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DISSEMINATION OF *MCR-1*-CARRYING *ESCHERICHIA COLI* IN SEAWATER AND SEWAGE WATER ACROSS LEBANON

by
TSOLAIRE GEORGE SOURENIAN

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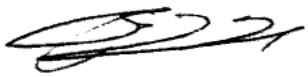

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ABSTRACT
OF THE THESIS OF

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Major: Food Safety

Title: Dissemination of *mcr-1*-carrying *Escherichia coli* in Seawater and Sewage Across Lebanon

Antibiotic resistance has become one of the core public health concerns. Excess use and misuse of antibiotics, especially the last resort antibiotic, colistin, led to emergence and dissemination of colistin resistant *Escherichia coli* carrying the *mcr-1* gene to different countries worldwide. Detection of *mcr-1* in humans and animals has a significant risk of contamination to seawater and sewage water. Many studies done in Lebanon illustrated the prevalence of *mcr-1* in the agricultural-environmental-human sectors; therefore, we first hypothesized the potential contamination of *mcr-1* alongside other antimicrobial resistance genes in the Mediterranean Sea water; along the Lebanese coastline.

Samples were collected from 22 different locations from North to South of the Lebanese coast. Approximately, 45.5% of the samples were contaminated with colistin-resistant *E. coli;* out of which a total of 16 isolates were *mcr-1* positive. The colistin minimum inhibitory (MIC) for these isolates ranged between 4 μg/mL and 32 μg/mL and antimicrobial susceptibility (AMR) testing showed that all the *E. coli* isolates were multidrug-resistant; showing resistance to at least 3 classes of antibiotics. The isolates were susceptible or exhibited intermediate resistance to carbapenems. Moreover, the extended-spectrum β-lactamase genes (ESBL), bla_{TEM}, were detected in five isolates. A variety of plasmid types were detected in the isolates, especially the ones responsible for the global dissemination of the *mcr-1*, IncX4. BOX- PCR showed that 87.5 % of the isolates were genotypically diverse. The *mcr-1*-carrying isolates can persist in the water milieu for more than 35 days. Based on these outcomes, we further hypothesized that the *mcr-1* positive isolates detected in seawater might be related to the direct discharge of sewage water. Samples were collected from 6 main sewage outfalls from North to South of Lebanon. The samples were 100% positive for colistin-resistant *E. coli*, yielding to 60 *mcr-1* positive isolates which were confirmed by PCR analysis. The colistin MIC for these isolates ranged between 8 μg/mL and above 640 μg/mL, and AMR testing showed that 3.3% of the *E. coli* isolates were (PDR), 20% XDR, and 76.6% were MDR. 78% of the plasmids were IncX4 type.

It is the first study to highlight the dissemination and characterization of *mcr-1*-positive *E. coli* in seawater and sewage in Lebanon.

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CHAPTER I

INTRODUCTION

Over the years, reports from different parts of the world have indicated the gradual decline in the efficacy of drugs prescribed to inhibit the growth of bacterial pathogens in the human body [1, 2]. The low effectiveness of drugs has been attributed to the continuous emergence of antibiotic-resistant microorganisms across the globe. According to the Centers for Disease Control and Prevention (2013), microorganisms have developed new mechanisms to transfer genes to resist various antibiotics. Recent studies have indicated that some multi-drug resistant strains of the bacteria that belong to the family *Enterobacteriaceae* produce enzymes that breakdown an antibiotic known as Carbapenem [1, 2, 3, 4]. Thus, the diseases caused by these bacteria fall under the difficult to treat categories. The rise in antibiotic resistance led to a documented increase in the cases of hospitalized infected individuals as well as increase in the rates of morbidity and mortality of among the affected individuals [1, 5, 6]. Although the rise in the occurrence of infectious diseases caused by microbes that belong to the family *Enterobacteriaceae* has become of concern of public health officials, there is limited information on the global spread of these microorganisms [7]. Hence, there is an urgent need to conduct further research studies to better understand and evaluate the epidemiological aspects (i.e. incidence, morbidity, and mortality) of the diseases caused by bacteria that are resistant to Carbapenem and other antibiotics worldwide. Some studies have documented the detection of different species of Gram-negative rods from the family *Enterobacteriaceae* that exhibit novel resistance mechanisms to the drug

Polymyxin E (also known as Colistin) [4, 5, 8]. This antimicrobial agent is used to control pathogens that are no longer susceptible to Carbapenem [4].

A. Colistin: The Last Resort Antibiotic

Polymyxins are antibiotics naturally produced by different species of *Paenibacillus*. There are five classes of polymyxins but only two are used therapeutically: polymyxin B and polymyxin E [9]. Colistin is a polypeptide of the group E polymyxin family, produced by *Paenibacillus polymyxa* ssp. *colistinus.* It was discovered in 1950 by Y. Koyama [10] and then became available in the 1950s for the treatment of Gram-negative bacilli infections [11,12].

Colistin works by binding to lipopolysaccharides (LPS), a component of the bacterial outer membrane, found only in Gram-negative bacilli. Its mechanism of action, not fully elucidated, can be explained by three distinct and concomitant modes: lysis of bacterial membranes (main mode), “vesicle-vesicle” contact, and formation of free radicals. These three mechanisms lead to the death of the bacteria [9]. Anti-toxin activity has also been found [13].

Its main described mechanism of action is as follows: polymyxins possess positive charges and then bind to negatively charged LPS. This interaction causes a displacement of the divalent cations (essentially Mg^{2+} and Ca^{2+}) responsible for the disorganization of the membrane structure allowing colistin to insert into the outer membrane. Colistin will then lead to an alteration in membrane permeability and the formation of pores, thus leading to leakage of the intracellular content, causing the death of the bacteria [11,14,15].

Colistin was used until the 1980s and was then excluded from therapeutic protocols due to the appearance of new molecules such as third generation Cephalosporins, but especially because of its renal toxicity [16]. Its use has remained exceptional in patients with cystic fibrosis to control infectious complications [17]. Since the emergence of multi-resistant bacteria, mainly *Pseudomonas aeruginosa*, *Acinetobacter baumannii,* and *K. pneumoniae* and the absence of new antibiotics effective against these bacteria, a renewed interest in the use of colistin has been observed since the 1990s [18]. In 2014, it reverted to an antibiotic prescribed for the treatment of severe human infections linked to bacteria resistant to all other treatment options [5]. Colistin is mainly used intravenously but a recent study has underlined the interest and the effectiveness of inhalation of colistin to treat respiratory infections [19].

In previous years, researchers suggested that the reduced sensitivity to Polymyxin E was caused by changes in the following gene regulatory systems: *pmrAB, phoPQ,* and *mgrB* [3]. However, it was recently discovered that the low susceptibility to this therapeutic drug is mediated by plasmids that harbor *mcr* genes [3]. After the first identification and molecular analysis of the first *mcr* genes, other *mcr* variants that have been reported around the world [7, 8, 6, 20, 21]. Moreover, the gene variants were detected in transferable plasmids such as IncI2, IncX4, IncF, IncP, IncY, and IncHI2. Many bacteria that harbor these plasmids have also been cultured from the following samples: uncooked and ready-to-eat food products and diverse sources of water such as rivers and seas [20, 21, 22]. The recent detection of Gram-negative bacteria that are not susceptible to colistin has been attributed to the intake of the antibiotic for the enhancement of growth in food animals [24]. Despite the isolation of bacteria with *mcr*

genes from different food and environmental sources, the importance of these reservoirs in the transmission of plasmids that harbor *mcr-1* among bacterial species has remained under investigated in developing countries.

B. *mcr-1* Gene: Overview

The first detection of *mcr-1* was documented in China in the year 2016 [25]. Ever since, more than 25 bacterial species with plasmid-borne *mcr-1* have been reported in different parts of Asia and other continents such as Africa, Europe, Oceania, South America, and North America [26]. Although most of the studies on plasmid-borne *mcr-1* have been carried out in China, bacterial isolates with this gene have also been discovered in Italy, the United Kingdom, and Spain. According to El Bediwi et al. (2019), the current documentation of the rising incidence of bacteria with *mcr* genes in various parts of the world may be attributed to the continuous administration of antibiotics in animal husbandry. Moreover, some researchers have reported that the rise in the number of cases of microorganisms with *mcr* genes worldwide may be due to the rise in international trade and the exportation of foods from countries with a high prevalence of these microbes [27, 28]. Another major factor that has led to the global spread of bacteria containing plasmids that harbor *mcr* genes is the long-term use of the antibiotic to inhibit the growth of microbes that exhibit low susceptibility to common antibiotics [22, 24, 29]. Other variants of *mcr-1* genes such as *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5*, *mcr-6*, *mcr-7*, and *mcr-8* have been identified worldwide. These genes are often carried by different plasmids. Although the IncHI2 plasmid has been identified frequently in different parts of Europe, the IncI2 plasmid is commonly found in Oceania, Asia, South America, and North America [26].

