AMERICAN UNIVERSITY OF BEIRUT

A NATIONWIDE ASSESSMENT ON THE OCCURRENCE OF PLASMID-BORNE MOBILE COLISTIN-RESISTANCE GENE, *MCR-1*, IN *ESCHERICHIA COLI* ISOLATED FROM LEBANESE RIVER WATER

by
JOUMAN WALID HASSAN

A thesis
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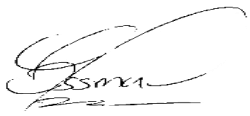

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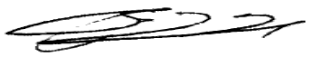

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AN ABSTRACT OF THE THESIS OF

Jouman Walid Hassan for Master of Science
Major: Food safety

Title: A Nationwide Assessment On The Occurrence Of Plasmid-Borne Mobile Colistin-Resistance Gene, *mcr-1*, In *Escherichia Coli* Isolated From Lebanese River Water

Colistin, a last resort antibiotic, is used to treat multidrug and extensively drug-resistant Gram-negative infections. However, colistin's excessive use has led to the emergence of the plasmid-borne mobile colistin-resistance gene; *mcr-1*. The dissemination of this transmissible genetic marker has been documented in different niches across the globe. Anthropogenic, industrial, and agricultural activities near freshwater resources have significantly polluted river water with antibiotic-resistant organisms and genes. This study aims to determine the dissemination of *mcr-1* alongside other antimicrobial resistance genes across 14 major perennial rivers and Ras El Ain water spring in Lebanon (45 sub-rivers). Samples were collected from three sites along the rivers in triplicates yielding 135 freshwater samples. Approximately 98.3% of the samples detected the presence of colistin-resistant *E. coli,* and a total of 116 *mcr-1* positive isolates were recovered from 27 sub-rivers. Other *mcr* genes (*mcr-2, mcr-3, mcr-4, mcr-6,* and *mcr-8*) were also reported in some of the isolates. The antimicrobial characteristics showed colistin's MIC ranging between 4-64 μg/ml and the phenotypic resistance reported against Penicillin (100%), Ampicillin (77%), Amoxicillin/Clavulanic acid (76%), Cefepime (25%), Cefotaxime (44%), Cephalexin (77%), Cefixime (39%), Doripenem (5%), Meropenem (3%), Imipenem (7%), Gentamicin (35%), Kanamycin (39%), Streptomycin (61%), Tetracycline (68%), Ciprofloxacin (30%), Norfloxacin (25%), Chloramphenicol (39%), and Trimethoprim/Sulfamethoxazole (57%). The majority of the isolate 94% were multidrug-resistant. Moreover, the extended-spectrum β-lactamase genes (ESBL), bla_{TEM}, bla_{SHV}, bla_{CTX-M} were detected in 48%,42%, 36%, of the isolates, respectively. Moreover, the carbapenem-resistance genes bla_{KPC}, bla_{OXA-48}, bla_{IPM} and bla_{NDM}, were also reported in 3%,1%,2% and 4%, respectively. Interestingly, 62% of the *mcr-1*-positive-*E. coli* samples harbored the Class-1 Integron gene. The plasmid harboring the *mcr-1* gene was successfully transformed into chemically competent JM109 *E. coli* cells. A variety of plasmid types were detected in the samples, especially the ones responsible for the global dissemination of the *mcr-1* gene, IncX4, IncI1 α, and IncI2. Remarkably, 54.3% of the isolates were genotypically diverse. Additionally, the *mcr-1* gene persisted in the water matrix for more than 127 days. These findings highlight the high prevalence of the *mcr* genes and other antibiotic resistance genes in Lebanese river water. This is the first study to highlight the dissemination and characterization of *mcr-1*-positive-*E.coli* in the Lebanese river water.

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To My
Beloved Family

CHAPTER I

INTRODUCTION

A. Antibiotics and Antibiotic Resistance: The Blessing and The Curse!

Antibiotics are low molecular weight compounds, naturally produced or derived from environmental fungi or microorganisms, to kill or inhibit the growth of bacterial cells [1, 2]. Antibiotics are classified based on their origin as; naturally occurring, semisynthetic, and synthetic and based on their biological activity as bactericidal or bacteriostatic [2]. The first antibiotic, penicillin, was discovered in 1928 by Alexander Fleming; and was derived from the fungus *Penicillium notatum*. This drug was utilized for therapeutic use in the 1940s [1]. After the discovery of penicillin, another breakthrough in medicine followed, which was the discovery of the first sulfonamide, Prontosil, in 1935 by Gerhard Domagk [2]. The use of these newly discovered agents revolutionized medicine since they did not only treat lethal bacterial infections and lowered mortality and morbidity rates, they also opened doors for new medicinal interventions like surgery and organ transplant [3]. Subsequently, different classes of antibiotics were then discovered and produced between 1949 and 1980; however, following this period an "antibiotic discovery void" followed where no novel successful antibiotics were developed [4]. Although many believed that antibiotics would put an end to bacterial infectious diseases, this was a farfetched dream due to the emergence of drug-resistant pathogens. The emergence of antimicrobial-resistant organisms was not a surprise; since Fleming, himself warned about this phenomenon even

before the introduction of penicillin [5]. This leaves us with a question, what is antimicrobic resistance? Furthermore, what are its respective consequences?

Antimicrobial resistance (AMR) is when a specific antibiotic is no longer effective in treating the bacterial infection it used to treat [2]. When microorganisms are challenged with an antibiotic, they devise ways and evolve to resist the stress of this agent and survive. Mechanisms that lead to antibiotic resistance can be summarized by the development of an efflux pump, enzymatic degradation of the drug, new metabolic pathways, alterations in the receptor site, and cell wall changes [2]. Bacterial cells acquire these resistance properties either by horizontal gene transfer (transformation, conjugation, and transduction) or by genetic mutations [2, 6].

The emergence of AMR has increased exponentially over the years due to the misuse and abuse of antibiotics in human medicine and agricultural practices [1]. Some of the factors that aggravated the emergence of AMR in humans is the lack of knowledge mainly reflected in prescribing antibiotics without confirming a bacterial infection [1, 7] and patient's non-compliance to prescriptions or tendencies to self-prescribe antibiotics [8-10]. These malpractices were not limited to the clinical context but were also documented in the agricultural sector, whereby these drugs were used for growth promotion and prophylaxis in animal farming [11]. These malpractices have detrimental effects since, pharmaceutical companies are no longer interested in investing in new antibiotics due to their short-lived effectiveness and high cost [12], leaving humanity to face complicated infections with very limited treatment options [2]. According to the Center for Disease Control and Prevention (CDC), AMR is one of the nation's grave public health threats [13]. The emergence of drug-resistant pathogens puts the lives of millions in jeopardy, whereby

it was estimated that in Europe and the US, nearly 50,000 people lose their lives prematurely due to AMR. Also, it was approximated by the World Health Organization (WHO) that 100 million individuals will die due to AMR by 2030 and that by 2050, 300 million people will be lost to resistant pathogens [14]. Besides, the unfathomable human loss, AMR has dire effects on the economy. It was estimated that between 2014 and 2050, the world will have immense economic losses estimated between $60 and $100 trillion US dollars as economic output, which commensurates to 1-year of global economic output. The gross domestic product (GDP) is believed to decrease by 2% to 3.5% in 2050. This financial loss is due to the high morbidity and mortality rates affecting people's productivity and extending their hospital stay, which in turn burdens governments with extra expenses on the healthcare system [15].

B. Colistin: A Brief History

The overuse of antibiotics in different settings has caused the rising tides of antimicrobial resistance, whereby the available antibiotics are no longer effective in treating severe infections. The emergence of multidrug-resistant pathogens (MDR), pathogens resisting at least three classes of antibiotics, and extensively drug-resistant Gram-negative infections (XDR), organisms resistant to all except one or two classes of antibiotics [16], necessitated the reintroduction of a potent disregarded antibiotic; colistin.

Colistin, polymyxin E, is an old antibiotic discovered in 1947 from the bacterium *Paenibacillus polymyxa var*. *colistinus* and was used in the clinical setting in 1959 to treat Gram-negative bacteria [17, 18]. Five groups of polymyxins have been discovered (A-E), yet only polymyxin E and B have been utilized in clinical practices [17]. Although these

drugs were highly effective in treating infections caused by *Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumonia, Escherichia coli, Enterobacter spp., Salmonella spp., Shigella spp., and Haemophilus influenzae* [17], in 1970 colistin was disregarded as a treatment option due to its severe nephrotoxicity and neurotoxicity and was replaced by safer drugs like cephalosporins [17, 19, 20]. However, colistin was reintroduced into the clinical setting with the rise of complicated infections [21] caused by carbapenem-resistant *Enterobacteriaceae* (CRE), carbapenem-resistant *Acinetobacter baumannii* (CRAB), multidrug-resistant *Pseudomonas* species, extensively drug-resistant (XDR) *Pseudomonas aeruginos*a, and (XDR) *Acinetobacter baumannii* [22, 23].

Colistin is found in two formulations: colistin sulfate and colistimethate sodium (CMS) a less toxic prodrug [17], and based on the manufacturer and mode of administration, the recommended dosage for humans vary between (4–6 mg/kg/day) divided to three doses or (2.5-5 mg/kg/day) in 2 doses [24].

Colistin is an amphiphilic, complex, multi-component, Penta-cationic decapeptide antibiotic [25]. It operates by disrupting the membrane's permeability through polar and hydrophobic interactions [26]. Colistin, a cationic antibiotic, electrostatically interacts with the anionic Lipopolysaccharide on the cell wall of Gram-negative bacteria. This interaction causes the displacement of the calcium (Ca++) and magnesium (Mg++) ions stabilizing the membrane, causing its increased permeability, and, in turn, the leakage of the cytoplasmic cell content, ensuring cells death [17].

Although colistin is our last resort antibiotic, its utilization has not been restricted to humans; however, it has been immensely used for decades in animal farming [27]. In animal farming, colistin is deployed for therapeutic purposes to treat gastrointestinal

infections caused by noninvasive *Enterobacteriaceae* in cattle, small ruminants, poultry, and pigs, and also to manage enteric diseases caused by *Salmonella* and *E. coli* in cases of postweaning diarrhea in piglets [11, 28]. Likewise, colistin has been administered orally to treat colibacillosis in poultry caused by enteropathogenic *E. coli* (APEC) [28]. Nevertheless, the utilization of colistin in animals was not purely therapeutic, whereby the usage of colistin for growth promotion and disease prevention was well established in the literature [28]. For example, 495 tons of polymyxins were orally given to swine and poultry in Europe in 2013 [28]. Additionally, colistin was the fifth most sold group of antimicrobial agents administered in Europe's agricultural practices in 2010 [29]. Moreover, China is globally the highest colistin producer and consumer (17.5 million tons) [30].

C. Colistin Resistance

Resistance to colistin was thought to be caused by chromosomal mutations that altered the Lipid A moiety of the Lipopolysaccharide, decreasing colistin's affinity to the LPS. Until 2015, these mutations were only vertically transmitted between bacterial progeny [21]. Nevertheless, a new discovery in China documented the presence of a plasmid-borne mobile colistin-resistance gene that was horizontally transmissible between bacterial cells. This new mobile genetic element was isolated from an *Escherichia coli* strain (SHP45) recovered from a pig sample in southern China [30]. This was the first description of the plasmid-borne mobile colistin-resistance gene, dubbed as *mcr-1*. This gene was able to laterally transmit between *Enterobacteriaceae* and other bacterial species.

The *mcr-1* gene encodes for the phosphoethanolamine transferase enzyme, which catalyzes the addition of a positively charged phosphoethanolamine to the lipid A; reducing the affinity of colistin to its targeted Lipopolysaccharide. The dissemination of *mcr-1* has been documented in more than 40 countries worldwide [31, 32]. Further research on this novel discovery, reported the detection of other *mcr* variants (*mcr-2* to *mcr-10*) [33, 34].

Several studies have reported the low prevalence of *mcr* genes in clinical samples as compared to isolates recovered from animal origin. This anticipated the theory that *mcr-1* has originated for animal farming due to the extensive use of colistin in animal production [35]. The mobilization of this genetic marker caused its worldwide dissemination to a multitude of hosts and environmental niches [11, 31], like recreational waters at public beaches in Brazil [36], fecal samples from otherwise healthy individuals [37, 38], and Rivers in China [39].

Subsequently, the unrestrained spread of the *mcr* genes to different bacterial hosts globally poses a significant public health risk and jeopardizes the effectiveness of a highly important last-resort antibiotic. Also, in 2012 the World Health Organization categorized colistin as the highest priority critically important medicine for humans [29]. Therefore, efforts to preserve the effectiveness of this critically important antibiotic and curtail the spread of resistance is of utmost importance. As a result, countries like European countries, China, Thailand, Japan, Brazilian, Indian, Malaysian, and Argentine, banned the use of colistin for growth promotion in animal husbandries [40].

D. The Worldwide Dissemination of The *mcr* Genes

In the section below, we will summarize the global dissemination of the *mcr* genes, with emphasis on the MENA region. The information presented below is from my published book chapter about the spread of the *mcr* gene in the clinical-agricultural-environmental continuum [41].

