicon plasmid (IncFIA, IncFII and IncI1γ) and two smaller ones.

Green circle: relaxase and replication initiation site; red circle: relaxase; yellow circle: replication initiation site; blue circle: contigs.

Chapter 4

Discussion

Antibiotic resistance is a worldwide concern and the spread of drug-resistant bacteria in different environments including water sources may constitute a route for the dissemination of drug-resistant pathogens (Egervärn et al, 2017). The prevalence of antibiotic-resistant bacteria in surface water in Lebanon is a growing concern with the lack of collection and domestic wastewater management strategies. According to the Central Administration of Statistics, 37% of the buildings in Lebanon are connected to a sewer network, the rest either use cesspools or septic tanks or release raw sewage directly into the environment (Ministry of Environment, ECODIT). Wastewater management is expensive and as a result community and hospital effluents are reaching surface water with the ultimate outcome being an increase in the microbial load and level of contamination (Houri & El Jeblawi, 2007).

Majority of the antibiotics we consume are metabolized with small amounts passing through wastewater to the environment. Wastewater introduced into the environment contributes to the spread of antibiotic resistance. In this study, we aimed at determining the role of antibiotic resistance determinants in the contamination of water supplies in Lebanon. Our results showed a common occurrence of bacterial contaminants in surface water including ESBL- and carbapenemase- producing *Enterobacteriaceae* and an increase in the risk of resistance genes with the increase in human population, population mobility and widespread lack of wastewater treatment.

The highest number of isolates was collected from El Qa'a refugee camp (28/91, 30.8%) compared to other locations. Displaced refugees are dispersed in 1700 areas in the six districts in Lebanon and no formal camps are present (Cherri, González & Delgado, 2016). However, Bekaa and North which include the most poor and vulnerable communities, host the largest numbers of refugee (341,333 and 245,292 respectively) (UNHCR, 2019). In addition, 30% of these refugees live in areas that were already densely populated thus creating issues of overcrowding, infrastructure damage, as well as insufficiency in access to water and sanitation services (UNOHCA, 2017). Thus, this increase in population to areas which are already vulnerable increased the pressure on different resources in Lebanon including water. The international institute of Environment and Development recently showed that since the beginning of the Syrian conflict a 30% increase in water demand was recorded. In addition, research published in 2014 by the Ministry of Environment showed that waste water pollution has increased by 33% since 2011 (UNOHCA, 2014) which can be explained by the increase in population around water sources.

The most commonly detected organism from the different locations was *E. coli* (36/91, 39%). This finding was in accordance with a previous study by Tokajian et al. (2018), assessing bacterial loads from sewage contaminated surface water samples from Lebanon. *E. coli* constituted 77.3% of the total number of isolates and was also the most prevalent organism from different water sources in Lebanon including wells, estuaries and river water (Diab et al., 2018). The second most prevalent organism was *Enterococcus* spp. (12/91, 13%) despite the fact that their presence in environmental water is not natural (Cabral, 2010). The presence of enterococci in water environments is usually due to

livestock, poultry, hospital and municipal sewage and wildlife faecal contamination (Byappanahalli, Nevers, Korajkic, Staley & Harwood, 2012). *K. pneumoniae* was also isolated from all locations except for El Qa'a refugee camp. *K. pneumoniae* was previously recovered from different water sources in Lebanon including wells, springs and estuaries (Diab et al., 2018). *K. pneumoniae* is a leading cause of community acquired pneumoniae, it can spread rapidly and often leads to nosocomial infections (Chung, 2016). Thus, the presence of this organism in surface water used for human consumption poses a major threat on the community. A previous study by Hafza et al. (2018) showed that *E. coli* has a fitness advantage over ESBL or non-ESBL producing *K. pneumoniae* and the presence of ESBL genes in the latter can cause a high fitness cost in the host bacterium. This can explain the lack of isolation of *K. pneumoniae* from El Qa'a refugee camp water samples which host the highest numbers of *E. coli*. This overall presence of *E. coli*, *K. pneumoniae* and *Enterococcus* spp. in our surface water clearly shows that untreated wastewater is still flowing into rivers across Lebanon, contributing to the increase in the prevalence of pathogenic organisms in surface water resources.

The most commonly identified ESBL gene in this study was $bla_{\text{CTX-M-15}}$ which was in line with recent studies from Lebanon that covered estuaries, wells and springs (Diab et al., 2018), and rivers (Tokajian et al., 2018). $bla_{\text{CTX-M-15}}$ is also the most common ESBL in Lebanon detected in isolates recovered from animals (Diab et al., 2018) as well as healthy and hospitalized patients (Moubareck et al., 2005). Surface water receiving untreated sewage and industrial water could be a factor contributing to the high occurrence of this resistant determinant (Houri & El Jeblawi, 2007). The dissemination of $bla_{\text{CTX-M-15}}$ was globally linked to the spread of the highly virulent ST131 *E. coli* (B2 phylogenetic group

and serotype O25:H4) and to ST405 (phylogroup D) (Coque, Baquero & Canton, 2008). The gene was linked to an IncF plasmid, which has a broad host range facilitating mobility and spread to other organisms (Villa et al., 2010; Zhao & Hu, 2012). In this study, all *E. coli* isolates belonging to either ST405 or ST131, harbored the $bla_{\text{CTX-M-15}}$ except for EC10 (ST131-B2-O24:H4) recovered from Saida. $bla_{\text{CTX-M-15}}$ was also detected in two out of four *K. pneumoniae* isolates belonging to ST4 and ST3483. CTX-M-15 producing *K. pneumoniae* was previously documented from an Indian riverine (Mondal, Siddiqui, Sultan & Haq, 2018) and an urban surface water source in Malaysia (Tissera & Lee, 2013). Co-occurrence of $bla_{\text{CTX-M-15}}$ with other ESBL genes ($bla_{\text{TEM-1}}$, $bla_{\text{SHV-1}}$ or $bla_{\text{OXA-1}}$) was also detected in 48% of the MDR isolates in this study. This phenomenon is common among Gram-negative organisms isolated from surface water (Chen et al., 2010; Zou et al., 2012; Mondal, Siddiqui, Sultan & Haq, 2018).