C. Transmission of Plasmids Harboring *mcr-1* Gene in *Escherichia coli*

The continuous use of Polymyxin E (Colistin) in animal farming has contributed significantly to the incidence of microbes with plasmids that harbor *mcr-1* [3, 30]. Some scientists have suggested that the visceral organs and feces of animals serve as source for the transmission of the microbes in food, the environment, and to humans [31, 29, 32]. According to El Bediwi et al. (2019), the food chain may facilitate the rapid spread of bacteria containing *mcr* genes. This was documented in the reports obtained from some of the research studies conducted in China [5, 26]. The result of the investigations indicated that the use of polymyxin E in aquaculture farming has led to the spread of bacteria that exhibit low sensitivity to the drug. Plasmids have also played an essential role in the global incidence of microorganisms that harbor *mcr* genes. These plasmids facilitate the rapid transmission of *mcr* genes in different reservoirs [9, 32]. A critical review of existing studies indicated that the most frequently detected plasmids that harbor *mcr* genes include IncX4, IncI2, and IncHI2. The IncI2 plasmid contains the genes that enable the microbe to survive in acidic conditions. Other genes on the plasmid also mediate the production of biofilm and sex pilus, which facilitate the transfer of genes by cell mating (known as conjugation). The genes facilitate the adherence of microbes to the surface of epithelial cells. Similarly, the IncHI2 plasmids contain genes that enable the microorganism to transfer the plasmid and other virulence factors at different temperature conditions [26, 30]. Furthermore, the genes located in the IncHI2 plasmid reduce the susceptibility of microorganisms to different broad-spectrum antibiotics [33, 34].

Many research findings have indicated that the major species of Gram-negative rods that harbor *mcr-1* are *Escherichia coli* (*E. coli*). Some of these Gram-negative rods

can also acquire extended-spectrum beta-lactamases (ESBLs), which confer resistance to antibiotics. Other acquired enzymes include AmpC beta-lactamases, carbapenemase, and metallo-beta-lactamase (MBLs). These molecules enable the bacteria to breakdown beta-lactam antimicrobial drugs [26, 34, 35]. Strains of *E. coli* that possess the ability to synthesize these enzymes are more likely to survive and transmit the *mcr-1* to other clones [36, 37].

D. The Prevalence of *E. coli* with *mcr-1* Gene in Seawater Worldwide

Some researchers have documented the isolation of bacteria with *mcr* genes in seawater samples from South America (30, 36). In Egypt, it is estimated that the prevalence of *E. coli* with *mcr-1* isolated from surface water is 16.6% [8]. Ahmed et al. (2019) reported that the occurrence of *mcr-1* in surface water may be attributed to the migratory activity of birds in the country. Shad (2019) also documented a similar suggestion in a mini-review on the global spread of microorganisms that possess low susceptibility to antibiotics. The author highlighted that the first case of the occurrence of *mcr-1* was reported in Ushuaia, Argentina. Shad (2019) also suggested that the detection of microbes with the *mcr-1* in this resort location may be due to the migration of gull species to and from different continents across the world. Moreover, some studies have indicated that *mcr-1* are associated with the transferable IncI2 plasmid found in the gull species [5, 6, 26, 38, 39].

A study conducted by Fernandes et al. (2016) indicated the presence of *E. coli* with *mcr-1* in seawater specimens of more than 10 different public beach locations in Sau Paulo, Brazil. Although all the isolates were not susceptible to meropenem and imipenem, only three exhibited resistance to polymyxin. These findings suggest that

these bacteria are can survive in several environments [40; 41]. *E. coli* has also been detected in well water specimens in China.

E. Detection of *mcr-1* in Seawater in Lebanon

Lebanon suffers from major environmental breaches and water contamination due to its debilitated infrastructure. The main sources of pollution in Lebanon are organic waste, raw sewage, and sewage from power plants, the main reservoirs of fecal contaminants and antibiotic resistance genes, neighboring the seashores or rivers, which ends up in the Mediterranean Sea.

Recently, the use of colistin-containing drugs in human medicine and animal farming in Lebanon have been documented through studies done in several settings. However, no studies evaluated the microbiological contamination of seawater with the fecal indicator *E. coli* harboring *mcr-1*. Therefore, my first study was to determine the dissemination and spread of the mobile colistin-resistant gene in Lebanese coastline; especially after its detection in poultry and livestock farms [42, 43, 44], irrigation water [44], and refugee camps sewage [45, 46].

Seawater samples were collected from 22 locations on the Lebanese coast from North to South [47]. 45.5% were positive for colistin-resistant *E. coli* which were further confirmed by PCR using16S-rRNA gene fragment. Another PCR analysis showed that the 16 colistin resistant *E. coli* isolates were positive for *mcr-1*, using a specific primer CLR5-F (5′-CGGTCAGTCCGTTTGTTC-3′) and CLR5-R (5′-CTTGGTCGGTCTGTAGGG-3’) which were also confirmed by commercial sequencing. colistin minimum inhibitory concentration (MIC) for the isolates ranged between 4 µg/mL and 32 µg/mL. The isolates expressed phenotypic resistance to

Penicillin (100% of isolates), Ampicillin (94%), Amoxicillin + Calvulanic acid (94%), Cefepime (13%), Cefotaxime (25%), Cephalexin (75%), Cefixime (19%), Gentamicin (13%), Kanamycin (38%), Streptomycin (69%), Tetracycline (81%), Ciprofloxacin (63%), Norfloxacin (13%), Trimethoprim-sulfamethoxazole (75%), and Chloramphenicol (63%). However, all the isolates were susceptible to Doripenem, Imipenem, and Meropenem. Therefore, the disk diffusion assay showed that all the *E. coli* isolates were multidrug-resistant. The plasmids of *mcr-1* positive isolates were extracted and confirmed that the gene was plasmid-born and transmissible. The plasmids belonged to different groups, mainly IncX4, which has been associated with the global dissemination of colistin resistance. Survival studies suggested that these isolates can persist for a long time in seawater after studying their fitness at different conditions which may allow the currents to disperse them beyond local waters. All these findings indicate that Lebanon is in need for serious investments in antimicrobial stewardship and surveillance in order to manage the dissemination of *mcr-1*, other antibiotic-resistance genes, and resistant bacteria to the Mediterranean Sea and, subsequently, to the surrounding areas.

CHAPTER II

DETECTION OF *MCR-1* IN SEWAGE IN LEBANON

Sewage management is one of the most core fundamentals of any country's ecological, social and economic ventures [48]. Among miscellaneous contaminants of sewage water, the microbial agents, especially the resistant ones, are becoming of critical concerns worldwide and their removal should be targeted throughout various wastewater treatment systems [49]. There are variety of biological contaminants in wastewater as such: Fecal coliforms, *Escherichia coli*, *Salmonella*, *Shigella*, *Vibrio cholerae*, diverse Parasite cysts and eggs, viruses and fungi. All of them can be hazardous to environmental and human health depending on the type and amount [50, 51]. There are two types of sewage: treated and untreated. Treated sewage is the one that has undergone treatment in a plant. Sewage goes through several stages in the treatment process to eliminate harmful bacteria, pollutants and contaminants. The final outcome of wastewater usually ends up in rivers or seas or be sometimes reused for irrigation and agricultural purposes. The other type of sewage is the untreated sewage, which contains harmful pathogens and other contaminants.

The use of antibiotics in humans and animals causes the secretion of residual drugs into environmental resources, whereby approximately 75%-90% of some of the antibiotics taken are excreted unmetabolized into the environment [52]. Countries with weakened infrastructure, dispose and release pollutants from households, hospitals, animal facilities and farms, and sewage water all into the environment, mainly in water resources [53]. This uncensored disposal contaminates water with antibiotic-resistant organisms and genes [53].