1. Asia

a. China

The first detection of the laterally transmissible *mcr-1* gene was reported in southern China. The gene was identified in an *Escherichia coli* recovered from a pig sample (SHP45). Further analysis of this genetic marker showed that it was plasmid-borne and elicited colistin resistance [30]. Following this discovery, samples collected from pigs (804), raw meat (523), and clinical isolates (902 *E. coli* and 420 *K. pneumonia*) were screened for the detection of the *mcr-1* gene. The genetic marker was highly prevalent in pigs 21% and was also detected in 15%, and 1.5% of the *E. coli* isolates recovered from raw meat, and clinical samples, respectively [42]. It is worth mentioning that *mcr-1*-positive-*K. pneumonia* was retrieved from 0.7% of the clinical isolates. Interestingly, the oldest *mcr-1*-positive-*E. coli* dates back to 1980 in China, highlighting that the gene has been present for a long time and was silently circulating to different niches worldwide. Another study reported the detection of *mcr-1*-positive-*E. coli* in 5.11% of chicken samples collected from 13 different provinces in China [39]. Also, a retrospective study illustrated an upward trend in the detection of *mcr-1*-positive-*E. coli* in chicken samples from 5.2% in 2009 to 30% in 2014 [32]. After this alarming increase, several studies documented the

occurrence of the *mcr-1* gene in animal farms in China. Notably, in 2018 the high prevalence of *mcr-1*-positive-*E. coli* was reported in pig's fecal matter, whereby 76.2% of the samples tested positive for this gene [43]. However, the prevalence of the *mcr-1* gene was still low in clinical settings. A retrospective analysis of samples collected from two Chinese hospitals identified the *mcr-1* gene in 1% of the *E. coli*, less than 1% in *K. pneumonia*, less than 1% in *Enterobacter cloacae*, and 1% in *Enterobacter aerogenes* isolates [38]. Furthermore, the first *mcr-1*-positive-*Salmonella* detected in China was in 2012. Notably, some isolates were not only resistant to colistin but also resisted *Salmonella's* drugs of choice, quinolones, and cephalosporins [44]. Moreover, the *mcr-1* gene was identified in multidrug-resistant *Shigella sonnei* isolates recovered from fecal samples of patients suffering from acute diarrhea or dysentery in China [45].

Remarkably, the *mcr-1* gene was also detected in environmental samples, whereby *K. pneumonia, Kluyvera spp.*, and *E. coli* isolates from the hospital's sewage water tested positive for the *mcr-1* gene [46]. These findings are of great importance since sewage water acts as a significant reservoir of antimicrobial-resistant genes and their dissemination. It is worth mentioning that *mcr-1* was also reported in vegetables and companion animals in China. Specifically, two *Raoultella ornithinolytica* and seven *mcr-1*-positive-*E. coli* strains were isolated from lettuce and tomato [47]. Additionally, 8.7% of *Enterobacteriaceae* isolates recovered from cats and dogs in Beijing harbored the *mcr-1* gene [48]. A worrying discovery documented the detection of a colistin and carbapenem resistance *E. coli* strain, positive for *mcr-1* and bla_{NDM-5}, recovered from a sick cat's rectal swab. Further analysis of this isolate showed that it resisted all antibiotics tested, including cephalosporins [49]. Other variants of the *mcr* gene have been reported in China. A study

on pigs and the farm's environment documented the detection of *mcr-3*-positive-*E. coli* isolates from flies and soil. Out of these *mcr*-3 positive samples, 80% were found to co-harbor *mcr-1* [50]. Recently, *mcr-8* was detected in a *K. pneumonia* isolate from livestock in China. This strain co-harbored the carbapenem-resistant gene bla_{NDM} [51]. Due to the wide distribution of *mcr* gene in various bacterial hosts, and niches in China, the Chinese government banned the use of colistin in animal feeds and limited its usage to humans [52].

b. Vietnam

In Vietnam, the *mcr* genes have been detected in humans, chickens, and local food products. In a study that screened fecal samples from humans and backyard chickens for *mcr-1*, the data revealed its detection in 20.6% and 59.4% of the samples, respectively. Likewise, this gene was reported in 12.8%, and 4% of the *E. coli* samples were isolated from chickens and farmers, respectively [53]. As for the hospital-acquired *E. coli*, the *mcr-1* gene was reported; however, it was shown to be carried chromosomally [54]. A study screened local foods (meat and seafood products) for the detection of the *mcr* genes, 97% of the colistin-resistant *E. coli* harbored the *mcr-1* genetic marker, and 3% were *mcr-3*-positive [55].

c. India, Thailand, Laos, and Malaysia

In India, the *mcr-1* gene was detected in both food products as well as clinical samples. A study conducted on Indian food documented three *mcr-1*-positive-*E. coli* isolates recovered from meat, fish, and raw vegetables [56]. As for the clinical

setting, *mcr-1* was reported in four *K. pneumonia* isolates*;* however, the analysis showed that the gene was chromosomal and not plasmid-borne [57].

In Thailand, three *mcr-3*-positive-*E. coli* isolates from blood and abscess samples and one *K. pneumonia* from a wound sample were retrieved from the clinical setting. Similarly, *mcr-1* was also detected in an *E. coli* isolated from a wound sample [58]. Interestingly, the *mcr-1* gene was detected in an *E. coli* isolated from asymptomatic people in Thailand [59]. Also, the mobile colistin resistance gene *mcr-1* and *mcr-3* were detected in flies [60].

In Laos, an *mcr-1* positive isolate was shown to have transferred from a pig to an asymptomatic farmer, further emphasizing the zoonotic potential of the *mcr* gene [61].

In Malaysia, a study reported six *mcr-1*-positive-*E. coli* from more than 900 bacterial isolates collected from the environment, humans, and animals, indicating the occurrence of *mcr-1* in different niches in Malaysia [62].

d. Japan, Korea, and Taiwan

In Japan, a study showed that 39 isolates retrieved from food-producing animals harbored the *mcr-1* gene (cattle, pigs, and broilers chickens) [63]. Also, the *mcr-1* gene was identified in four *E. coli* isolates (ST782 and ST456), as well as one *K. pneumonia* (ST1296), recovered from five patients in Okinawa, Japan [64].

In South Korea, the mobile-colistin-resistance gene was detected in food-producing animals, whereby 11 *mcr-1*-positive-*E. coli* were detected in chicken's fecal samples and carcasses and in a diseased pig [65]. Moreover, the *mcr-1* gene was reported in an *E. coli* sample from a hospitalized patient. Additionally, *mcr-1* and *mcr-3* were co-harbored in

a multidrug-resistant *E. coli* from a healthy pig [66]. Notably, a study suggested the possible transfer of *mcr-1*-positive-*E. coli* from chickens to humans in South Korea [67]. The emergence of mobile colistin resistance in South Korea was speculated to be associated with the increased annual consumption of antibiotics in animal production [65].

In Taiwan, an *E. coli* strain isolated from a patient with a urinary tract infection was resistant to all antibiotics and was found positive for *mcr-1*. The isolate co-harbored *mcr-1* and the bla_{NDM-9} genes, limiting treatment options for the patient [68].

2. Europe

a. Belgium

In Belgium, a surveillance study on colistin-resistant *E. coli* samples from 53 porcine and 52 bovine suffering from diarrheal diseases was done in 2011 [69]. The data illustrated that 12.5% of the isolates harbored the *mcr-1* gene, out of which 11.5% were bovine, and 13.2% porcine. Furthermore, *mcr-2* was detected in the *mcr-1* negative isolates and was harbored on an IncX4 plasmid. The high prevalence of *mcr-2* (20.1%) in porcine isolates was speculated due to the high transferability frequencies of the IncX4 plasmid as compared to the IncFII harboring *mcr-1* [33, 69]. In 2017, a novel *mcr* variant was detected in *E. coli* recovered from pigs suffering from postweaning diarrhea. Belgian pigs were sampled and screened; *mcr-4* was detected in 13.3% of the colistin-resistant isolates [34].

b. Denmark

In Denmark, an *mcr-1*-positive-*E. coli* was isolated from a human's bloodstream infection, co- harboring beta-lactamase genes. Also, 5 *E. coli* isolates recovered from

chicken samples harbored the *mcr-1* gene. Further analysis of the isolates identified that they belonged to an ST131, which is associated with human's urinary tract and bloodstream infections and is rarely detected in chickens [70]. This highlights the anthropogenic transmission of these strains to animals. In 2017, a study detected the cooccurrence of *mcr* and ESBL genes. The results identified an *mcr*-3-positive-*E. coli* sample from a hospitalized patient with pyelonephritis. This was the first detection of *mcr-3* outside of Asia [71]. However, it is worth mentioning that the patient had a previous travel history to Thailand, which suggests that he might acquired the gene abroad. A study conducted in 2016, identified *mcr-1* in 4 *Salmonella* Typhimurium isolates from humans. One of the *mcr-1*-positive-*Salmonella* carriers had a previous travel history to Thailand [72]. Similarly, A study reported the detection of 10 *Salmonella* isolates harboring *mcr* genes, one harboring both *mcr*-1 and *mcr-3*, while the other strains were only *mcr-3* positive [73]. Notably, these isolates were retrieved from people which a previous travel history to Asia. Therefore, most of the *mcr-3* positive strains were associated with patients who visited Asia, indicating the acquisition of this gene during travel. This further suggests the vital role of travel and human movement in the transfer and dissemination of these resistance genes to different parts of the world [74].

c. Italy

The lax rule of law regarding colistin's use is evident in Italy, and Spain since their consumption of colistin is estimated to be 20 mg/kg in biomass, which is a marked difference in comparison to other northern European countries [75]. A study on postweaning pigs treated with colistin determined the high prevalence of *mcr-1* in *E. coli*

on their premises, whereby 72.5% of the *E. coli* isolates were positive for this gene. It is worth mentioning that another *mcr* gene was detected in the pig isolate in Italy; an *mcr-4*-positive-*Salmonella enterica* serovar Typhimurium [34]. The relatively high prevalence of *mcr-1* in pigs in Italy might be associated with the excessive use of colistin in animal production in this country [75]. As for the clinical setting, a variant of the *mcr-1* gene was detected in an Italian hospital. The isolate was an *mcr-1.2* carbapenem-resistant *K. pneumonia,* isolated from a leukemic child [76]. This was the first description of an MDR carbapenem-resistant *K. pneumonia* (ST525) harboring *mcr-1.2* [76]. Another study reported the presence of the *mcr-1* variant (*mcr-1.2*) in an *E. coli* isolated from human blood samples [77].

d. Spain

In Spain, the *mcr-1* gene and its variants have been documented in clinical, environmental, and animal samples. In 2019, 15 *mcr-1*-positive-*E. coli* clinical isolates were retrieved from a Spanish hospital [78]. A report in 2019, identified five multidrug-resistant (MDR) *mcr-1*-positive-*E. coli* isolates from patients with adult acute myeloid leukemia (AML). This poses a significant threat since leukemic patients have low immunity and are highly susceptible to bacterial infections [79]. As for the environmental detection, the *mcr-1* gene was documented in 33.3% of the samples recovered from sewage water. The isolates were *E. coli (n=9),* and *K. pneumonia (n=1)*. These stains co-harbored $bla_{CTX\text{-}M\text{-}55}$ and $bla_{TEM\text{-}1}$ and were resistant to different classes of antibiotics [80]. Moreover, a study revealed that 23 commensal *E. coli* isolates from pig samples harbored *mcr-1* [81]. Additionally, nine *mcr-1*-positive strains, out of which five were *E.*

coli were recovered from turkey's feces (n =1), and swine's feces (n = 4), also four *Salmonella* isolates were retrieved from swine's lymph nodes [82]. Interestingly other *mcr* genes were detected in postweaning pigs in Spain. Notably, nine isolates harbored *mcr-4 [34]*. Additionally, enteropathogenic *E. coli* isolates from postweaning swine were collected and screened, and the results documented the high prevalence of *mcr-4* (n=102 isolates), *mcr-1* (n= 37), and *mcr-5 (n=5)* in these samples. The latter was the first detection of *mcr-5* in Spain. Notably, different *mcr* genes; for example, *mcr-1/mcr-4*, *mcr-1/mcr-5*, and *mcr-4/mcr*-5 were shown to co-occur in the same isolates [83]. Furthermore, screening of young calves in Spain resulted in the isolation of 6 *E. coli* that were positive for *mcr-1*, and one of the isolates was positive for both *mcr-1* and *mcr-3* [84]. Taken together, data suggest that Spain is one of the European countries with a relatively comprehensive detection of *mcr* genes in humans, animals, and the environment. Also, different *mcr* varients (*mcr-1,mcr-3, mcr-4, mcr-5*) were detected and were associated with diverse bacterial species. Perhaps these observations could collectively be associated with Spain's overall excessive consumption of colistin [75, 78].

e. Germany

In Germany, a study was done to screen for the presence of the *mcr-1* gene in samples collected from humans, animals, and the environment. Out of the 577 isolates, four *mcr-1*-positive *E. coli* were retrieved from swine samples (n=3) and a human sample (n=1). The latter *E. coli* isolate was also carbapenem-resistant and harbored the bla_{KPC-2} gene [85]. Another study collected 10,609 *E. coli* from different farms, slaughterhouses, and retail meat products and screened them for the presence of *mcr-1*. Out of the 505 phenotypically

colistin-resistant isolates, 402 harbored *mcr-1*. The highest prevalence of *mcr*-1 was observed in turkeys (11.8%), broiler chickens (6.7%), and chicken meat (4.3%) [86]. In contrast, a relatively lower prevalence of *mcr-1* was noted in veal calves (2.4%), while only three *mcr-1* positive isolates were detected in layer chickens. Interestingly, *mcr-1* was discovered in a sample from 2010 in Germany, indicating the gene's silent presence.

f. Netherlands

In the Netherlands, the utilization of colistin is relatively low in both humans and animals, whereby polymyxins accounted for 0.4% of the antibiotics administered to broilers in 2014 [87]. A similar percentage (0.1%) was observed in primary care, and hospital settings, where polymyxins accounted for 0.3% of all systemic antimicrobials used [87]. Consequently, a low prevalence of *mcr-1* was reported in a retrospective screening of 2,471 *Enterobacteriaceae* from retail chicken meat, hospitalized patients, clinical cultures, and healthcare-associated outbreaks. Only 3 ESBL-producing *E. coli* isolates from retail chicken meat were found to harbor *mcr-1* [87]. A different study screened ESBL-producing and colistin-resistant *E. coli* samples collected from 9 travelers, 1 to 2 weeks after their return to the Netherlands for *mcr-1* [88]. The *mcr-1* gene was found in 6 of the *E. coli* isolates. It is noteworthy that these samples tested negative for the *mcr-1* gene before travel; highlighting the role of travel in the acquisition of the gene [88]. The low prevalence of the *mcr-1* gene in animals was confirmed by a study that screened *E. coli* and *Salmonella* isolates collected from food animals in slaughterhouses between 2002 and 2014. The data documented the presence of *mcr-1* in only 0.3% of *E. coli* tested [81].