In addition to $bla_{\text{CTX-M-15}}$, IncF plasmids have been implicated in the spread of other resistance determinants including TEM, OXA, CMY and KPC (Carattoli, 2011), which were detected in this study from all different sites. $bla_{\text{TEM-1}}$ was the most frequently detected gene. $bla_{\text{TEM-1}}$ was frequently isolated from sewage and river water in Poland (Korzeniewska, Korzeniewska & Harnisz, 2013), China (Shi et al., 2019) and from river water samples from Lebanon (Tokajian et al., 2018). The presence of this gene indicated the contamination of surface waters by anthropogenic antibiotic-resistant organisms (Shi et al., 2019) or by hospital effluents since they constitute a major source of bla_{TEM} gene (Narciso-da-Rocha et al., 2014). The IncI1 plasmid type was previously reported in organisms recovered from humans and animals (Rozwandowicz et al, 2018). This plasmid type is known to carry multiple resistance genes including $bla_{\text{CMY-2-like}}$ genes (Lorme et al.,

2018). We detected two isolates that were resistant to all tested cephalosporins and recovered from the El Qa'a refugee camp. Both carried *bla*$_{\text{CMY-42,}}$ and a *bla*$_{\text{CMY-2-like}}$ gene. The nucleotide sequence of CMY-42 differed by two amino-acids from that of CMY-2 and is linked to a decrease in susceptibility to third and fourth generation cephalosporins (Hentschke et al., 2011). This variant was previously isolated from urban water sources in India (Bajaj, Singh, Kanaujia & Virdi, 2015) and patients from Egypt and Spain (Helmy & Wasfi, 2014; Pitart et al., 2015).

Moreover, NDM-5 producing *E. coli* (EC23, ST361) was also recovered from a sample collected from the El Qa'a refugee camp and it was carried on a conjugative plasmid of type IncFIA, IncFII and IncI1γ. NDM-5 was first detected in a clinical *E. coli* isolate in 2011 from the United Kingdom (Hornsey, Phee & Wareham, 2011). In 2018, *bla*$_{\text{NDM-5}}$ was isolated from an Indian hospital sewage water (Parvez & Khan, 2017). Parvez & Khan showed an association between *bla*$_{\text{NDM-5}}$ and *bla*$_{\text{CMY-42}}$ which was also the case in this study. Recently from Lebanon, NDM-1 producing *Enterobacter cloacae* was reported from hospital wastewater (Daoud et al., 2018). Altogether these findings indicated that carbapenem resistance genes from clinical settings are being transferred into the environment and may constitute a source of human infection with carbapenem-resistant *E. coli* through the food chain.

B2 was the most common detected phylogroup, representing 36.1% (n=13) of all recovered *E. coli* in this study, followed by type A (n=10, 27.8%). *E. coli* strains mainly fall into seven phylogroups (A, B1, B2, C, D, E and F) (Clermont et al., 2013). Phylogroups B2 and D, mainly being extra-intestinal pathogens, are known to be more virulent than groups A and B, which represent mainly the intestinal pathogens (Clermont

et al, 2013). On the other hand, phylogroups A and B1 showed higher resistance profiles than B2 and D (Chakraborty et al., 2015). A recent study by Diab et al. (2018) revealed that in Lebanon phylogroup A was the most prevalent while phylogroup B2 was the least. These differences between studies may be explained by the differences in water sources (from wells, springs, estuaries or rivers) and geographical locations.

During 2017, the total recovered bacteria from all water sources during the winter season was 46 compared to 24 recovered in the summer. Fecal contamination is usually more common during wet seasons (Kostyla, Bain, Cronk & Bartram, 2015). This could be linked to the failure of sewage systems to contain water levels following intense rainfall (Chu et al., 2014) or the resuspension of intertidal sediment containing high levels of bacterial contaminants (Ferguson, Moore, Getrich & Zhowandai, 2005). This overall increase in contamination of surface water during heavy rainfall seasons could lead to an increase in incidences of diarrheal infections which is usually more common during rainy seasons (Alexander & Blackburn, 2013). However, the data we have was not enough to infer any seasonal impact on the overall quality.

It's noteworthy that we were able to isolate one *Pseudomonas otitidis* (designated as PO1) in this study from Nahr Al-Awali in Saida. *P. otitidis* was also previously isolated from water sources in Mexico (Rodríguez-Verdugo, Souza, Eguiarte & Escalante, 2012). Pseudomonads are widespread microorganisms recovered from different natural (soil, water, plants) and clinical settings (Tan et al, 2015). *P. otitidis* however, is a recently described organism associated with hospital acquired infections including otic and non-otic ones (Lee et al., 2016). PO1 was found to carry $bla_{\text{POM-1}}$ gene encoding for resistance to carbapenems and other β-lactams (Thaller et al, 2010).

Highlighting main sources of pollution and their occurrences is important to understand the patterns of dissemination. To our knowledge, this is the first comprehensive study determining the role of mobile genetic elements in the spread of resistant determinants within surface water resources in Lebanon. Bacterial diversity, resistance genes and plasmid content. The prevalence of MDR bacteria in natural aquatic environments calls for investing more into wastewater treatment.

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