A recent report by the European Medicines Agency (EMA) showed that Spain is one of the European countries with higher levels of use of colistin (> 120 tons) in food-producing animals. In the analysis of Lekunberri et al, also reported an increase of in the total number of the *mcr-1* gene in the past 5 years. Since wastewater treatment plants (WWTPs) in Spain obtain sewage containing antibiotic-resistant bacteria and antibiotic residues from various sources, these facilities represent 'hotspots' for the survival and spread of antibiotic resistance genes, which may be subsequently released to receiving environments. The abundance of the *mcr-1* gene was significantly higher in raw samples, than in treated samples in winter seasons [54]. In China, the presence of *mcr-1* and carbapenemase genes in *Enterobacteriaceae* (*E. coli* and bla_{NDM}-1-carrying *E. cloacae* and *C. freundii*) was confirmed in sewage water samples which were collected from 5 tertiary hospitals. The *mcr-1* and carbapenemase genes have arisen in various aquatic environments, including rivers, seepage, well water and wastewater treatment plants [55].

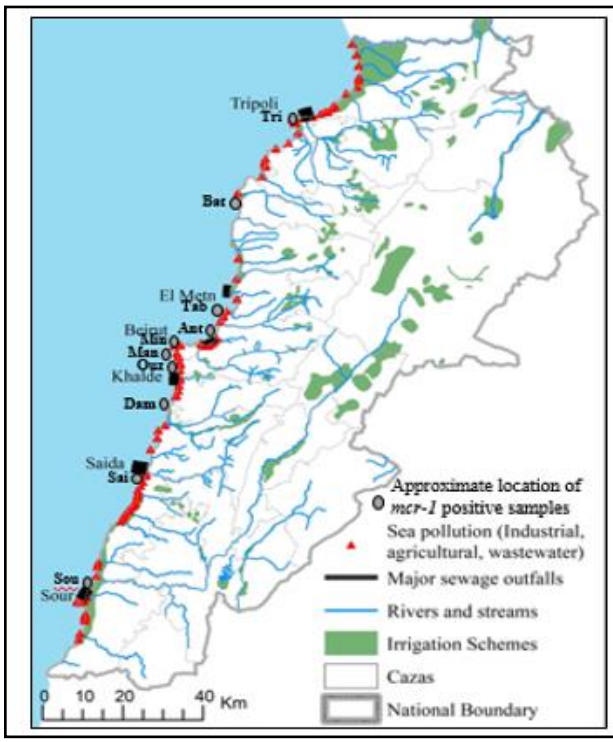
In Lebanon, due to rapid population growth, demands on water resources for industrial, households, agricultural and commercial use are increasing. Domestic wastewater in Lebanon during the war years (1975-1990), was discharged without any treatment directly into the sea. Present estimates indicate that 35 to 50% of the untreated urban sewage water is infiltrated to the aquifers because of shortage in discharge networks and WWTPs and then pumped for domestic use and irrigation. In fact, most villages lack sewage infrastructure except for the traditional household septic tanks or the method of draining wastewater into boreholes in bedrock which will eventually reaches the groundwater [56]. Currently, the use of non-conventional water in Lebanon is being practiced mainly in agriculture. For example, in central Bekaa valley as well as

in other agricultural areas in Lebanon, sewage water is being used to irrigate vegetables; even those which are normally consumed as raw [57]. There are no studies that assessed the microbiological contamination of sewage with *E. coli* harboring the *mcr-1* gene in Lebanon. Therefore, it was important concern to investigate the dissemination and spread of *mcr* in sewer outfalls and to validate its direct impact on the Mediterranean Sea [47].

A. Materials and Methods

1. Sample Collection

Sewage water samples were collected from 6 main sewer outlets from different locations across Lebanon (Figure 1).



Sewage Sources	Coordinates
Tripoli	34.456500, 35.865477
Tabarja	34.021530, 35.623467
Antelias	33.9180211, 35.5834501
Manara	33.899822, 35.469969
Saida	33.588900, 35.386554
Sour	33.273979, 35.195182

Figure 1 The Approximate Locations of Major Outfalls in Lebanon with their Corresponding Coordinates

Samples were collected in duplicate; directly from the outfalls with disposable sterile 100 mL sample cups (2 cups from each source). Samples were then placed individually in the plastic sample bags and were transferred to the laboratory in a water cooler with ice for analysis within 12 h of collection.

2. Bacterial Isolation

The duplicate sewage samples from each source were pooled. Due to the viscosity and the turbidly of the sewage samples, each sample was diluted 100 times. A volume of 1 mL of the pooled sample was mixed with 99 mL sterile autoclaved water, then filtered through 0.22-µm Millipore membranes (S-Pak®). Each membrane was placed on an *Escherichia coli* selective medium plates (RAPID' *E. coli* 2 agar; Bio-Rad) supplemented with 4 µg/mL of colistin (Sigma-Aldrich, USA). The samples were incubated at 44°C for 18-24 hours. Based on the phenotype, 10 *E. coli* colonies were selected and isolated from each sample plate. Therefore, a total of 60 isolates were purified and stored in 1 mL LB (Luria Bertani-Sigma) broth with 0.5 mL of 80 % glycerol and preserved at -80°C for further analysis.

3. Polymerase Chain Reaction

a. <u>DNA Extraction of the Sewage Isolates</u>

The DNA of sewage water isolates was extracted and used to run PCR (Polymerase Chain Reaction) analysis. One to two bacterial colonies were suspended in 100 µl of DNase free water (Sigma) in a 0.2 mL PCR tubes, then placed in PCR machine programmed to reach 99°C for 13 minutes. The tubes were placed on ice directly after the run for few minutes and then centrifuged for 2 minutes at 14000 rpm.

The supernatant containing the genomic DNA was removed and transferred into a new sterile tube and stored at -20 °C [47].

b. Detection of the *mcr-1*

The extracted DNA was used to screen for the *mcr-1* using specific primers CLR5-F (5′-CGGTCAGTCCGTTTGTTC-3′) and CLR5-R (5′-CTTGGTCGGTCTGTAGGG-3') [63]. Into each 0.2 mL PCR reaction tube, 4 µL of the master mix (Solis-5x FIREPol® Master Mix Ready to Load) were added to 12 µL of DNase free water followed by 0.5 µL of each of the forward and reverse primers. Then 3 µL of genomic DNA were added. The PCR analysis was programmed for 38 cycles using a thermal cycler (VWR, USA): denaturation step at 95°C for 1 minute, annealing at 52°C for 45 seconds, elongation at 72°C for 1 minute with a final extension for 10 minutes at 72°C. The amplified DNA was screened using a 1% agarose gel stained with 5 µL ethidium bromide (Bio-Rad, USA) and separated using gel electrophoresis for 45 minutes at a constant voltage of 100V. The bands were visualized by the gel imaging system Chemi-Doc (Bio-Rad, USA) reader. The size (309 bp) of the *mcr-1* was checked compared to a 100 bp DNA (Solis-Ready to load) ladder as reference.

c. Detection of the Antimicrobial Resistance Genes

Antimicrobial resistance (AMR) genes, *bla*-$_{TEM}$, *bla*-$_{CTX-M,}$ *bla*-$_{SHV}$, were examined by PCR to detect resistance to β-lactam antibiotics. Also, carbapenemase genes, *bla* $_{NDM}$, *bla* $_{OXA-48}$, *bla* $_{IMP}$ and *bla* $_{KPC}$ were used to determine resistance to carbapenem antibiotics, and Class 1 Integron to investigate the presence of these elements, using specific primers (Table 1). A volume of 4 µl of Master Mix (Solis-5x FIREPol® Master Mix Ready to Load) were added to 12 µL of DNase free water followed by 0.5 µL of each specific forward and reverse primers. Then 3 µL of

extracted DNA were added to the mix. The PCR conditions of each primer are indicated in the Table 1. PCR reactions were placed in a thermocycler for 38 cycles. Then the corresponding gene amplicons were separated through 1% agarose gel stained with ethidium bromide using gel electrophoresis for 45 minutes at 100V.

4. Sequencing of mcr-1 Positive Isolates

Commercial sequencing was performed on 18 representative *mcr-1* positive isolates from sewage water (3 isolates per source) to confirm the observed *mcr-1* signal. The amplified *mcr-1* fragments were purified using the QIAquick® PCR Purification Kit (50) as per the protocol provided by the kit insert and sent to be sequenced at laboratory of the University of Saint Joseph (USJ, Beirut, Lebanon).