Another study reported the detection of *mcr-1* in 1% of *Salmonella* isolates from poultry meat [89]; also 0.3% of fecal samples from healthy animals were *mcr-1* positive [81].

g. Switzerland

In Switzerland, two *E. coli* associated with two different bacteremia cases harbored the *mcr-1* gene. Analysis of the strains showed that both harbored the beta-lactamase genes [90]. Subsequently, a retrospective study from human blood cultures identified that *mcr-1* was absent in *E. coli, K, pneumonia,* and *Salmonella* serovar enteritidis samples [90]. Still, mcr-*1*-positive-*E. coli* isolates (belonging to ST10 and ST5) were identified in fecal samples from two Swiss travelers returning from India [91]. Also, the screening of 2,049 non-duplicate urine samples identified *mcr-1* in an *E. coli* strain (ST428), which was previously associated with broiler breeder infections [92]. The *mcr-1* gene was not only identified in *E. coli* but interestingly, the first case of *mcr-1*-positive-*Salmonella enterica subsp.* enterica was isolated from the blood of a 77 years-old male patient in Switzerland [93]. Additionally, *mcr-1* was reported in six colistin-resistant *E. coli* isolates from Germany's imported retail chicken meat [91]. Furthermore, the gene was detected in ESBL-producing *Enterobacteriaceae* isolate from the river Birs (n=1) and imported vegetables from Thailand and Vietnam (n=2) [94]. These findings are of notable importance because they highlight 1) potential environmental (river water) contamination with *mcr*-1 2) the role of food imports in transmitting the gene to other countries.

h. France

In France, the plasmid-borne mobile colistin-resistance was reported in humans,

animals, and food products from different bacterial species, including *K. pneumonia, E. coli, Enterobacter cloacae,* and *Salmonella spp.* [95]. Notably, high frequencies of colistin-resistance and *mcr-1* were identified in ESBL-producing *E. coli* retrieved from veal calves suffering from diarrheal disease, whereby (21%) tested positive for *mcr-1* [96]. Furthermore, a study to evaluate colistin resistance in commensal *E. coli* isolates from French livestock identified *mcr-1* in pigs (0.5%), broiler chickens (1.8%), and turkeys (5.9%) [97]. Additionally, the *mcr-1* gene was identified in food products, whereby 4 *Salmonella* strains isolated from sausages, retail chicken products, and ready-to-eat pies were positive for the *mcr-1* gene [88]. The *mcr-1* was also identified in two colistin-resistant *K. pneumonia* isolated from humans in France [61]. A similar study detected *mcr-1*-positive ESBL-producing *K. pneumonia* from a patient with fungal meningitis [98]. Additionally, *mcr-1* was recognized in an ESBL-producing *E. cloaca* isolated from 50-year-old Algerian women hospitalized in France. Given that *mcr-1*-positive-*E. cloacae* have been reported in Asia, the gene and bacterium might have been acquired outside of France. Regardless, this suggested that people's movement (travel) can potentially contribute to the dissemination of these genetic markers [95].

i. Portugal

In Portugal, the relatively high prevalence of colistin-resistance is due to the utilization of colistin in animal food production. A study reported *mcr-1*-positive- *Salmonella*, from pigs, pork products, and human samples [99]. A different study, detected 98 *mcr-1* positive isolates (*E. coli and K. pneumonia)* retrieved from 100 pigs from two farms [100]. A nationwide study in Portugal screened

1,840 *Enterobacteriaceae* isolates for *mcr-1*, the samples were collected from food-producing animals (bovine, swine, and poultry), meat (cow and pig), meat products, and animal feed. The gene was detected in 8% of the *E. coli* and 0.47% of the *Salmonella* enterica isolates [101]. Notably, 45.7% of the *mcr-1*-positive-*E. coli* were either ESBL or harbored the plasmid-mediated AmpC beta-lactamase co-producers. Furthermore, *mcr-1* was detected in *E. coli* retrieved from turkeys (27%), swine (10.1%), swine meat (5.1%), and broilers (2%) [101]. A significant finding detected *mcr-1* in carbapenem-resistant *K. pneumonia* from a hospitalized patient in Portugal [102]. Accurately, 24 carbapenemase-producing and *mcr-1*-positive *K. pneumonia* isolates were retrieved from 16 hospitalized patients. Given that colistin is administered to treat carbapenem-resistant bacteria, the occurrence of *mcr-1* in these aforementioned isolates is of paramount importance.

j. The United Kingdom

In the United Kingdom, *mcr-1*-positive-*Salmonella* and *E. coli* were detected from human and food samples [103]. A retrospective analysis of 24,000 *Enterobacteriaceae* samples identified *mcr-1* in 15 *Salmonella,* and *E. coli* isolates. It is worth mentioning that six *mcr-1*-positive-*Salmonella* isolates were recovered from patients who recently visited Asia. Additionally, two *mcr-1*-positive-*S. Paratyphi B var Java* were detected in poultry meat imported from the European Union (EU) [103]. Similarly, the detection of *mcr-1* was reported in two isolates (*E. coli and an S.* Typhimurium var Copenhagen) associated with a pig farm in the UK [104].

k. Poland

In Poland, *mcr-1* was identified in a hospitalized patient with a urinary tract infection and pneumonia. *mcr-1* was harbored in an *E. coli* (ST617) isolate. Further analysis demonstrated that it also resisted different antibiotics, including cephalosporins, penicillin, amoxicillin, clavulanic acid, tetracyclines, and aztreonam [105].

l. Sweden

In Sweden, the *mcr-1* gene was detected from a human sample. Two *mcr-1*-positive *E. coli* isolates were recovered from humans' fecal samples; however, the individuals' travel histories showed that they traveled to Asia. Therefore, the gene might have been acquired outside Sweden [106].

m. Finland

In Finland, the use of colistin is restricted to carbapenem-resistant pathogens [107]. A prospective study screened fecal samples from healthy volunteers. A single *E. coli* isolate harbored the *mcr-1* gene[107]. This highlighted that *mcr-1* could be detected in countries with limited use of colistin.

n. Norway

A retrospective study detected the presence of *mcr-1* in a nonpathogenic *E. coli* isolate from a patient’s fecal matter. The patient's travel history showed that he traveled to India and suffered from a traveler's diarrhea [108]. Additionally, *mcr-1* was discovered in an *E. coli* ST10 previously found in seawater samples [109]. The source of this gene has not

been identified; however, it is speculated that it might be due to fecal contamination from ship toilets, migratory birds, or nearby farms.

3. Americas

In the United States, colistin has not been broadly advertised for animal use [110]. After the detection of the *mcr-1* gene in China, a study evaluated its presence *in Enterobacteriaceae*. Two thousand three cecal samples were collected from cattle, swine, turkey, and chicken from slaughterhouses in the United States and were genetically tested for the presence of *mcr-1* [111]. Two *E. coli* isolates from swine harbored *mcr-1* on an IncI2 plasmid. In Eastern Canada, the *mcr-1* gene was found in 60% of swine in confinement facilities [112].

As for Latin America, samples were collected from Venezuela, Argentina, Ecuador, and Brazil, and the *mcr-1* gene was detected *in E. coli* isolates recovered from human and animal samples [113, 114]. For instance, 19.5% of the chicken meat samples were positive for *mcr-1* in Brazil [115]. In Ecuador, 3.4% of the isolates carried *mcr-1* [115]. Furthermore, in Mexico, the *mcr-1* gene was reported in an *E. coli* collected from a cancer patient [113]. Additionally, *mcr-1*-positive-*E. coli* (ST44) were recovered from pigs' stool in Mexico. As for Colombia, 513 out of the 5887 *E. coli* isolates were colistin-resistant. The screening for the *mcr-1* gene indicated that it is harbored chromosomally in two of these isolates whereby the rest were plasmid-borne [116]. In Venezuela, the first report of *mcr-1* was in two *E. coli* isolates collected from a 43-year-old man and a pig [117]. Notably, the human isolate co-harbored several genes that elicit resistance to

different antibiotics like aminoglycosides, beta-lactams, macrolides, sulfonamides, tetracycline, trimethoprim, and fluoroquinolones.

4. Africa: South Africa

The data about mobile-colistin-resistance in South Africa is scarce; only two manuscripts reported the detection of *mcr-1* in both chickens and humans. The first study detected seven clonally unrelated *mcr-1*-positive-*E. coli* isolated from hospitalized and community patients in South Africa. Notably, one of the isolates co-harbored the floR gene, encoding florfenicol-resistance. Florfenicol is an antibiotic used in animal production, which might suggest the zoonotic potential and origins of this *E. coli* strain [118]. A second study identified 19 Avian Pathogenic *E. coli* (APEC) recovered from broiler chicken's air sacs in 2015, which tested positive for *mcr-1* [119]. The latter has two significant ramifications, 1) *mcr* might emerge in novel zoonotic strains, and 2) the genes will spread to strains that might affect animal health and production, causing severe economic and food security issues.

5. Australia

The utilization of colistin in the clinical and agricultural settings in Australia is limited as compared to bordering countries [120]. Despite the limited usage of colistin, two *mcr-1-positive-E. coli* were identified from patients in New South Wales. The plasmids harboring the *mcr-1* were identical to previously identified plasmids from the Middle East and Asia. Although the two patients did not travel outside Australia, the low prevalence

of *mcr-1* and colistin resistance in *Enterobacteriaceae* in Australia, suggested that the gene might have originated from outside [120].

E. MENA Region

This section will focus on the detection and dissemination of the *mcr* genes in the MENA regions. Although data is scarce in this part of the world, several studies have reported the occurrence of the *mcr* gene and its variants in different bacterial strains and various settings.

1. Humans

In Algeria, the mobile-colistin resistance gene was identified in three clinical cases. The first was from a sperm sample of a 29-year-old male. The gene was harbored by an ST405 *E. coli* isolate. Further analysis of the isolate showed it was an ESBL producer and resistant to cephalosporins, tetracycline, and fluoroquinolones [121]. The second case was detected from a urine sample of an 18-year-old hospitalized patient, with no travel history outside Algeria. Likewise, the retrieved *mcr-1*-positive-*E. coli* belong to ST405 [122]. Recently, a variant of the *mcr* gene was reported in Algeria. A *Klebsiella pneumonia* isolate recovered from the oral cavity of a 25-year-old hospitalized man with lymphoma harbored *mcr*-8. The patient had no travel history and never received colistin treatment. This isolate harbored various resistance genes (bla_{OXA-48}) and extended-spectrum β-lactamase genes like $bla_{CTX-M-15}$. The whole-genome analysis showed that the *mcr-8* detected in Algeria is highly identical to the *K. pneumonia* isolate detected in pig's feces in China [123].

Lebanon is a country that heavily relies on colistin to treat complicated human infections; therefore, the dissemination of the *mcr* gene in the clinical context poses a significant public health risk [124]. A recent study assessed the prevalence of plasmid-borne colistin-resistance in *Enterobacteriaceae*; nine hundred eighty-eight clinical isolates were collected from the Lebanese university's bacterial bank and screened for *mcr-1*. Six *mcr-1*-positive-*E. coli* samples were detected from the 36 colistin-resistant isolates. All the *mcr-1* positive isolates resisted all classes of β-lactam antibiotics, except carbapenems [125]. Moreover, in a routine screening for fecal matter collected from diapers from 2-year-old toddlers' in the community, *Proteus mirabilis*, an enteric bacterium, was isolated. Although *Proteus* is an intrinsically colistin-resistant opportunistic pathogen, the isolate harbored the *mcr-1* gene and had an MDR profile resisting at least three classes of antibiotics. This was the first detection of *mcr-1* in an intrinsically resistant pathogen in the Lebanese population. This finding is of utmost importance since organisms that are intrinsically resistant to colistin are not screened for the *mcr* genes, and they can silently spread this gene to other bacterial cells [126].

As for Egypt, the *mcr-1* gene was detected in two clinical samples derived from hospitalized patients. One *E. coli* retrieved from the sputum of an ICU patient with bacteremia harbored the *mcr-1*. The patient's records showed that he had no previous travel history, which suggests the local acquisition of the gene [127]. Another detected the presence of *mcr-1* in cancer patients in Egypt. Samples were collected from 450 cancer patients, the majority of the isolates recovered were *K. pneumonia* (n=234), *E. coli* (n=200), and *Enterobacter* (n=16). None of the patients had a previous medical history of colistin's intake. Forty isolates were colistin-resistant, out of which 18 were resistant to carbapenems.

Two of the strains tested positive for the *mcr-1*. Cancer patients are prone to bloodstream infections as well as other infections due to their compromised immunity. Therefore, detecting the *mcr-1* gene alongside carbapenem resistance makes treatment and surgery impossible for these individuals. Thus, it becomes a necessity to monitor the spread of the *mcr-1* gene in clinical settings [128].

In Saudi Arabia, several variants of the *mcr* gene were reported. *mcr-5* and *mcr-8* were described for the first time in the MENA region. A study found *mcr-5* in an *E. coli* isolate with no further details about the molecular qualities of this strain [129]. A different study identified *mcr-1* in a multidrug-resistant *E. coli* recovered from a patient's blood sample. This isolate was both colistin and carbapenem-resistant, co-harboring $bla_{NDM,}$ and *mcr-1* [130]. This is highly problematic since colistin is given as salvage therapy when cephalosporins and carbapenems fail [131]. A retrospective study on uropathogenic *E. coli* in Saudi Arabia detected *mcr-1* in an ST131 isolate. This *E. coli* was retrieved from a 2-year-old and was shown to resist different classes of antibiotics like aminoglycosides penicillin, chloramphenicol, streptomycin, sulfonamide tetracycline, and trimethoprim [131]. Recently, a study documented the detection of *mcr-8* in *Klebsiella pneumonia* in Saudi Arabia. This isolate co-harbor both the *mcr-1* and *mcr-8* gene and was isolated from the sputum of a 60-year-old patient. The antimicrobial analysis showed that the isolate was also resistant to fluoroquinolones cephalosporins and aminoglycosides [132].

In Morocco, two *mcr-1* positive isolates from two Moroccans who went on a religious pilgrim in Mecca (Saudi Arabia). The origin of acquisition of the *mcr-1* gene is still unknown, but crowded areas and religious events act as a potential hotspot for the transmission and dissemination of mobile-colistin resistant genes [133].

In Sudan, one study assessed the presence of *mcr-1* in clinical samples from Khartoum. Out of the Fifty *Enterobacteriaceae* isolates gathered, seven were reported as *mcr-1* positive [134].

In Qatar, a study detected the *mcr-1* gene in an ST95 *E. coli* isolate from a patient with subarachnoid hemorrhage; analysis of the isolate showed that it was multidrug-resistant [135]. Additionally, *mcr-1* was detected in *E. coli* from humans isolates in Qatar [136].

In Sultanate Oman, twenty-two colistin-resistant *E. coli* isolates recovered from the clinical setting were screened for *mcr-1* and *mcr-2* [54]. One *mcr-1* positive *E. coli* ST10 was detected from a male's blood culture with comorbidities [137].

In UAE, the mobile genetic marker was recovered from a blood sample of a hospitalized patient in 2013. Two other *mcr-1* isolates were also detected from a blood sample and a wound infection in 2012 [130].

In Jordan, one study reported the presence of *mcr-1* in humans' samples. Out of the 1000 Gram-negative isolates screened, the pooled *E. coli* and *Klebsiella* isolate from Jordan were positive for *mcr-1* [130]. More studies are needed to assess the burden of *mcr* in humans in Jordan.

In Iran, most studies have associated colistin resistance with chromosomal mutations; however, two studies recently identified the presence of *mcr-1*. *E. coli* and *K. pneumonia* isolates from different clinical samples were screened for *mcr-1*, eight tested positive for *mcr-1* [138]. This was the first detection of *mcr-1* in Iran. Similarly, a study screened for both *mcr-1* and *mcr-2* in 60 *K. oxytoca* samples isolated from the clinical settings. PCR analysis showed that four isolates were *mcr-1* positive, and one was *mcr-2*.

The isolates were resistant to colistin, carbapenem, and other antibiotics like cephalosporins, ciprofloxacin, tetracycline, trimethoprim-sulfamethoxazole [139].

In Kuwait, no mention of the detection of the *mcr* gene or its variants in the literature.

2. Animals and Animal Products

The MENA regions face many challenges regarding their feeble infrastructure, weak antimicrobial stewardship, and political and economic instability. Also, the lax implementation of laws on the utilization of colistin in the agricultural sector has posed a significant public health risk due to the dissemination of the *mcr* genes in food-producing animals in different countries. These mobile genetic markers have been detected in Algeria, Lebanon, Egypt, Tunisia, Morocco, Qatar, and Turkey.

Algeria is a country were colistin is frequently used in animal husbandries [140]. The first detection of the *mcr-1* gene was from a poultry farm; the gene was detected in an *E. coli* isolate recovered from the chicken's fecal matter [30]. Another study detected the gene in 5 out of the 503 samples [141]. Similarly, the high prevalence of *mcr-1* (20.8%) was documented in samples collected from chicken farms and slaughterhouses (n=120) from three different regions in Algeria. The high occurrence of the *mcr-1* gene in the poultry sector highlights the critical role poultry plays in the carriage of *mcr-1* [140]. Poultry farms can play a significant role in contaminating nearby natural resources because manure is utilized as a biofertilizers in many sustainable agricultural practices.

Lebanon is challenged by the lack of monitoring and control over the purchase and utilization of antibiotics in animals. A study showed that colistin, a last-resort antibiotic,

was easily accessible in veterinary pharmacies without the need of a prescription [124]. This uncensored use of colistin has caused the high emergence and spread of the mobile resistance gene *mcr-1* in Lebanese poultry and swine farms. In 2018 a study on a pig farm in the South of Lebanon detected *mcr-1* in 23 (23.7%) of the *E. coli* isolates. It is worth mentioning that 98% of *E. coli* were also ESBL producers [142]. Other studies also monitored the occurrence of this mobile gene in poultry farms. Fecal samples (n=982) were collected from poultry farms in 7 districts across Lebanon. The *mcr-1* gene was detected in one ESBL-producing *E. coli* isolated from a sample from Sidon [143]. An Additional study reported a much higher prevalence of *mcr-1* across three major chicken broiler farms. Ninety-three fresh fecal samples were collected, and ninety colistin-resistant *E. coli* isolates were recovered. All the isolated *E. coli* were multidrug-resistant. Also, 98% of the isolates harbored the *mcr-1* gene, while the other 2% were negative for *mcr-2* and *mcr-3* [144]. Lebanon reports the highest prevalence of the *mcr-1* gene in poultry farms worldwide. This highlights two significant issues in Lebanon; 1- Colistin is being extensively misused in the agriculture sector, 2- High prevalence of the *mcr-1* gene in livestock and poultry, poses a significant public health risk for its vital role in transmitting these organisms to humans directly or indirectly.

In Egypt, the mobile-colistin resistant gene was detected from a cow suffering from subclinical mastitis. The gene was harbored by an *E. coli* isolate ST10; further analysis showed that the isolate resisted other antibiotics like tetracyclines, ampicillin, amoxicillin, chloramphenicol, aminoglycosides, and quinolones [145]. As for poultry, the *mcr-1* gene was identified in 7.9% of the *E. coli* isolates collected from chicken's fecal samples from 48 farms across the Delta Nile in 2018. These samples yielded 63 *E. coli,* whereby 12.5%

were extended-spectrum beta-lactamases, and 1.8% were both ESBL and carbapenem-resistant [146]. The detection of *mcr-1* and other antimicrobial genes in poultry farms near a significant water source, the Nile Delta, poses a significant threat since these farms can easily contaminate the river. Also, this river has a substantial role in irrigation and recreational activities, thus posing a major threat to the safety of crops and the wellbeing of the community. Besides, another study highlighted the detection of *mcr-1* in sausage meat in Egypt. The *E. coli* isolate was multidrug-resistant, resisting beta-lactams, tetracyclines sulfonamides, and quinolone antibiotics [147]. The *mcr-1* gene was also detected in a famous ready-to-eat food in Egypt, Karisha cheese. Four *E. coli* isolates were positive for the *mcr-1* gene [148].

In Tunisia, only two studies on poultry farms were done to screen for *mcr-1*. The first study collected 52 fecal samples from healthy birds from three different Tunisian farms. Thirty-seven extended-spectrum beta-lactamase *E. coli* isolates were recovered, and the prevalence of the *mcr-1* gene in farms A, B, and C was 20%,17%, and 83%, respectively. Interestingly, the birds originated from France, which highlights the role of trade and animal movement in the transfer of mobile-colistin resistance [149]. The other study confirmed the previous finding and detected two *mcr-1*-positive-*E. coli isolates* from the one hundred thirty-seven chicken fecal samples collected from three industrial farms in North Tunisia [150].

In Morocco, *mcr-1* was identified in a poultry farm, whereby 560 *E. coli* samples were collected from septicemic broilers and analyzed. The data reported 3 *mcr-1* positive isolates [151].

In Qatar, a study in 2017 collected 172 fecal samples from 2 broilers farms and one live bird market. The data revealed that 15.6% of the 90 *E. coli* isolates were *mcr-1* positive. Further analysis specified that 2.2% were also ESBL producers and that 33% were MDR [136].

In Turkey, the *mcr-1* gene was detected from retail poultry samples collected from two provinces (n=80) and screened for the *mcr-1* gene in *E. coli*. The gene was detected in 4 *E. coli* strains that belonged to three distinct MLST sequences [152].

3. Environmental Continuum

After the detection of the *mcr-1* in humans and animals, some countries pushed their research further and tested environmental samples to identify the occurrence of these genes.

In Algeria, environmental samples from agricultural sites in Oran, northwest Algeria were collected from agrarian soil (n=22), irrigation water (n=10), and animal manure (n=8) from eight different agricultural sites. The work was done on eight colistin-resistant *E. coli* isolates, *mcr-1* was detected in 6/8 samples from soil (n=3), irrigation water (n=1), and horse manure(n=1), and *mcr-3* was identified in 2/8 of the isolates from agricultural land. Further antimicrobial analysis of these isolates indicated that they resisted other antibiotics like amoxicillin, amoxicillin-clavulanate, ticarcillin, nalidixic acid, ciprofloxacin, gentamicin, trimethoprim-sulfamethoxazole, rifamycin [153]. This study highlights the importance of farmlands as potential reservoirs for the mobile-colistin resistant gene. Another study in Algeria detected the *mcr-1* gene in wild animals in 2018. Fecal samples from the Barbary Macaques in Algeria identified the presence of an *mcr-1*-

positive *E. coli* (1/86) [154]. The presence of this mobile genetic marker in wild animals is significant; since wildlife can spread this gene into the environment and water sources through animal waste. Correspondingly, the presence of *mcr-1* was documented in 2 *E. coli* (ST23, and ST115) from seawater sources in Algeria. These isolates were recovered from highly contaminated beaches. Water sources are a primary vehicle for the spread of these genes locally and to different surrounding countries [155]. Therefore, *mcr-1* and *mcr-3* were detected in the Algerian environmental samples, which requires continuous monitoring and evaluation to prevent the spread of these genes to different niches.

As for Lebanon, the *mcr-1* gene was detected in irrigation, sea, and refugee sewage water. Irrigation water samples (n=27) from 2 major agricultural areas, in the Beqaa valley, and the South of Lebanon were collected. A total of 22 *mcr-1*-positive-*E. coli* isolates were recovered. Additional antimicrobial analysis indicated that all the strains had an MDR profile resisting at least four classes of antibiotics. Unfortunately, two colistin-carbapenem resistant isolates were detected, whereby they co-harbored the *mcr-1* and bla_{NDM} and $bla_{OXA\text{-}48}$ genes [156]. After, the detection of *mcr-1* in irrigation water it became a necessity to test seawater for this genetic marker. Seawater samples were collected from 22 locations spanning the Lebanese coastline and screened for the detection of *mcr-1*-positive-*E. coli.* Colistin-resistant *E. coli* was detected in 45.5% of the samples and 16 *mcr-1 E. coli* isolates were retrieved. All the *mcr-1* positive isolates were multidrug-resistant and whole-genome analysis revealed that some of the isolates harbored between 9 and 17 additional antibiotic-resistant genes [157]. Interestingly, the isolates persisted for 30 days at room temperature, which suggests their ability to persist in water and contaminate neighboring countries. Also, the first detection of *E. coli* harboring *mcr-1* in Syrian refugee camps was reported in

Lebanon. Twelve composite samples were collected from drinking, well, and sewage water in two different refugee campsites in Lebanon. All the colistin-resistant *E. coli* isolates (n=36) harbored the *mcr-1* gene. Some of the colonies isolated from drinking water were genotypically identical to the sewage isolates, suggesting possible cross-contamination between these two sources. It is worth mentioning that 6% of the isolates were also carbapenem-resistant [158]. Not only *E. coli* was identified in these water samples, but eight *mcr-1*-positive-*Proteus mirabilis* isolates were recovered. All the strains were multidrug-resistant [159]. The detection of the *mcr* gene in different niches in Lebanon, calls for immediate action and more robust research in this area to identify the true dissemination of this gene.

In Egypt, a single study focused on environmental contamination with the *mcr-1* and *mcr-2* genes. Fecal samples (n=140), residing birds (n=8), migratory water fouls (n=60), surface water samples (n=20), and stool samples from healthy farms (n=50) were collected. *Escherichia coli, Klebsiella pneumonia, Klebsiella oxytoca, and P. aeruginosa* isolated were screened for *mcr-1* and *mcr-2*, the data reported *mcr-1* in indweller birds, migratory birds, water sources, and humans in 10.4%, 20%,16.6%, and 9.6%, while *mcr-2* in 1.4%, 3.6%, 11.1%, and 9.6% of the samples, respectively [160]. This study highlights the importance of looking into the environment to check the dissemination of the *mcr* genes.

CHAPTER II

RIVERS

The high prevalence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) Gram-negative bacterial infections made treatment options limited and, in many cases, nonexistent [2]. The unprecedented escalation of complicated pathogens, that resist all available antibiotics, led to the reintroduction of colistin, a last-resort antibiotic, into the clinical setting [32]. Colistin, polymyxin E, was utilized to treat resistant infections; however, its unguided use in humans and animal farming triggered the emergence of colistin-resistant pathogens [11]. Initially, colistin resistance was attributed to chromosomal mutations in the *pmrAB, pmrD, phoPQ*, and *mrgB* genes, which altered the Lipid A moiety decreasing colistin's affinity and effectiveness [31]. Nevertheless, the newly discovered mobile colistin-resistance gene, *mcr-1*, posed a significant public health risk and substantially jeopardized colistin's efficiency, for its ability to laterally transmit between bacterial progeny through horizontal gene transfer [32]. After the detection of the *mcr-1* gene in *E. coli* in a pig sample from Southern China [30], efforts were put to detect the dissemination of this genetic marker in different niches and matrices worldwide. Although *mcr-1* was detected in more than 40 countries in different samples, rivers, and freshwater resources were not exhaustively studied [32].

The use of antibiotics in humans and animals causes the secretion of residual drugs into environmental resources, whereby approximately 75%-90% of the antibiotics taken are

excreted unmetabolized into the environment [161]. The release of excessive concentrations of antibiotic residues selects for resistant bacterial cells by killing the susceptible ones [39]. 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 [162]. This uncensored disposal contaminates water with antibiotic-resistant organisms and genes [162, 163]. Furthermore, antibiotic-resistant genes can persist in the water milieu and horizontally transfer to new bacterial hosts [50]. Several studies have reported the detection of genes encoding resistance to tetracyclines, aminoglycosides, macrolides, chloramphenicol, vancomycin, sulfonamides, trimethoprim, and β-lactams in surface and groundwater [164]. Therefore, the detection of *mcr-1* in freshwater samples alongside other resistance genes is highly problematic since rivers play a substantial role in the food chain and human activities in different parts of the world. Hence, the aquatic environment can be a hotspot for this gene and its dissemination to different niches, which will in turn affect human directly or indirectly. Only a few studies in China, Switzerland, and Italy reported the detection of *mcr* in freshwater samples.