5. Antimicrobial Susceptibility Testing

Antimicrobial analysis was done to all sewage water *mcr-1* positive isolates using the Disc Diffusion Sensitivity method. Fresh colonies were suspended individually in 5 mL in Muller Hinton (MH) broth (Bio-Rad). Bacterial suspensions were spread with a sterile cotton swab on Mueller-Hinton Agar (MHA) plates after adjusting the optical density to 0.05 at OD_{600} and 20 commercially available antibiotic discs were placed on the agar; four antibiotic discs per plate, and incubated at 37°C for 18-24 hours [47]. The antibiotics used were Penicillin (PEN), Ampicillin (AMP), Amoxicillin +Clavulanic acid (AMC), Cefepime (FEP), Cefotaxime (CTX), Cephalexin (LEX), Cefixime (CFM), Doripenem (DOR), Meropenem (MEM), Imipenem (IPM) Gentamicin (GEN), Kanamycin (KAN), Streptomycin (STR), Tetracycline (TET), Ciprofloxacin (CIP), Norfloxacin (NOR), Trimethoprim-Sulfamethoxazole (SXT),

Chloramphenicol (CHL), Colistin (COL). Erythromycin (ERY) for quality control, because *E. coli* is intrinsically resistant to this antibiotic. Antibiotic susceptibility and resistance were determined by measuring the diameter of the zone of inhibition around each antibiotic disc and comparing it to clinical breakpoint standards. The results were classified as per the guidelines of the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility (EUCAST) [58,59].

6. Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) for colistin was done to all *mcr-1*-positive *E. coli* detected in sewage water to determine the susceptibility to colistin [60]. 180 µL of bacterial suspension previously adjusted to 0.05 at OD_{600} were added to a 96-microtiter plate and 20 µL of prediluted colistin (Sigma-Aldrich, USA) were added to the each well of different concentration ranging from1 µg/mL to 640 µg/mL. The microtiter plates were incubated at 37°C for 18-24 hours. The plates were analyzed with the Infinite M200PRO microplate reader at $\lambda = 600$ nm. Isolates having a colistin breakpoint >2µg/mL were considered resistant to colistin as per EUCAST recommendation [61].

7. Plasmid Transformation

Plasmid extraction was done on 12 *mcr-1*-positive *Escherichia coli* isolates from each sewage sources using the ZymoPURE^{TM} Plasmid Mini Prep Kit (100 Preps, USA) following the steps provided by the kit. Competent *E. coli* JM109 cells were used as recipient of the genetic material. 50 µL of competent cells were gently mixed with 10 µL of the extracted plasmid and incubated on ice for 30 minutes. After the incubation,

the cells were heat-shocked by immersing 2/3 of the tube in the water bath at 42℃ for 2 minutes, followed by another incubation on ice for 90 seconds. After incubation on ice, 940 µL of freshly prepared LB broth were added to the cells and incubated in a shaking incubator for 1 hour 45 minutes at 37°C. The tubes were centrifuged for 2 minutes at 14000 rpm, and 900 µL of the supernatant were discarded. The pellet was resuspended in the remaining 100 µL of LB and transferred on RAPID' *E. coli* 2 plates supplemented with 2 µg/mL of colistin. The plates were incubated at 37° C for 18-24 hours. The transformants were harvested and further examined for *mcr-1* as well as for their MIC and antimicrobial properties as described above [47].

8. BOX-PCR Analysis

The BOX-A1R oligonucleotide (5'CTACGGCAAGGCGACGCTGACG-3') was used to generate BOX-PCR profiles for all the *mcr-1* positive isolate to identify their genetic diversity [64]. All the PCR reactions were done in volumes of 25 µL, containing 17.5 µL of DNase free water (Sigma), 4 µL of ready to load Master Mix (Solis), 0.5 µL of the single BOX primer, and 3 µL of bacterial DNA. The amplifications were performed in the thermal cycler (VWR) programmed for an initial denaturation step for 2 minutes at 94℃ followed by 35 cycles of 30 seconds at 94℃ followed by annealing at 50℃ for 1 minute and an elongation step for 8 minutes at 65°C. The final elongation for 10 minutes at 72℃. The amplified DNAs were migrated in a 1.5% agarose gel stained with ethidium bromide using gel electrophoresis at 100 V for 75 minutes.

9. Plasmid Typing

The PCR Based Replicon Typing Kit 2.0 (Diatheva) PBRT kit was used on 18 *mcr-1*-positive-*E.coli* isolates of sewage (same isolates which were sequenced) to determine the incompatibility plasmid types. The PBRT kit is composed of 8 amplification mixes of which amplifies three or four targets, allowing to detect a total of 28 replicons (Table 2). As per manufacturer's recommendations, 1 µL of DNA was added to 24 µL of each PCR mix solution. The amplifications were performed in the thermal cycler (VWR) programmed for an initial denaturation step for 10 minutes at 95°C followed by 30 cycles of 60 seconds at 95°C followed by annealing at 60°C for 30 seconds and an elongation step for 60 seconds at 72°C. The final elongation for 5 minutes at 72°C. 5 µL of the amplification products were resolved on 2.5% agarose gel stained with ethidium bromide and electrophorized for 45 minutes at 100 Volts and visualized using the Chemi-Doc gel reader (Bio-Rad-USA) [62].

Table 1 List of Primers Corresponding to Target Genes and their PCR Conditions

Gene/ primer sequence	**PCR conditions for 38 cycles**	**Amplicon size**	**Reference**
bla-$_{TEM}$ Forward: 5'-GCGGAACCCCTATTTG-3' Reverse: 5'-ACCAATGCTTAATCAGTGAG-3'	95°C for 1minute 56°C for 45 seconds 68°C for 1 minute	963 bp	[65]
bla-$_{CTX-M}$ Forward: 5'- ATGTGCAGYACCAGTAARGTKATGGC-3' Reverse: 5'- TGGGTRAARTARGTSACCAGAAYCAGCGG-3'	95°C for 30 seconds 57°C for 45 seconds 72°C for 1 minute	593 bp	[66]
bla -$_{SHV}$ Forward: 5'- CACTCAAGGATGTATTGTG-3' Reverse: 5'- TTAGCGTTGCCAGTGCTCG-3'	95°C for 1minute 56°C for 45 seconds 68°C for 1 minute	822 bp	[67]
bla-$_{NDM}$ Forward: 5'-GGTTTGGCGATCTGGTTTTC-3' Reverse: 5'- CGGAATGGCTCATCACGATC-3'	95°C for 1minute 56°C for 45 seconds 68°C for 1 minute	621 bp	[68]
bla-$_{OXA-48}$ Forward: 5'-GCTTGATCGCCCTCGATT-3' Reverse: 5'-GATTTGCTCCGTGGCCGAAA-3'	95°C for 1minute 56°C for 45 seconds 68°C for 1 minute	281 bp	
bla-$_{IPM}$ Forward: 5'-TGAGCAAGTTATCTGTATTC-3' Reverse: 5'-TTAGTTGCTTGGTTTTGATG-3'	95°C for 1minute 56°C for 45 seconds 68°C for 1 minute	740 bp	
bla-$_{KPC}$ Forward: 5'-CATTCAAGGGCTTTCTTGCTGC-3' Reverse: 5'-ACGACGGCATAGTCATTTGC-3'	95°C for 1minute 56°C for 45 seconds 68°C for 1 minute	538 bp	
Class 1 Integron Forward: 5'-GGCATCCAAGCACAAGC-3' Reverse: 5'-AAGCAGACTTGACTGAT-3'	95°C for 30 seconds 55°C for 45 seconds 65°C for 1 minute	Variable	[69]

Table 2 Replicons Detected by PBRT 2.0

Multiplex	Target name	Amplicon Size (bp)
M1	HI1	534
	HI2	298-308
	I1-α	159
M2	M	741
	N	514
	I3	316
	B/1	159
M3	FIB	683
	FIA	462
	W	242
M4	L	854
	P	534
	X4	284
	I1-γ	161
M5	T	750
	A/C	418
	FIIS	259-260
M6	U	843
	X2	370
	R	251
	FIIK	142-148
M7	Y	765
	X3	376
	FIC	262
	K	160
M8	HIB-M	570
	FIB-M	440
	FII	258-262

B. Results

Typical colistin-resistant *Escherichia coli* colonies were detected in 6 sewage sources (100%). A total of 60 colistin-resistant *E. coli* colonies were successfully retrieved (10 colonies per sample) and screened for the detection of the *mcr-1* using specific CLR5 primers (Figure 2).