In Switzerland, the screening of 21 rivers and lakes reported *mcr-1* in extended-spectrum beta-lactamase (ESBL) *Escherichia coli* isolate from the river Birs. Further analysis of the isolate identified that it was multidrug-resistant, resisting at least three classes of antibiotics [165]. Furthermore, the screening of Oltrepò Pavese, Pavia Italy, which supports many agricultural and industrial activities, was done. Water samples were collected from streams, wells, and wastewater treatment plants to monitor the dissemination of resistance genes. High levels of resistance genes were detected mainly in wastewater

treatment plants. As for the river isolates, 62.1% were extended-spectrum beta-lactamase (ESBL) producers. PCR analysis detected an *E. coli* harboring *mcr-1.2* and co-harboring $bla_{CTX\text{-}M}$ and bla_{SHV} [77]. The multilocus analysis reported that the *mcr-1*-positive-*E. coli* belonged to ST10, which was previously reported in humans and animals, suggesting the possible anthropogenic or agricultural contamination of the river with this transmissible genetic marker. IncX4 plasmid harbored that *mcr-1.2* gene, which is responsible for the worldwide dissemination of *mcr-1* [77].

As for China, the *mcr* gene was detected in a chief water system in Northern China, Haihe River [166]. This river flows through extensive agricultural and livestock areas before it discharges into the Bohai Sea, making it a critical site to screen for *mcr* [166, 167]. *mcr-1* was detected in all the samples collected, with higher loads in samples near the estuary; thus, confirming that water contaminated with sewage, livestock, and industrial wastes can be a potential hotspot for *mcr-1.* Also, the *mcr-1* gene was isolated from 11 *E.coli* and 2 *Klebsiella Pneumonia* isolates [166]. Another study on the Funan River, which is heavily impacted by anthropogenic and animal contaminates, was done [162]. Thirty water samples were collected from densely populated areas and screened for the *mcr* genes. The genes were detected in 24 isolates: 17 *Escherichia coli* and 1 *Enterobacter cloacae* harboring *mcr-1*, and 2 *Aeromonas veronii* and 4 *Aeromonas hydrophila* carrying *mcr-3*. Further analysis showed that 87.5% of the *mcr* positive isolates were MDR. Interestingly carbapenem-resistance genes were also detected in the river samples [162]. Moreover, samples collected from humans, animals, animal products, rivers, lakes, and a fountain in the Zhejiang University hospital's surroundings were tested in China [168]. The results document the detection of ten *Aeromonas* isolates harboring *mcr-3* (*mcr-*

3.3 and *mcr-3.13* to *3.18)*. The samples were isolated from human rectal swabs (n=2), pork (n=1), chicken meat (n=3), and the aquatic environment (n=4). This highlights the abundance of *mcr-3* in the aquatic environment and suggests the possible transfer of bacterial cells harboring the *mcr-3* gene to humans [168]. Furthermore, a study in China collected 22 samples from the Yangtze River (Nanjing section), nearby wastewater treatment plants (WWTP), and drinking water treatment plants and screened them for the detection of the colistin-resistance gene *mcr-1* and the carbapenem-resistant gene bla_{NDM-1} [169]. The data showed that these genes were detected in all the investigated samples. Also, it reported that the treatment used in the WWTP was not efficient in decreasing the *mcr* gene's load in the effluent and sludge. The river samples demonstrated higher loads of *mcr-1* and bla_{NDM-1} as compared to WWTP, which was attributed to the effluent's discharge into the river which was impacted by other contaminants. Unfortunately, the gene was also detected in the drinking water plant, illustrating that treatment was not effective in removing these genes. Therefore, *mcr-1* and bla_{NDM-1} were transmitted from the WWTP to the river then eventually to drinking water, putting the lives millions in jeopardy [169]. Therefore, the prevalence of *mcr* in rivers poses a significant public health risk to human life. As seen above, only a handful of studies investigated the prevalence of *mcr* in river water, and none of these studies were done in the MENA region. Hence the aim of this study was to detect the occurrence of *mcr-1* in freshwater samples in Lebanon.

Lebanon, a middle eastern country located on the Mediterranean basin, is rich with its water resources; however, it suffers major environmental breaches and water contamination due to its frail infrastructure and governmental oversight. Sewage outlets dispose of raw sewage directly into water resources, contaminating them with fecal

pollutants and antibiotic resistance genes. Few studies tackled freshwater quality in Lebanon, looking into the chemical and microbiological properties of eight perennial rivers during the dry season [170]. However, no studies assessed the microbiological contamination of river water with the fecal indicator *E. coli* harboring antimicrobial genes, especially the *mcr-1* gene. Therefore, it became a necessity to determine the dissemination and spread of the mobile colistin-resistant gene in Lebanese rivers; especially after its detection in poultry and livestock farms[142, 143, 156], irrigation water [156], refugee camp's sewage, and drinking water [158, 159], and recently in seawater [157] in Lebanon. Therefore, this nationwide study aims to investigate 14 perennial rivers and a water spring in Lebanon to identify the prevalence and dissemination of the *mcr-1* gene and other antibiotic resistance genes in these freshwater resources.

CHAPTER III

MATERIAL AND METHODS

A. Sample Collection

Freshwater samples were collected from 14 perennial rivers and a water stream in Lebanon, spanning five districts: North, South, Beqaa, Mount Lebanon, and Beirut from May 2019 to July 2019. The freshwater resources were chosen based on their significant role in irrigation and recreational activities. Samples were collected in triplicates from three distant locations across the river: upstream, middle, and downstream, 7 to 42 km apart depending on the length of the river. For the litany river, since it is the longest river in Lebanon, three middle samples were gathered. A total of one-hundred thirty-five freshwater samples from 45 sub-rivers were collected in 1 Liter sterile Nalgene® water bottles and transported to the laboratory in a water cooler at (2-5°C). Samples were processed within 16 hours of collection.

B. Bacterial Isolation

Two volumes of water, 100 ml, and 250 ml were filtered through 0.22-µm Millipore® membranes (Sigma-Aldrich). The membranes were enriched on an *Escherichia coli* selective medium (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. The *E. coli* colonies were then enumerated. The suspected purple colonies, 1 to 3 colonies per sample, were further purified, yielding 116 phenotypically colistin-resistant *E. coli* isolates.

The colonies recovered were purified and stored in 1 ml Luria Bertani (LB) broth with 0.5 ml of 80 % glycerol and preserved at -80°C for further analysis [156, 158, 159].

C. Polymerase Chain Reaction

Genomic DNA was used as a template for the PCR reaction and extracted from the bacterial colonies by boiling. One to two bacterial colonies were suspended in 100 µl of DNase free water, then placed in a water bath at 95°C for 15 minutes until the cells completely lysed. The tubes were centrifuged for 2 minutes at 14000 rpm. The supernatant containing the genomic DNA was collected and transferred into a new sterile tube and stored at -20 °C.

1. Detection of the mcr-1 gene

The extracted DNA was used to screen for the *mcr-1* gene using specific forward and reverse primers CLR5 primers (CLR5-F 5′-CGGTCAGTCCGTTTGTTC-3′) and CLR5-R (5′-CTTGGTCGGTCTGTA GGG-3’). The reactions were prepared as follows, 3 µl of genomic DNA was added to a 20 µl PCR reaction tube containing, 0.5 µl of each of the forward and reverse primer, 4 µl of master mix (5x FIREPol® Master Mix Ready to Load), and 12 µl of DNase free water. The PCR analysis consisted of 38 cycles in the thermal cycler (VWR) including a denaturation step at 95°C for 1 minute followed by annealing of the primers at 56°C for 45 seconds and an extension cycle for 1 minute at 72°C with a final extension for 10 minutes at 72°C (Table 1). The *mcr-1* gene fragment’s size is 309 bp. The amplified gene product was inoculated in a 1% agarose gel stained with

ethidium bromide and subjected to electrophoresis for 35 minutes at a constant voltage of 100V. The gels were visualized by the gel imaging system GelDoc reader.

2. Detection of the other mcr genes mcr-2 to mcr-8

The DNA templates were also screened for other *mcr* genes (*mcr-2, mcr-3, mcr-4, mcr-5, mcr-6, mcr-7, mcr-8*). The list of specific primers and PCR conditions used are listed in (Table 1**)**. All the PCR reaction consisted of 3 µl of the DNA template, 4 µl of Master Mix (5x FIREPol® Master Mix Ready to Load), 0.5 µl of each of the forward and reverse gene-specific primers, and 12 µl of DNase free water. The PCR reactions were placed in a thermocycler for 38 cycles. The corresponding gene amplicons were visualized by a 1% agarose gel stained with ethidium bromide and electrophoresed for 35 minutes at 100V.

3. Detection of the antimicrobial resistance genes

PCR analysis was used to screen for the presence of beta-lactam genes*:* bla_{TEM}, bla_{CTX-M}, bla_{SHV}, bla_{KPC}, bla_{IMP}, bla_{NDM}, $bla_{OXA-48,}$ and Class 1 Integron using specific primers (Table 1). The PCR reactions were prepared as follows 3 µl of DNA template, 0.5 µl of the specific forward and reverse primers, 4 µl of Master Mix (5x FIREPol® Master Mix Ready to Load), and 12 µl of DNase free water. The PCR conditions are in (Table 1). PCR reactions were placed in a thermocycler for 38 cycles. Then the corresponding gene amplicons were visualized by running the templates in a 1% agarose gel stained with ethidium bromide and electrophoresed for 35 minutes at 100V.

D. Antimicrobial Analysis

All the *E. coli* isolates were subjected to antimicrobial analysis using the Disc Diffusion Sensitivity method. The optical density of the samples was adjusted to 0.05 at OD_{600}, using Muller Hinton broth as a diluent. The bacterial suspension was spread using a sterile cotton swab on freshly prepared Mueller-Hinton Agar (MHA) plates. Then twenty commercially available antibiotic discs were added to the plates, four antibiotics per plate, and incubated at 37°C for 18-24 hours. 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, since *E. coli* is intrinsically resistant to colistin. Antibiotic susceptibility and resistance were determined by measuring the diameter of the zone of inhibition around each antibiotic disc and comparing it to the 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)[171, 172].

E. Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) for colistin was done to all *mcr-1*-positive-*E. coli*, based on CLSI recommendations, to determine the susceptibility/resistance profile against colistin [17]. The wells of a 96-microtiter plate were inoculated with 100 µl

of bacterial suspension adjusted at an optical density of 0.05 at OD_{600} and challenged with 20 µl of prediluted colistin (Sigma-Aldrich, USA) of different concentration ranging from 1 µg/ml to 64 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 [173].

F. Plasmid Transformation:

Plasmid Extraction was done on 22 *mcr-1*-positive-*Escherichia coli* isolates from different geographic locations using the QIAGEN® Plasmid Mini Kit (25) following the manufacture's recommendations. The extracted plasmids were stored at -20°C for further analysis.

Competent *E. coli* JM109 cells were used as the plasmid's recipient. 50 µl of competent cells were mixed with 10 µl of the extracted plasmid and incubated on ice for 30 minutes. After the incubation, the cells were heat-shocked by placing them in a water bath at 42°C for 2 minutes, followed by a 90-second incubation on ice. After the heat-shock, 940 µl of freshly prepared LB broth was added to the cells and incubated in a shaking incubator for 1 hour 45 minutes at 37°C. Next, the tubes were centrifuged for 2 minutes at 14000 rpm, and 0.9 ml of the supernatant was expelled. The pallet was resuspended in the remaining 0.1 ml LB and then spread on a RAPID' *E. coli* 2 plate supplemented with 2 µg/ml of colistin. The Plates were incubated at 37° C for 18-24 hours. The Transformants were harvested and further analyzed for *mcr-1* as well as checked for their MIC and antimicrobial properties [144, 158, 159].

G. BOX-PCR

Repetitive sequence-based PCR typing with the BOX-A1R primer (5'-CTACGGCAAGGCGACGCTGACG-3') was carried for all the *mcr-1* positive isolates to determine their genotypic diversity. All the PCR reactions were performed in a 25 µl PCR reaction tube, containing 3 µl DNA template, 0.5 µl Box-A1R primer, 4 µl Master Mix (5x FIREPol® Master Mix Ready to Load), and 17.5 µl DNase free water. The amplifications were done based on the following conditions: an initial denaturation step for 2 minutes at 94°C followed by 38 cycles each consisting of 30 seconds at 94°C followed by annealing at 50°C for 1 minute and an extension for 8 minutes at 65°C. The final elongation was for 8 minutes at 65°C. Fingerprints were interpreted visually and analyzed on a 2% agarose gel stained with ethidium bromide and electrophoresed at 100 V for 75 minutes.

H. Plasmid Typing

The PCR Based Replicon Typing Kit 2.0 (Diatheva) PBRT kit was used on 10 *mcr-1*-positive-*E. coli* isolates to determine the incompatibility plasmid types, as per manufacturer's recommendations. The visualization of the amplified DNA product was done by inoculating the amplified product into a 2.5% agarose gel stained with ethidium bromide and electrophorized for 45 minutes at 100 Volts [174].

I. Survival Assay

To determine the persistence of the *mcr-1* gene in river water. Freshwater samples were autoclaved and sterilized by multiple autoclave cycles. Then three *mcr-1* positive

isolates from different river samples were each inoculated in a 600 ml microcosm of sterile water. The optical density of the bacterial suspension was adjusted to 0.1 using the spectrophotometer, and the samples were incubated at room temperature on a magnetic stirrer. Periodically 10 ml of the bacterial suspension was serially diluted and filtered using membrane filtration and cultured on RAPID' *E. coli* 2 agar supplemented with 4µg/ml of colistin. The *E. coli* isolates were then enumerated and frequently checked for the preservation of the *mcr*-1 gene using PCR analysis [156].

J. Commercial Sequencing for *mcr-1* gene fragment

Commercial sequencing was performed on 18 *mcr-1* positive isolates, to confirm the observed *mcr-1* gene signal. The amplified *mcr-1* gene fragments were purified using the QIAquick® Gel Extraction Kit (50) and QIAquick® PCR Purification Kit (50) and sent to be sequenced commercially.

Table 1. List of primers and PCR conditions

Gene/ primer sequence	**PCR conditions for 38 cycles**	**Amplicon size**	**Reference**
mcr-1 gene CLR5-F 5′-CGGTCAGTCCGTTTGTTC-3′ CLR5-R 5′-CTTGGTCGGTCTGTA GGG-3’	95°C for 1 minute 56°C for 45 second 72°C for 1 minute	309 bp	[30]
mcr-2 gene MCR-2F 5’-GCGATGGCGGTCTATCCTGTAT-3’ MCR-2R 5’-TGCGATGACATGGGGTGTCAGC-3’	95°C for 1 minute 55°C for 45 seconds 72°C for 1 minute	378 bp	[175]
mcr-3 gene MCR-3F 5’-TATGGGTTACTATTGCTGG-3’ MCR-3R 5’-CTACCCTGATGCTCATCG-3’	95°C for 1 minute 55°C for 45 seconds 72°C for 1 minute	814 bp	
mcr-4 gene MCR-4F 5’-GTCATAGTGGTATAAAAGTACAG-3’ MCR-4R 5’-CCACCGTCTATCAGAGCCAAC-3’	95°C for 1 minute 55°C for 45 seconds 72°C for 1 minute	664 bp	
mcr-5 gene MCR-5F 5’-GCGGTTGTCTGCATTTATCAC-3’ MCR-5R 5’-CTTTGAAAACCTGTCTTCGGCA-3’	95°C for 1 minute 55°C for 30 seconds 72°C for 1 minute	1042 bp	
mcr-6 gene MCR-6F 5’-GTCCGGTCAATCCCTATCTGT-3’ MCR-6R 5’-ATCACGGGATTGACATAGCTAC-3’	95°C for 1 minute 55°C for 45 seconds 72°C for 1 minute	556 bp	
mcr-7 gene MCR-7F 5’-TGCTCAAGCCCTTCTTTTCGT-3’ MCR-7R 5’-TTCATCTGCGCCACCTCGT-3’	95°C for 1 minute 55°C for 45 seconds 72°C for 1 minute	892 bp	
mcr-8 gene MCR-8F 5’-AACCGCCAGAGCACAGAATT-3’ MCR-8R 5’-TTCCCCCAGCGATTCTCCAT-3’	95°C for 1 minute 60°C for 45 seconds 72°C for 1 minute	667 bp	
Bla$_{TEM}$ Forward: 5’-ACCAATGCTTAATCAGTGAG-3’ Reverse: 5’-GCGGAACCCCTATTTG-3’	95°C for 1minute 55°C for 45 seconds 68°C for 1 minute	963 bp	[176]
bla-$_{CTX-M}$ Forward: 5’-ATGTGCAGYACCAGTAARGTKATGGC-3’	95°C for 1minute	593 bp	[177]

Gene/ primer sequence	**PCR conditions for 38 cycles**	**Amplicon size**	**Reference**
Reverse: 5' - TGGGTRAARTARGTSACCAGAAYCAGCGG-3'	60°C for 45 seconds 68°C for 1 minute		
bla-SHV Forward: 5'- CACTCAAGGATGTATTGTG-3' Reverse: 5'- TTAGCGTTGCCAGTGCTCG-3'	95°C for 1minute 55°C for 45 seconds 68°C for 1 minute	822 bp	[179]
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	[180]
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	[181]

CHAPTER IV

RESULTS

Freshwater samples were aseptically collected from 14 perennial rivers and Ras el Ain water spring across five districts in Lebanon. These water resources are heavily impacted by anthropogenic, industrial, and animal wastes making them suitable for sampling and analyzing. In addition to their significant role in agriculture, these water resources are characterized by their persistent flow throughout seasons, making them an important vehicle of transmitting contaminants and genes to various niches. The samples were uniformly collected from three distant locations across the rivers, upstream, middle and downstream, 7 to 42 km apart in triplicates, yielding one-hundred thirty-five freshwater samples (Table 2).

Major River	River source	River Middle			River end
Hasbani	Hasbaya	Ibel el Saqi	Wazzini		
Litani	Nabaa Al Litani	Bar Elias	Jamraqa	Mazraat Tamrah	Qassemia
Awali	Barouk	Besri			Awali
Zahrani	Nabaa el Tassi	Habbouch			Zahrani
Tyre	Nabaa Ras el Ain				
Damour	Nabaa el Safaa	Jesir el Qadi			Damour
Naher el Kaleb	Faraya	Jeita			Naher el Kaleb
Beirut	Majdel Tarshish Aintoura	Beirut			Beirut Port
Naher Ibrahim	Afqa	Yahshoush			Naher Ibrahim
Assi	Ain el Zarqa	Bejaji			Laboueh
Bared	Fnaideq	Oyoun el Samak			Naher el Bared
Kbir	Wadi Khalid	Heker el Daher			Arida
Oustoueni	Aine el Tine	Khraybet el Jundi			Oustoueini
Abou Ali	Bcharre	Zgharta			Abou Ali
Naher el Jouaz	Tannourine	Kaftoun			Jaouz

Table 2. The names of the major rivers and sub rivers of the sampling sites

Two volumes of the water were filtered through a 0.22-µm Millipore membrane and placed on an *E. coli* selective media supplemented with colistin. Typical colistin-resistant *Escherichia coli* colonies were detected in 43 sub-rivers (98.3%), with counts varying between 1 to 10^4 CFU/100 ml. The highest count was observed in Beirut river, 10^4 CFU/100ml. Only Nabaa Al Tassih in the South and Fnadiq River in the North showed no detectable colistin-resistant *E. coli* on the plates (Figure 1).

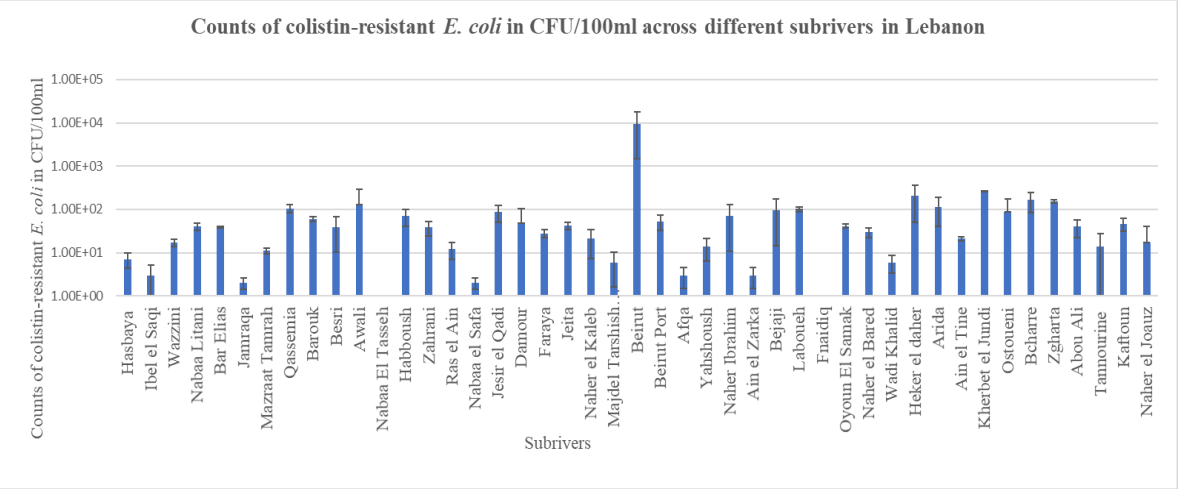

Fig. 1. The counts of colistin-resistant *E. coli* in CFU/100ml across different sub rivers in Lebanon

E. coli was successfully recovered from 27 sub rivers with a 60% retrieval rate from different geographic locations. Although, more samples showed growth of colistin-resistant *E. coli* isolation was challenging due to the high bacterial counts, and other bacterial contaminates on the plate. A total of one hundred sixteen colistin-resistant *E. coli* colonies were collected (1-3 colonies per sample) and screened for the detection of the *mcr-1* gene using specific CLR5 primers (Table 1).

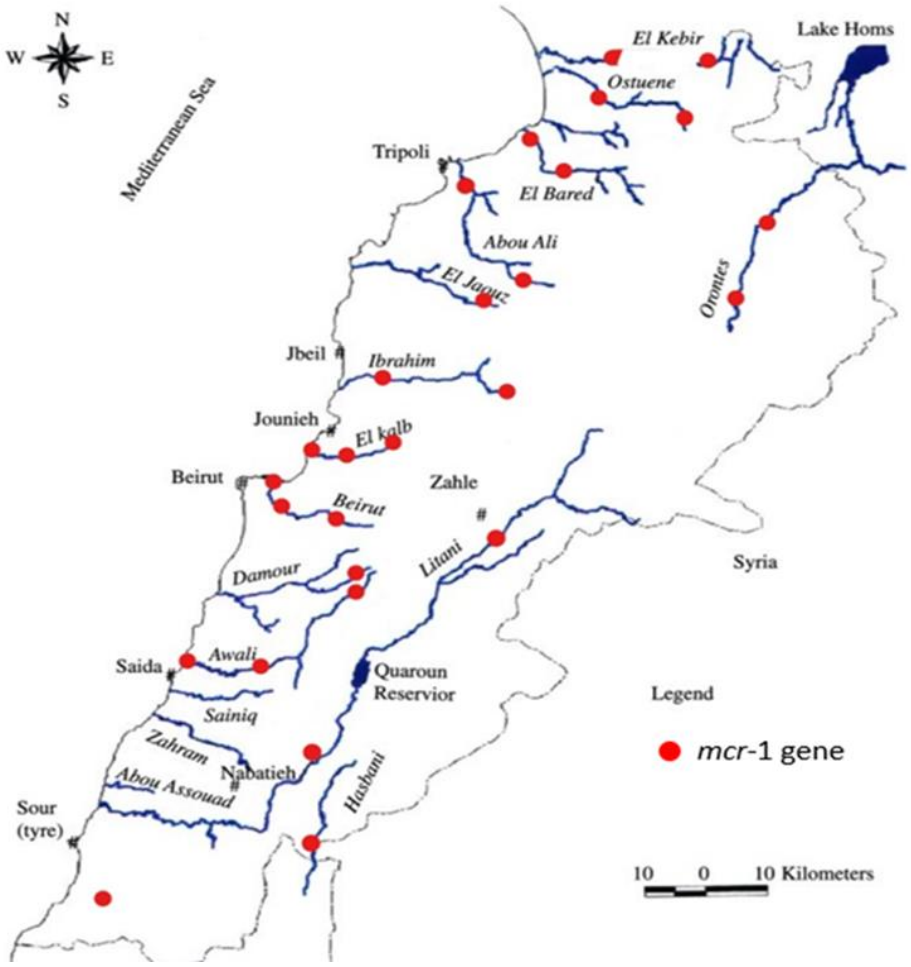

Fig. 2. The approximate geographic disrtibution of the *mcr-1*-positive-*E. coli* isolates across rivers in Lebanon

The *mcr-1* gene was detected in all the colistin-resistant *E. coli* strains in different rivers across Lebanon (Figure 2). The amplified *mcr-1* gene fragments were purified using the PCR purification kit and sent out to be commercially sequenced for 15.5% of the samples. The isolates showed 100% homology to the previously reported *mcr-1* gene (Figure 3).

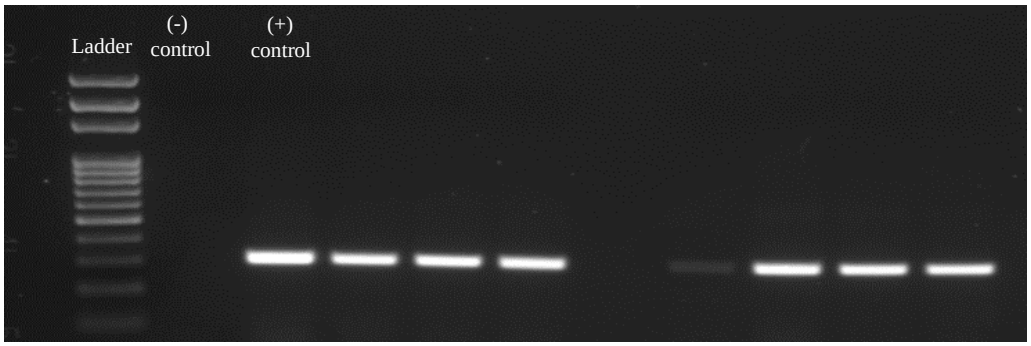

Fig. 3. Agarose gel of the amplified *mcr-1* gene fragment (309 bp)

Additionally, the isolates were screened by PCR for other *mcr* genes (*mcr-2* to *mcr-8*) using specific primers (Table 1). Some of the *mcr-1* positive isolates co-harbored *mcr*-2 (n=19), *mcr*-3 (n=10), *mcr*-4 (n=2), *mcr*-6 (n=1), and *mcr*-8 (n=9) (Figure 4, Table 4). These signals were not confirmed by commercial sequencing, and further analysis should be done.

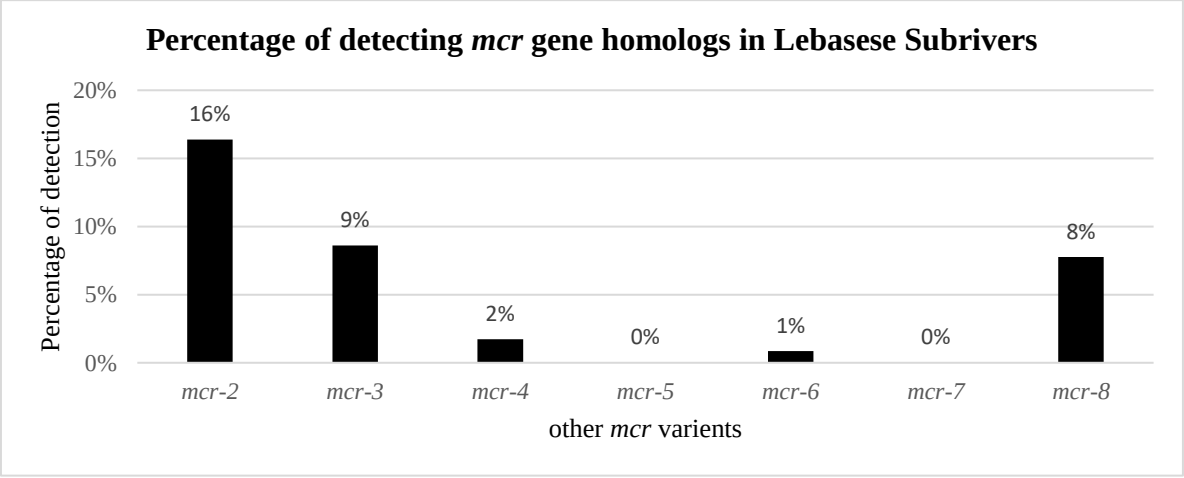

Fig. 4. The percentages of detecting *mcr* gene homologs in Lebanese river samples

Colistin's minimum inhibitory concentration (MIC) was ≥ 4 μg/ml for all the *mcr-1* positive isolates confirming the colistin-resistant profile, and the values ranged between 4 and 64 μg/ml, with the majority of samples having a MIC ≥8 μg/ml (Figure 5, Table 4).