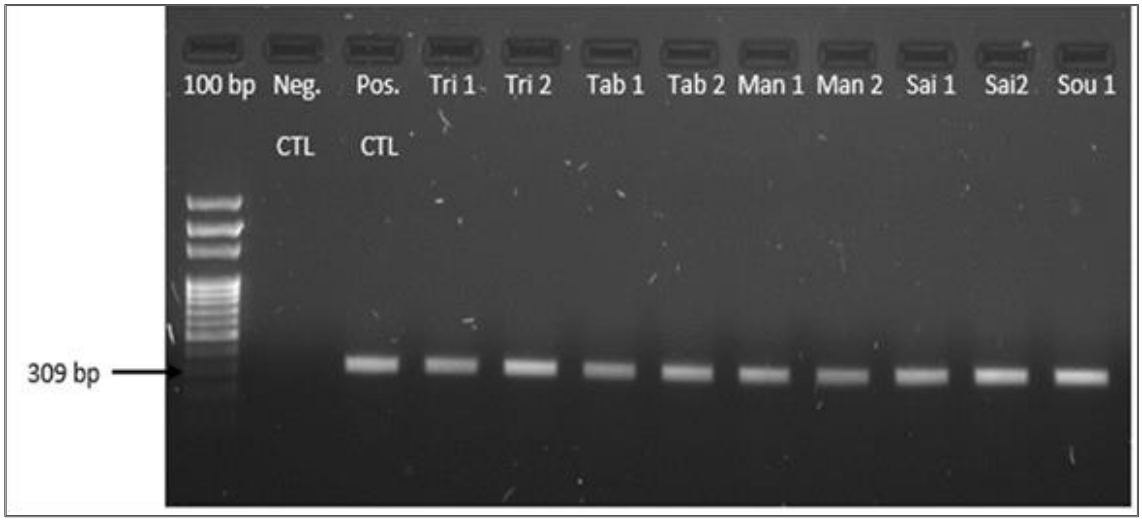


Figure 2 Polymerase chain reaction (PCR) amplification of target *mcr-1* gene.

Three representative amplicons of the *mcr-1* fragments (n=18) were selected from each source and were purified and sequenced. Sequences alignment using the BLAST function (NCBI, National Center for Biotechnology Information, U.S. National Library of Medicine- USA) revealed that all of the sequenced fragments were comparable to the internationally reported *mcr-1* bacteria available in databanks (http://www.ncbi.nlm.nih.gov/).

We were also interested whether these isolates were resistant to multiple antibiotics in addition to colistin. Antimicrobial susceptibility testing by the disk diffusion assay showed that all of the *E. coli* isolates were multidrug-resistant (resistant to at least three antibiotic classes). The isolates expressed phenotypic resistance against

Penicillin (100%), Ampicillin (90%), Amoxicillin/Clavulanic acid (93%), Cefepime (27%), Cefotaxime (48%), Cephalexin (68%), Cefixime (43%), Doripenem (32%), Meropenem (28%), Imipenem (17%), Gentamicin (28%), Kanamycin (47%), Streptomycin (98%), Tetracycline (100%), Ciprofloxacin (67%), Norfloxacin (58%), Trimethoprim/Sulfamethoxazole (90%) and Chloramphenicol (77%) (Figure 3). The antimicrobial profile showed that 76.6% of the isolates were multidrug- resistant (MDR). It is worth mentioning that 20 % of the isolates were extensively drug resistant (XDR), susceptible to only one or two antimicrobial categories, and significantly 3.3% of the isolates were pen-drug resistant (PND), resistant to all 20 antibiotics mentioned (Table 4).

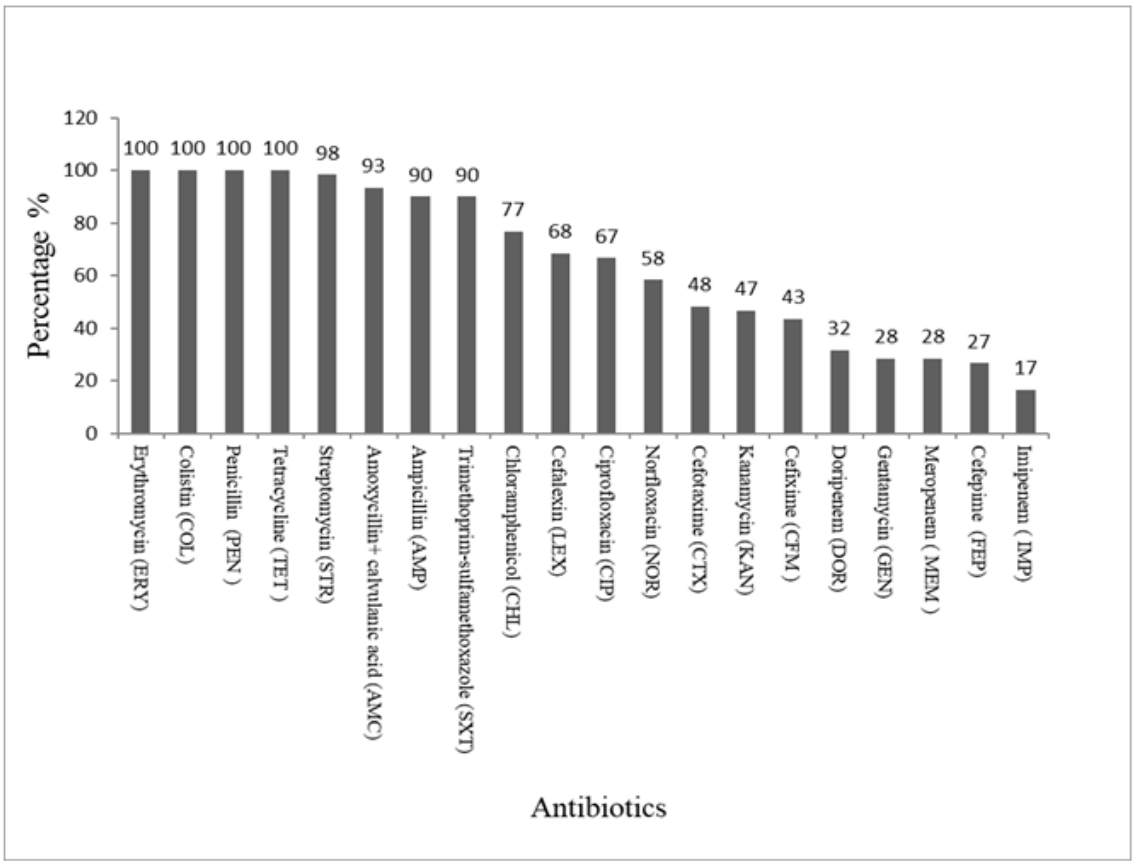


Figure 3 Percentages of Antibiotic Resistance of the *mcr-1*-positve-*E coli* Isolates

Using broth microdilution method, the minimum inhibitory concentration (MIC) of colistin was ≥ 8 µg/mL for all the *mcr-1* positive isolates confirming the colistin-resistant profile, and the values ranged between 8 and ≥ 640 µg/mL, where 50% of isolates had MIC 16 µg/mL and 13.3% had MIC 28 µg/mL. It is worth to mention that 18.3% had MIC ≥ 640 µg/mL (Figure 4, Table 4).