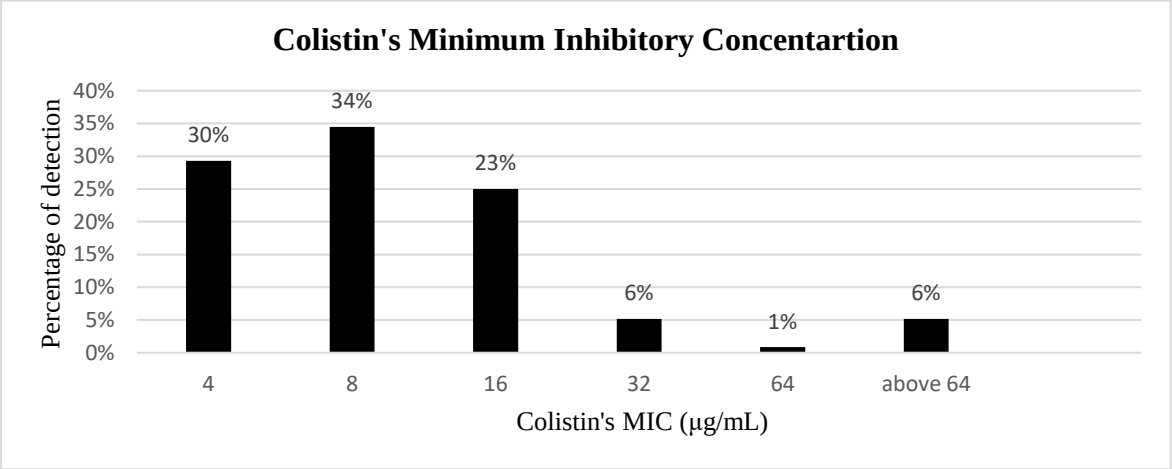

Fig. 5. Percentages of the Minimuim Inhibitory Concentration (MIC) of the *mcr-1*-positive-*E. coli* isolates recovered from the rivers

Moreover, the isolates expressed phenotypic resistance against Penicillin (100%), Ampicillin (77%), Amoxicillin/Clavulanic acid (76%), Cefepime (25%), Cefotaxime (44%), Cephalexin (77%), Cefixime (39%), Doripenem (5%), Meropenem (3%), Imipenem (7%), Gentamicin (35%), Kanamycin (39%), Streptomycin (61%), Tetracycline (68%),

Ciprofloxacin (30%), Norfloxacin (25%), Trimethoprim/Sulfamethoxazole (57%) and Chloramphenicol (39%) as shown by the disc diffusion assay (Figure 6).

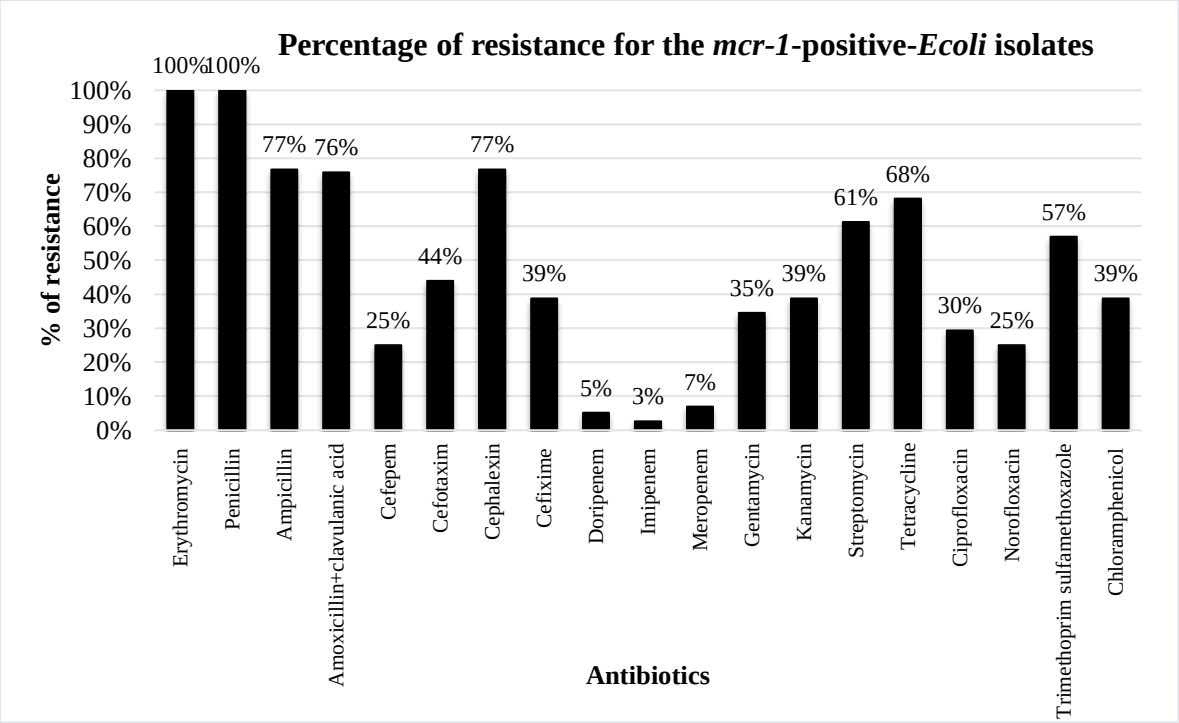

Fig. 6. Percentages of resistance for the *mcr-1*-positve-*E. coli* in river isolates

Interestingly, 94% of the isolates were multidrug-resistant, resisting at least three classes of antibiotics. It is worth mentioning that 10% of the isolates were both colistin and carbapenem-resistant, expressing resistance against doripenem, meropenem, and imipenem (Table 4).

To determine the antibiotic resistance genes harbored by the *mcr-1* positive isolates, the samples were screened for beta-lactam genes using specific primers (Table 1). Nearly half of the isolates harbored bla_{TEM} bla_{SHV} and $bla_{CTX\text{-}M,}$ 48%,42%, 36%, respectively (Figure 7).

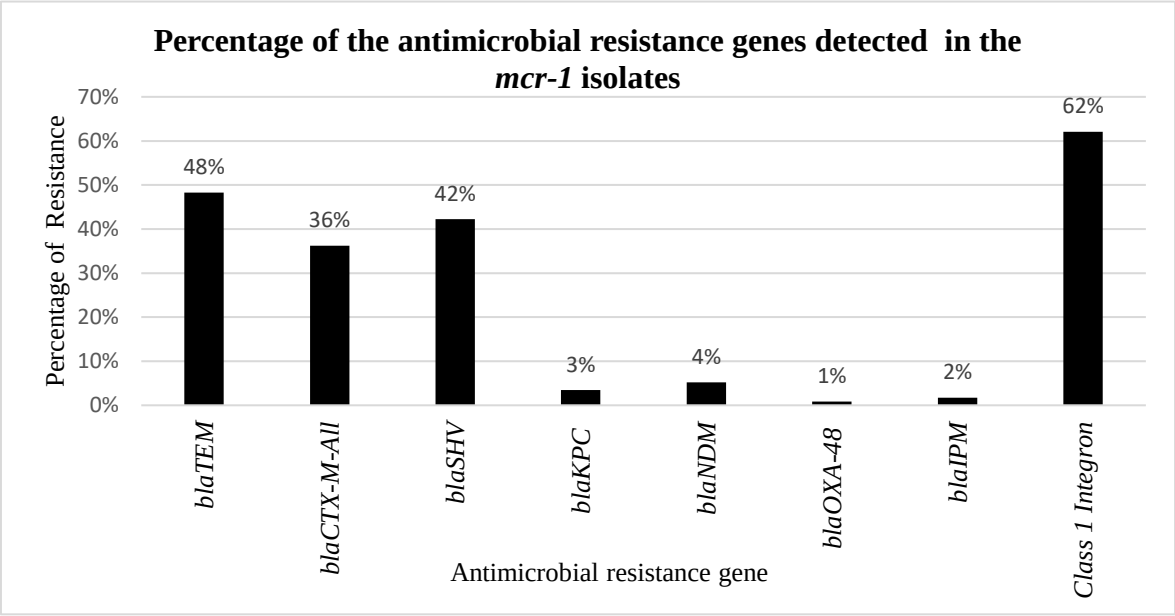

Fig. 7. Percentages of detecting other antimicrobial resistance genes in the *mcr-1* isolates recovered from the rivers in Lebanon

It is worth mentioning that 62% of the isolates harbored the Class 1 Integron gene. Also, some isolates co-carried the carbapenem-resistance genes encoding resistance against imipenem, doripenem, and meropenem alongside the *mcr-1* gene. While some isolates only harbored one of the resistance genes bla_{IPM}, bla_{KPC}, bla_{NDM}, or bla_{OXA-48}, others co-harbored bla_{NDM}/bla_{KPC} (Barouk river) (Table 4). Box PCR fingerprint analysis demonstrated that the 116 *mcr-1*-positive-*E. coli* isolates were highly diverse and belonged to 63 distant genotypes. Approximately 54% of the isolates were genotypically different from each other.

The *mcr-1* gene was successfully transformed into chemically competent *E. coli* JM109 recipient cells (QIAGEN) using the heat-shock method in all the tested samples (n=22). All the transformants harbored the *mcr-1* gene, as shown by PCR analysis, confirming the plasmid-borne nature of the gene. Also, colistin's MIC of the *E. coli* JM109

before the transformation was < 0.25 μg/mL; however, the transformants' MIC varied between 2-8 μg/mL, thus confirming they are colistin-resistant. Moreover, in some of the transformants, resistance to other antibiotics was observed, like resistance to Cephalosporins, Tetracycline, Aminoglycoside, Fluoroquinolone, Chloramphenicol, and Sulfonamides, indicating that these genes might be plasmid-borne as well. None of the transformants expressed resistance to carbapenem.

Additionally, various plasmid types were identified in 8.6% of the *mcr-1*-positive-*E. coli* isolates using the PBRT kit: IncI1 α, IncI2, IncX1, IncX4-FIBM; IncFIB, IncFII, IncFIBM; IncX4; IncFIIK-FII; IncFIIK; IncFIBKN, IncFII; IncX4-FII (Table 3).

Table 3 The various plasmid types recovered for the *mcr-1* positive isolates

Sample ID	**Plasmid Type**
Wazzani river 1 (1)	$IncI_1\ \alpha$, IncI2,IncX1,IncX4-FIBM
Bar Elias river 1	IncFIBM
Bejaji river 1	IncFIB,IncX1,IncFII
Wadi Khalid river 1 (2)	IncFIBM
Barouk river 1 (3)	IncFIB, IncFIBM
Zgharta river 2 (2)	IncX4
Aine Tineh river 2 (2)	IncX4
Beirut river 1 (1)	IncFIIK-FII
Bcharre river 3	IncFIIK,IncFIBKN, IncFII
Awali river 2 (1)	IncI1 α, IncI2,IncX4-FII

Three *mcr*-1 positive isolates recovered from Bcharre (Bch 3), Ain el Tine (ATM 2 (2)), and Zgharta (Zgh 2 (2)), were inoculated in 600 ml of the sterile river water and monitored for the persistence of the *mcr*-1 gene. The data showed that the *mcr*-1 gene persisted for ≥ 127 days.