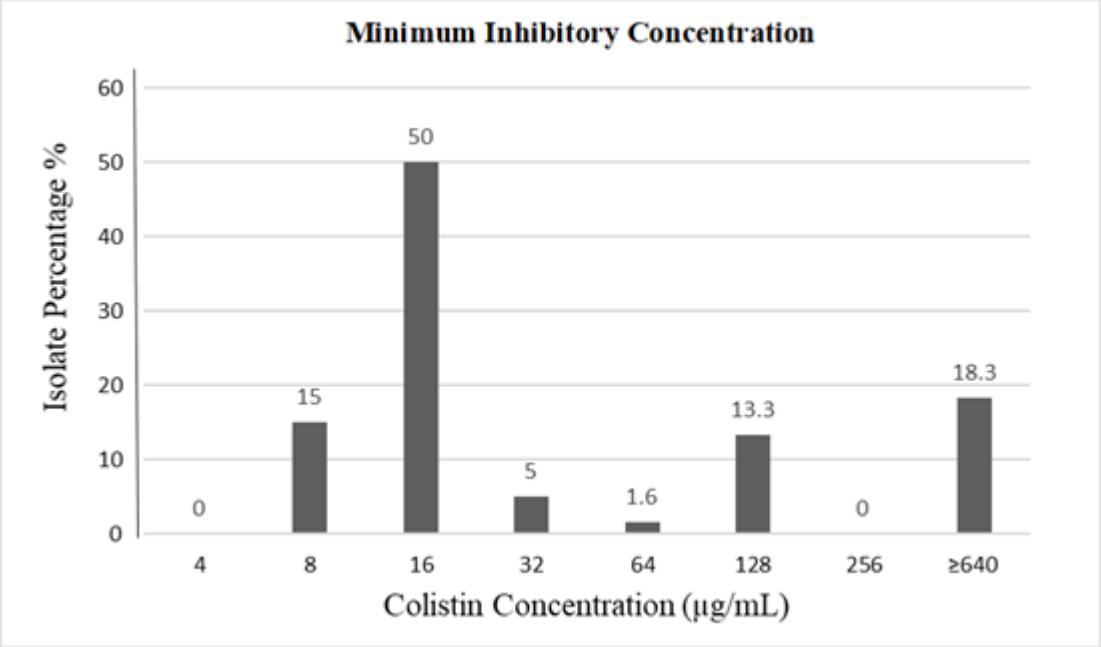


Figure 4 Minimum Inhibitory Concentration (MIC) of the *mcr-1*-positive-*E.coli* Isolates Recovered from the Sewage Outlets in Lebanon

We further investigated other antibiotic resistance genes that might be harbored by the *mcr-1* positive isolates. The isolates were checked for beta-lactam genes using specific primers (Table 1). Out of 60 isolates, 17 isolates (28%) from different sewage sources harbored bla_{TEM}, 1 isolate from Tabarja source (Tab8) was positive for bla_{SHV} and 12 isolates (20%) were $bla_{\text{CTX-M}}$ positive (Table 4). Notably, 80% of the isolates also harbored the Class 1 Integron gene. Only 1 isolates (Ant8) was positive for $bla_{\text{OXA-48}}$.

Box PCR fingerprinting confirmed that the 60 *mcr-1*-positive-*E.coli* isolates were highly diverse. They belonged to at least 10 different genotypes. The results showed that out of 60 isolates, 17 (28 %) had a genotype different from another group consisting of 7 isolates (12%). Another 6 isolates (10%) were totally different form the stated groups. Based on these results, there was a notable genotypic diversity in the isolates.

Plasmids were extracted from the isolates (ZymoPURE, USA) based on antimicrobial profile and Box PCR outcomes (n= 12, 2 isolates from each sewage source) and were successfully introduced into *E. coli* JM-109 by the heat-shock method. All the transformants were colistin-resistant since colistin MIC was between 4 µg/mL and 8 µg/mL, and they were *mcr-1* positive, confirming the plasmid-borne nature of the gene. We further investigated the resistance profile of these transformants to ascertain their gene allocation on the plasmid as well. As a result, resistance to some antibiotics was observed including resistance to Cephalosporins, Tetracycline, Aminoglycosides, Fluoroquinolone, and Sulfonamides. Yet, none of the transformants expressed resistance to Carbapenems.

PCR-based replicon typing (PBRT) analysis showed that the 30% of the plasmids in the original isolates belonged to different groups, including IncF, IncFII, IncFIIS, IncHI1, IncHI2, IncI2, IncI1α, Inc B/O, IncN, IncX1, IncX2, IncX3 and IncX4 (Table 3).

Table 3 The Different Plasmid Types Identified in *mcr-1* Positive Sewage Isolates (n=18)

Sources	Isolates	Replicon	Inc Group
Tripoli	Tri 3	FIB, X1, FII, X4	IncF, IncX1, IncFII, IncX4
	Tri 4	FIB, X1, FII, X4	IncF, IncX1, IncFII, IncX4
	Tri 10	HI2, FIB, FIIS, FII	IncHI2, IncF, IncFIIS, IncFII
Tabarja	Tab 5	FIB, FII, X4	IncF, IncFII, IncX4
	Tab 6	HI2, B/O, FIB, FIIS, FII, X4	IncHI2, Inc B/O, IncF, IncFIIS, IncFII, IncX4
	Tab 9	I1α, X1, FII	IncI1α, IncX1, IncFII
Antelias	Ant 1	I1α, X1, FII, X4	IncI1α, IncX1, IncFII, IncX4
	Ant 6	I1α, R, X1, FII, X4	IncI1α, IncX1, IncFII, IncX4
	Ant 10	I1α, HI1, HI2, X3, FIIS, R, X1, FII, X4	IncI1α, IncHI1, IncHI2,IncX3, IncFIIS, IncX1, IncFII, IncX4
Manara	Man 1	HI2, I2, FIB, X1, X4	IncHI2, IncI2, IncF, IncX1, IncX4
	Man 4	FIIS, X4	IncFIIS, IncX4
	Man 6	I1α, X4	IncI1α, IncX4
Saida	Sai 1	HI1, FIB, FIIS, FII, X4	IncHI1, IncF, IncFIIS, IncFII, IncX4
	Sai 2	HI1, HI2, I2, FIB, R, FII, X4	IncHI1, IncHI2, IncI2, IncF, IncFII, IncX4
	Sai 8	HI1, I2, FIB, R, FII	IncHI1, IncI2, IncF, IncFII
Sour	Sou 3	HI2, I2, N, FIB, R, FII, X4	IncHI2, IncI2, IncN, IncF, IncFII, IncX4
	Sou 4	FIB, FIA, FII, X4	IncF, IncFII, IncX4
	Sou 7	HI2, R	IncHI2

Table 4 Antimicrobial resistance profile of *mcr-1*-positive colistin-resistant Escherichia coli (n = 60) isolated from the sewage water outlets along the coast of Lebanon

Location[a]	*E. coli* Isolate Identification Code	Antibiotic Resistance Profile[b]	Intermediate Resistance Profile	Colistin MIC (μg/ml)	Detected Genes
Tripoli	Trip1	PEN-AMP-CTX-DOR-STR-TET-SXT-CHL	IPM	128	*mcr-1*, Class 1 Integron
	Trip2	PEN-AMP-STR-TET-SXT-CHL	IPM-DOR-MEM	128	*mcr-1*, Class 1 Integron
	Trip3	PEN-AMP-AMC-FEP-CTX-LEX-CFM-KAN-STR-TET-CIP-NOR-SXT-CHL	_	> 640	*mcr-1*, Class 1 Integron
	Trip4	PEN-AMP-AMC-FEP-CTX-LEX-CFM-IPM-DOR-MEM-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	_	> 640	*mcr-1*, Class 1 Integron
	Trip5	PEN-AMP-AMC-CTX-LEX-DOR-STR-TET-SXT-CHL	FEP-KAN-IPM	128	*mcr-1*, bla_{TEM}, Class 1 Integron
	Trip6	PEN-AMP-STR-TET-SXT-CHL	CTX-IMP-MEM	64	*mcr-1*
	Trip7	PEN-AMP-AMC-STR-TET-SXT-CHL	FEP-IPM-DOR	128	*mcr-1*, Class 1 Integron
	Trip8	PEN-AMP-AMC-STR-TET-SXT-CHL	FEP-CTX-KAN-IPM	128	*mcr-1*, bla_{TEM}
	Trip9	PEN-AMP-AMC-STR-TET-SXT-CHL	CTX-KAN-DOR-MEM	128	*mcr-1*, bla_{TEM}, Class 1 Integron
	Trip10	PEN-AMP-AMC-LEX-KAN-STR-TET-CIP-SXT-CHL	NOR-MEM	128	*mcr-1*, Class 1 Integron
Tabarja	Tab1	PEN-AMP-AMC-LEX-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	CTX	16	*mcr-1*, Class 1 Integron
	Tab2	PEN-AMP-AMC-LEX-STR-TET-CIP-SXT-CHL	CTX-CFM-KAN-NOR	> 640	*mcr-1*, bla_{TEM}, Class 1 Integron
	Tab3	PEN-AMC-AMP-LEX-KAN-STR-TET-CIP-SXT-CHL	CTX-NOR	16	*mcr-1*, Class 1 Integron
	Tab4	PEN-AMP-AMC-LEX-CFM-STR-TET-CIP-SXT-CHL	CTX	32	*mcr-1*, bla_{TEM}, Class 1 Integron
	Tab5	PEN-AMP-AMC-FEP-CTX-LEX-CFM-DOR-MEM-GEN-KAN-STR-TET-SXT-CHL	CIP	> 640	*mcr-1*, bla_{TEM}, Class 1 Integron
	Tab6	PEN-AMP-AMC-CTX-LEX-CFM-KAN-STR-TET-CIP-NOR-SXT-CHL	_	16	*mcr-1*, bla_{TEM}, Class 1 Integron
	Tab7	PEN-AMP-AMC-CTX-LEX-CFM-KAN-STR-TET-CIP-NOR-SXT-CHL	_	16	*mcr-1*, Class 1 Integron
	Tab8	PEN-AMP-AMC-FEP-CTX-LEX-CFM-KAN-STR-TET-CIP-NOR-SXT	_	16	*mcr-1*, bla_{CTX}, bla_{SHV}
	Tab9	PEN-AMP-AMC-FEP-CTX-LEX-CFM-KAN-STR-TET-CIP-NOR-SXT	_	16	*mcr-1*, bla_{CTX}, Class 1 Integron
	Tab10	PEN-AMP-AMC-STR-TET-SXT-CHL	CTX-KAN-CIP	128	*mcr-1*
Antelias	Ant1	PEN-AMP-AMC-FEP-CTX-LEX-CFM-IPM-MEM-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	DOR	16	*mcr-1*, bla_{TEM}, bla_{CTX}
	Ant2	PEN-AMP-AMC-FEP-CTX-LEX-CFM-DOR-MEM-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	IPM	16	*mcr-1*, bla_{TEM}, bla_{CTX} Class 1 Integron
	Ant3	PEN-AMP-AMC-CTX-LEX-CFM-IPM-DOR-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	FEP	16	*mcr-1*, bla_{TEM}, bla_{CTX} Class 1 Integron
	Ant4	PEN-AMP-AMC-CTX-LEX-CFM-IPM-MEM-STR-TET-CIP-NOR-CHL	FEP-DOR-KAN	16	*mcr-1*, bla_{CTX}
	Ant5	PEN-AMP-AMC-FEP-CTX-LEX-CFM-IPM-DOR-MEM-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	_	32	*mcr-1*, bla_{CTX}, Class 1 Integron
	Ant6	PEN-AMP-AMC-FEP-CTX-LEX-CFM-DOR-MEM-GEN-KAN-STR-TET-CIP-NOR-CHL	IPM	> 640	*mcr-1*, bla_{CTX}, Class 1 Integron
	Ant7	PEN-AMP-AMC-FEP-CTX-LEX-CFM-DOR-MEM-GEN-KAN-STR-TET-CIP-NOR-CHL	DOR	8	*mcr-1*, bla_{CTX}, Class 1 Integron
	Ant8	PEN-AMP-AMC-FEP-CTX-LEX-CFM-DOR-MEM-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	IPM	> 640	*mcr-1*, bla_{CTX}, bla_{OXA-48} Class 1 Integron
	Ant9	PEN-AMP-AMC-FEP-CTX-LEX-CFM-DOR-MEM-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	IPM	> 640	*mcr-1*, bla_{CTX}, Class 1 Integron
	Ant10	PEN-AMP-AMC-FEP-CTX-LEX-CFM-DOR-MEM-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	IPM	> 640	*mcr-1*, bla_{TEM}, bla_{CTX}

Location[a]	*E. coli* Isolate Identification Code	Antibiotic Resistance Profile[b]	Intermediate Resistance Profile	Colistin MIC (µg/ml)	Detected Genes
Manara	Man1	PEN-AMP-FEP-CTX-LEX-CFM-MEM-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	FEP-DOR	640	*mcr-1,* Class 1 Integron
	Man2	PEN-AMC-FEP-CTX-LEX-IPM-DOR-MEM-STR-TET-CIP-NOR-SXT	AMP-CFM-KAN-CHL	8	*mcr-1,* Class 1 Integron
	Man3	PEN-AMP-AMC-CTX-LEX-IPM-DOR-MEM-STR-TET-CIP-NOR-SXT	FEP-CFM-IPM-KAN	16	*mcr-1,* Class 1 Integron
	Man4	PEN-AMC-FEP-CTX-LEX-CFM-IPM-DOR-MEM-GEN-STR-TET-CIP-NOR-SXT	AMP-KAN	16	*mcr-1,* Class 1 Integron
	Man5	PEN-AMC-CTX-LEX-CFM-DOR-MEM-STR-TET-CIP-NOR-SXT	AMP-FEP-IPM-KAN	8	*mcr-1,* Class 1 Integron
	Man6	PEN-AMC-FEP-CTX-LEX-CFM-IPM-DOR-MEM-KAN-STR-TET-CIP-NOR-SXT-CHL	AMP-GEN	8	*mcr-1,* Class 1 Integron
	Man7	PEN-AMP-AMC-CTX-LEX-CFM-TET-SXT	FEP-IPM-DOR-KAN-STR-CIP	8	*mcr-1,* Class 1 Integron
	Man8	PEN-AMC-AMP-LEX-KAN-STR-TET	CTX-DOR-MEM-SXT	8	*mcr-1,* Class 1 Integron
	Man9	PEN-AMP-AMC-STR-TET-CIP-NOR-SXT	CTX	16	*mcr-1,* Class 1 Integron
	Man10	PEN-AMC-CTX-LEX-KAN-STR-TET-CIP-NOR-SXT	AMP-FEP-CFM-IPM-DOR-MEM	16	*mcr-1,* Class 1 Integron
Saida	Sai1	PEN-AMP-AMC-LEX-GEN-STR-TET-CIP-NOR-SXT-CHL	CTX-KAN	16	*mcr-1,* Class 1 Integron
	Sai2	PEN-AMP-AMC-LEX-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	FEP-CTX	16	*mcr-1, bla*$_{TEM}$, Class 1 Integron
	Sai3	PEN-AMP-AMC-LEX-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	_	16	*mcr-1,* Class 1 Integron
	Sai4	PEN-AMP-AMC-LEX-STR-TET-SXT	FEP-CTX-KAN-CIP	16	*mcr-1*
	Sai5	PEN-AMP-AMC-LEX-GEN-STR-TET-CIP-NOR-SXT-CHL	KAN	16	*mcr-1,* Class 1 Integron
	Sai6	PEN-AMP-AMC-STR-TET-SXT	CTX	16	*mcr-1,* Class 1 Integron
	Sai7	PEN-AMC-LEX-GEN-STR-TET-CIP-NOR-SXT-CHL	AMP-FEP-KAN	16	*mcr-1,* Class 1 Integron
	Sai8	PEN-AMP-AMC-LEX-KAN-STR-TET-CIP-NOR-SXT-CHL	FEP-CFM	16	*mcr-1,* Class 1 Integron
	Sai9	PEN-AMP-AMC-STR-TET-SXT	KAN-CIP	16	*mcr-1,* Class 1 Integron
	Sai10	PEN-AMP-AMC-STR-TET-SXT	_	8	*mcr-1,* Class 1 Integron
Sour	Sou1	PEN-AMP-AMC-LEX-STR-TET-CIP-NOR-SXT	CTX-DOR-KAN	16	*mcr-1, bla*$_{TEM}$, Class 1 Integron
	Sou2	PEN-AMP-AMC-LEX-STR-TET-CIP-NOR-SXT	_	128	*mcr-1,* Class 1 Integron
	Sou3	PEN-AMP-AMC-CTX-LEX-CFM-STR-TET-CIP-NOR-SXT-CHL	KAN	128	*mcr-1, bla*$_{TEM}$, Class 1 Integron
	Sou4	PEN-AMP-AMC-LEX-CFM-STR-TET-SXT-CHL	KAN	16	*mcr-1, bla*$_{TEM}$, Class 1 Integron
	Sou5	PEN-AMP-AMC-KAN-STR-TET-CIP-SXT	_	16	*mcr-1,* Class 1 Integron
	Sou6	PEN-AMP-AMC-STR-TET-CIP-NOR-SXT	_	8	*mcr-1, bla*$_{TEM}$
	Sou7	PEN-AMC-AMP-CTX-LEX-CFM-KAN-STR-TET-CHL	FEP-IPM-DOR-CIP	16	*mcr-1*
	Sou8	PEN-AMP-AMC-STR-TET-SXT	KAN	16	*mcr-1, bla*$_{TEM}$
	Sou9	PEN-AMP-AMC-LEX-STR-TET-CHL	CIP	8	*mcr-1,* Class 1 Integron
	Sou10	PEN-AMP-AMC-LEX-STR-TET-SXT-CHL	_	16	*mcr-1*

MIC, minimum inhibitory concentration; ARG, antimicrobial resistance gene; PEN, penicillin; AMP, ampicillin; FEP, cefepime; CTX, cefotaxime; LEX, cefalexin; CFM, cefixime; STR, streptomycin; TET, tetracycline; CIP, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole; NOR, norfloxacin; AMC, amoxicillin/ clavulanic acid; KAN, kanamycin; GEN, gentamicin; CHL, chloramphenicol; DOR, doripenem; IPM, imipenem; MEM, meropenem;

[a] Locations refer to the name of the coastal area in Lebanon. Locations are arranged geographically, from North to South of the Lebanese coast.

[b] Antimicrobial agents in the resistance profile are arranged according to the order of antibiotics/classes listed in the Clinical and Laboratory Standards Institute (CLSI) guidelines.

C. Discussion

The world is now facing a challenging threat from the emergence of bacteria that are resistant to almost all available antibiotics [13]. The main concern is that the world is running out of possible alternatives that can be used to treat resistant pathogens, indicating that antibiotic resistance could become a global disaster that needs a urgent attention [70].

With the increase of multidrug-resistant pathogens, colistin, the last-resort antibiotic, was reintroduced in the clinical practices [63]. However, resistance to colistin emerged due extensive use, abuse and misuse of this antibiotic in animal husbandry, veterinary medicine, agricultural practices and in clinical sectors [71]. Recently, a mobile plasmid-borne colistin-resistance gene, *mcr-1*, was discovered which could be laterally transmitted between bacteria [63].

Since then, the dissemination of *mcr-1* has been reported in many different countries worldwide and species, and from a variety of origins. Many studies have highlighted the role of wastewater as a substantial environmental reservoir of antimicrobial resistance (AMR) bacteria and antimicrobial resistant genes (AMR). Although the treatment process might reduce these genes, they can persist can be spread among microbial communities in the environment through horizontal gene transfer [72]. The increasing prevalence of antibiotic-resistant bacteria in water resources is associated with anthropogenic wastes from health care sectors, domestic and industrial effluents, and agricultural contaminants [73].

A study done in Spain highlighted the detection of colistin resistance gene, *mcr-1*, in 29 *E. coli* cells and a *K. pneumonia* isolated from two WWTPs in Barcelona, Spain [74]. China detected *mcr-1* in 9 *E. coli* isolates from the sewage samples collected from

five tertiary hospitals in Beijing, China [55]. Our study also emphasized the presence of colistin resistant *mcr-1* gene in sewage water in Lebanon and the results apparently exceeded those reported worldwide; although very few reported the prevalence of *mcr-1* in wastewater. The detection of this gene poses an essential risk for the direct and indirect transmission of colistin resistance bacteria that might be pathogenic to humans.

It is well known that the infrastructure and the wastewater management in Lebanon remain under construction; leaving behind partial or even non-functional treatment plants where the effluent end up in the sea [75]. Thus, sewage is considered a significant vehicle of transportation of antibiotic-resistance gene and antibiotic residues from one environment to another. Previous studies in Lebanon have detected *mcr-1*-positive *E. coli* in poultry, irrigation water, and other niches, perhaps confirming the extensive use of colistin in medical and agricultural practices in this country [42, 44, 45, 46, 47, 76]. Additionally, the detection of *mcr-1* positive *E. coli* in the Mediterranean Sea [47] might indicate the spread of the gene through the direct outfall of sewage across the Lebanese coastline. All sewage samples yielded colistin-resistant *E. coli*. The results were not surprising given the lack of sewage treatment and the wide use of colistin in Lebanon.

All the colistin-resistant isolates were positive for the *mcr-1* gene (100%), highlighting the wide environmental dissemination of this genetic marker in Lebanon. The MIC analysis of the *mcr-1* positive isolates showed that 50% of the isolates had MIC 8 µg/mL. This shows that the dominant population of *E. coli* in sewage water are highly resistant to colistin. Also, the antimicrobial properties of the sewage samples showed resistance against Penicillin, Ampicillin, Amoxicillin-Clavulanic acid, Cefepime, Cefotaxime, Cephalexin, Cefixime, Doripenem, Meropenem, Imipenem,

Gentamicin, Kanamycin, Streptomycin Tetracycline, Ciprofloxacin, Norfloxacin, Trimethoprim-Sulfamethoxazole, and Chloramphenicol. Remarkably, some isolates were colistin and carbapenem-resistant at the same time. All the isolates were multidrug-resistant (MDR). Therefore, these findings are highly problematic since colistin is given as last resort therapy when cephalosporins and carbapenems fail [77, 78]. This high prevalence of MDR *E. coli*, harboring the *mcr- 1*, emphasizes the extensive usage of antibiotics in humans and animals in Lebanon.

Molecular analysis for the antimicrobial-resistant genes determined that the majority of the samples co-harbored extended-spectrum beta-lactamase genes bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, eliciting resistance against beta-lactam antibiotics. The most prevalent gene was bla_{TEM}. These findings corroborate previous results, showing the high prevalence of the bla_{TEM} in ESBL-producing Gram-negative bacilli in Lebanon [43]. Notably, none of the isolates harbored bla_{IMP}, bla_{KPC}, bla_{NDM}, or $bla_{\text{OXA-48}}$, although the carbapenem-resistant genes, bla_{KPC} were previously documented in Lebanon in seawater [47] and camp drinking and sewage water [45]. The Class 1 Integron gene was reported in 80% of the isolates, which highlights the ability of these bacterial isolates to evolve, acquire, and express different resistance genes [79].

The *mcr-1* gene was confirmed to be plasmid-borne and transmissible. PCR-Based Replicon Typing analysis also showed that the plasmids in the original seawater isolates belonged also to different groups, including IncFII, IncFIIK, IncHI2, IncI2, IncK IncX2, and IncX4 [47]. It is worth mentioning that IncI2, IncI1 α, and IncX4 plasmid are responsible for the worldwide dissemination of the *mcr-1* gene [47,76].

This is the first report of *mcr-1*-positive-*E.coli* in sewage water in Lebanon as well as the MENA region. The prevalence of the *mcr-1* gene reported is among the

highest worldwide, urging to implement policies to limit the use of colistin. The detection of the *mcr-1* in wastewater samples poses a significant risk to the Mediterranean Sea and neighboring countries. Therefore, there should be a proper implementation of waste treatment strategies in Lebanon to limit the contamination of seawater.

CHAPTER III

CONCLUSION

It has been estimated that the rising tide of multidrug resistant strains of Gram-negative bacteria is an evolving jeopardy to humans and veterinary medicine. In the recent decades, this represented an alarming issue especially with the rapid emergence and spread of antimicrobial resistance to the last resort antibiotic, colistin. The discovery of transferable plasmids that confer resistance to colistin caused by the *mcr-1* gene, has led to more challenges. This study is the first to detect of *mcr-1*-positive bacteria in sewage water in Lebanon indicating the possibility of environmental dissemination of this gene. Despite the health risks posed by the occurrence of *Escherichia coli* that harbor *mcr-1* genes, there is a paucity of information on the global prevalence and antibiotic resistance of the *E. coli* harboring *mcr-1* isolated from sewage water. Taken together, the contamination of the Lebanese coastline is of a major concern to the surrounding Mediterranean basin countries.

From this study, we were able to assess that the *mcr-1*-positive isolates that were detected in the seawater and sewage water were highly related indicating that the contamination could be the direct discharge of the sewage. For that reason, new strategies for the treatment of wastewater need to be taken by activating the sewage treatment plants in Lebanon to prevent the dissemination of multidrug-resistant bacteria in the environment. Therefore, there is an urgent need to reconsider antimicrobial stewardship and limit the use of colistin in agricultural and veterinary practices in order to restrict access to important antibiotics and control the proliferation of *mcr-1* resistant *E. coli* in Lebanon and worldwide.

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