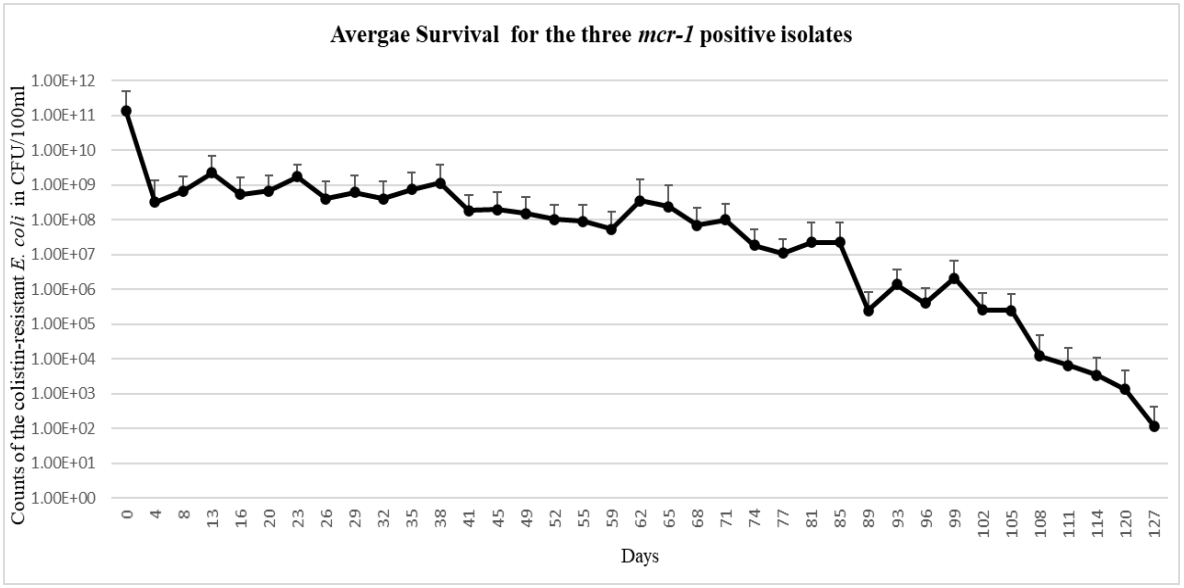

Fig. 8. The average survival for the three *mcr-1* positive isolates in sterile river water

Table 4 Antibiotic resistance profile of the *mcr-1*-positive colistin-resistant *E. coli* isolates recovered from freshwater samples in Lebanon

District[1]	River[2] Name	Sample[3] ID code	Colistin MIC (µg/mL)	Resistance profile[4]	Intermediate Resistance Profile	Antimicrobial[5] Resistance Genes
South Lebanon	Wazzini River	**Waz 1 (1) ***	16	PEN-AMP-AMC-FEP-CTX-LEX-CFM-GEN-KAN-TET-CIP-SXT-CHL		*mcr-1; bla$_{TEM}$; bla$_{CTX-M}$;* Class 1 Integron
		Waz 1 (2) *	16	PEN-AMP-AMC-CTX-LEX-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL		*mcr-1; mdfA*
		Waz 1 (3) *	16	PEN-AMP-AMC-LEX-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL		*mcr-1; bla$_{TEM}$;* Class 1 Integron
		Waz 2 (1) *	16	PEN-AMP-AMC-CTX-LEX-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL		*mcr-1; bla$_{TEM}$; bla$_{SHV;}$ bla$_{CTX-M}$;* Class 1 Integron
		Waz 2 (2) *	16	PEN-AMP-CTX-LEX-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL		*mcr-1; bla$_{TEM}$; bla$_{CTX-M}$;* Class 1 Integron
		Waz 2 (3) *	16	PEN-AMP-AMC-CTX-LEX-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL		*mcr-1; bla$_{TEM}$; bla$_{SHV}$; bla$_{CTX-M}$;* Class 1 Integron
		Waz 3 (1) *	4	PEN-AMP-AMC-FEP-CTX-LEX-CFM-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL		*mcr-1.1; bla$_{TEM-141}$; bla$_{CTX-M-3}$; aac3-IId; ant2''-Ia; ant3''-Ia; aph3'-Ia; catA1; floR; fosA3; mdfA; sul1; sul2; tetA;* Class 1 Integron
		Waz 3 (3) *	4	PEN-AMP-LEX-GEN-KAN-TET-CIP-NOR-SXT-CHL	CTX-DOR	*mcr-1; bla$_{TEM}$; bla$_{CTX-M}$;* Class 1 Integron

[1] Samples were collected across 5 districts in Lebanon, spanning the North to the South
[2] Names of the sub rivers where samples were collected.
[3] Isolates with a (*) are multidrug resistant, resisting at least three classes of antibiotics
[4] Antibiotic resistance profile of the *mcr*-1-positive colistin-resistant *E. coli* isolated from 14 major rivers and a water spring divided into 45 sub rivers in Lebanon spanning 5 districts. Penicillin (PEN), ampicillin (AMP), amoxicillin + clavulanic acid (AMC), cefepime (FEP), cefotaxime (CTX), cephalexin (LEX), cefixime (CFM), doripenem (DOR), gentamicin (GEN), kanamycin (KAN), streptomycin (STR), tetracycline (TET), ciprofloxacin (CIP), norfloxacin (NOR), trimethoprim-sulfamethoxazole (SXT), and chloramphenicol (CHL). The antibiotics in the resistance profile were arranged according to the order of antibiotics/classes listed in the CLSI guidelines.
[5] Highlighted isolates are isolates that underwent whole genome sequencing, data not discussed.

	Awali River	**Aw 2 (1) ***	8	PEN-AMP-AMC-CTX-LEX-CFM-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	FEP	*mcr-1; bla_{TEM}; bla_{SHV}; bla_{CTX-M}; Class 1 Integron*
		Aw 3 (2) *	8	PEN-AMP-AMC-FEP-CTX-LEX-CFM-GEN-KAN-STR-TET-CIP-NOR-SXT	DOR-IPM-MEM-CHL	*mcr-1; bla_{TEM}; bla_{SHV};* Class 1 Integron
		Aw 3 (3) *	above 64	PEN-AMP-AMC-CTX-LEX-IPM-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	FEP-CTX-CFM	*mcr-1; bla_{TEM}; bla_{SHV}; bla_{CTX-M}; bla_{OXA-48};* Class 1 Integron
	Besri River	**Bes 3 (1) ***	4	PEN-STR		*mcr-1; mcr-8*
	Ras El Ain River	**RA 2 (1)**	16	PEN		*mcr-1; bla_{TEM}; bla_{SHV},* Class 1 Integron
		RA 2 (2) *	8	PEN-AMP-AMC-CTX-LEX-STR-TET	CFM-DOR-IPM	*mcr-1; mcr-2; bla_{TEM}; bla_{SHV};* Class 1 Integron
		RA 2 (3) *	4	PEN-AMP-AMC-LEX-GEN-STR-TET-CIP-NOR-SXT-CHL	FEP-CTX-CFM-IPM	*mcr-1; bla_{SHV}; bla_{CTX-M}*
	Mazraat Tamrah River	**MT 1 (1) ***	8	PEN-AMP-AMC-CTX-LEX-CFM	STR	*mcr-1; bla_{SHV}; bla_{CTX-M};* Class 1 Integron
		MT 1 (2) *	16	PEN-AMP-AMC-FEP-CTX-LEX-STR-TET-SXT	CFM	*mcr-1; bla_{TEM}; bla_{CTX-M};* Class 1 Integron
		MT 1 (3) *	16	PEN-AMP-AMC-FEP-CTX-LEX-CFM-STR-TET-SXT		*mcr-1; bla_{SHV}; bla_{CTX-M}*; Class 1 Integron
		MT 2 (1) *	16	PEN-AMP-AMC-FEP-CTX-LEX-CFM-STR-TET-SXT	KAN	*mcr-1; mcr-3; bla_{TEM}; bla_{SHV}; bla_{CTX-M};* Class 1 Integron
		MT 2 (2) *	16	PEN-AMP-AMC-CTX-LEX-STR-TET-SXT	FEP-CFM-IPM-GEN	*mcr-1; mcr-3; bla_{SHV}; bla_{CTX-M};* Class 1 Integron
		MT 3 (1) *	4	PEN-AMP-AMC-LEX-STR-TET-CIP-SXT	KAN-NOR	*mcr-1; bla_{CTX-M};* Class 1 Integron
		MT 3 (2) *	4	PEN-AMP-AMC-LEX-STR	CTX-DOR	*mcr-1; bla_{TEM}; bla_{SHV}; bla_{CTX-M};* Class 1 Integron

Mount Lebanon	Nabaa El Safa	**Safa 1 (1) ***	8	PEN-AMP-GEN-TET-SXT-CHL	CFM-KAN-STR-NOR	*mcr-1; bla$_{SHV}$;* Class 1 Integron
		Safa 1 (2) *	16	PEN-AMP-AMC-CTX-LEX-CFM-DOR-STR-TET	FEP-MEM-KAN	*mcr-1; bla$_{TEM}$; bla$_{SHV}$;* Class 1 Integron
		Safa 1 (3) *	8	PEN-AMP-AMC-LEX-IPM-STR-TET-CIP-NOR-SXT	FEP-CTX-CFM-DOR-MEM-KAN	*mcr-1;mcr-2; bla$_{SHV}$; bla$_{KPC}$;* Class 1 Integron
		Safa 2 (1) *	8	PEN-AMP-AMC-FEP-CTX-LEX-MEM-STR-TET-CIP-NOR-SXT	CFM-DOR-IPM-KAN	*mcr-1;mcr-2; bla$_{SHV}$; bla$_{CTX-M}$; bla$_{NDM}$;* Class 1 Integron
		Safa 2 (2) *	above 64	PEN-AMC-FEP-CTX-LEX-CFM-GEN-KAN-STR-TET-CIP-NOR-SXT	AMP	*mcr-1, aadA5; aph 3''-Ib; aph 6-Id; dfrA17; mdfA; mphA; sul1; sul2; tetA;* Class 1 Integron
		Safa 2 (3) *	16	PEN-AMP-AMC-FEP-LEX-CFM-GEN-KAN-TET-CIP-NOR-SXT-CHL	STR	*mcr-1;* Class 1 Integron
		Safa 3 (1)*	4	PEN-AMP-AMC-CTX-LEX-KAN-TET-SXT-CHL	DOR-IPM-STR	*mcr-1; bla$_{SHV}$; bla$_{CTX-M}$;* Class 1 Integron
		Safa 3 (2) *	4	PEN-AMP-LEX-STR-TET-CIP-NOR-SXT		*mcr-1; mcr-8; bla$_{SHV}$;* Class 1 Integron
	Nabaa El Barouk	**Bar 1 (2) ***	4	PEN-AMP-AMC-FEP-CTX-LEX-CFM-STR-TET-CIP-NOR-SXT	FEP-KAN-IPM	*mcr-1; bla$_{TEM-1B}$; bla$_{CTX-M}$; aph 3''-Ib; aph 6-Id; dfrA5 mdfA; sul2; tetA;* Class 1 Integron
		Bar 1 (3) *	8	PEN-AMP-AMC-FEP-CTX-LEX-CFM-DOR-MEM-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	IPM	*mcr-1; bla$_{TEM}$; bla$_{IPM}$;* Class 1 Integron
		Bar 2 (1) *	4	PEN-AMP-AMC-LEX-CFM-DOR-GEN-KAN-STR-TET	FEP-CTX-IPM-CHL	*mcr-1; bla$_{TEM}$; bla$_{SHV}$;* Class 1 Integron
		Bar 2 (2) *	8	PEN-AMP-STR-TET-SXT-CHL		*mcr-1; bla$_{SHV}$;* Class 1 Integron
		Bar 3 (1) *	16	PEN-AMP-AMC-CTX-LEX-CFM-MEM-GEN-KAN-STR-TET-CIP-SXT-CHL		*mcr-1;mcr-2; mcr-8; bla$_{TEM}$; bla$_{CTX-M}$; bla$_{NDM}$;* Class 1 Integron

		Bar 3 (2) *	8	PEN-AMP-AMC-LEX-IPM-GEN-STR-TET-SXT	FEP-CTX-DOR-KAN-CIP	*mcr-1;mcr-2; bla$_{TEM}$; bla$_{SHV}$; bla$_{KPC}$; bla$_{NDM}$;* Class 1 Integron
		Bar 3 (3) *	4	PEN-AMP-AMC-LEX-GEN-KAN-STR-TET-CIP-SXT-CHL	FEP-DOR-IPM-NOR	*mcr-1; bla$_{TEM}$; bla$_{SHV}$;* Class 1 Integron
	Majdel Tarchich Anitoura River	**MTA 3***	8	PEN-AMP-AMC-LEX-CFM-STR-TET-SXT-CHL		*mcr-1; mcr-2; bla$_{ACT-12}$; fosA2; oqxA; oqxB*
	Naher El Kaleb	**Klb 1 (1) ***	16	PEN-AMP-AMC-FEP-CTX-LEX-CFM-TET-CIP-SXT	STR-NOR	*mcr-1; mcr-3; bla$_{CTX-M}$; bla$_{SHV}$*
		Klb 1 (2) *	8	PEN-AMC-FEP-CTX-LEX-CFM-SXT	STR	*mcr-1; mcr-3; bla$_{CTX-M}$; bla$_{SHV}$*
		Klb 3 (1) *	4	PEN-AMC-STR-TET-CIP-SXT	KAN	*mcr-1; bla$_{SHV}$;* Class 1 Integron
		Klb 3 (2) *	8	PEN-AMP-LEX-STR-NOR	TET	*mcr-1; mcr-3;* Class 1 Integron
	Jeita River	**Jei 2 (1) ***	32	PEN-AMP-AMC-LEX-CFM-GEN-KAN-STR-TET-SXT-CHL	CTX	*mcr-1; bla$_{TEM}$*
		Jei 2 (2) *	8	PEN-AMP-AMC-LEX-GEN-KAN-STR-TET-SXT-CHL		*mcr-1; bla$_{TEM}$*
		Jei 2 (3) *	16	PEN-AMP-AMC-CTX-LEX-CFM-GEN-KAN-STR-TET-SXT-CHL		*mcr-1;mcr-2; bla$_{TEM-1B}$; bla$_{CMY-101}$; aph3''-Ib; aph6-Id; dfrA5; mdfA; qnrB21; sul2; tetB*
	Faraya River	**Far 1 (1) ***	4	PEN-AMP-AMC-LEX-CFM	CTX-KAN-STR	*mcr-1; bla$_{TEM}$; bla$_{SHV}$*
		Far 1 (2)	16	PEN	STR	*mcr-1; bla$_{SHV}$;* Class 1 Integron
		Far 1 (3) *	16	PEN-AMP-AMC-LEX		*mcr-1*
		Far 2 (1) *	4	PEN-AMP-AMC-CTX-LEX	CFM-IPM-STR	*mcr-1; bla$_{SHV}$;* Class 1 Integron
		Far 2 (2) *	4	PEN-AMP-AMC-CTX-LEX-TET	CFM-IPM-STR	*mcr-1; bla$_{ACT-12;}$ bla$_{ACT-14}$; fosA2; fosA; oqxA; oqxB;* Class 1 Integron

		Far 3 (1) *	4	PEN-AMP-AMC-LEX-TET		*mcr-1; mcr-8; bla$_{TEM}$;* Class 1 Integron
		Far 3 (2) *	4	PEN-AMC-LEX-TET-SXT	STR	*mcr-1*
		Far 3 (3) *	4	PEN-AMC-LEX	AMP-MEM-IPM-STR	*mcr-1;* Class 1 Integron
	Yahshoush River	**Yah 2 (1) ***	8	PEN-AMP-AMC-FEP-CTX-LEX-CFM-KAN-STR-TET-SXT	GEN-CIP-NOR-CHL	*mcr-1; bla$_{TEM}$; bla$_{SHV}$*
		Yah 2 (2) *	8	PEN-AMP-AMC-CTX-LEX-CFM-STR-TET-SXT	FEP-KAN	*mcr-1; bla$_{TEM}$; bla$_{SHV}$;* Class 1 Integron
		Yah 3*	4	PEN AMP-AMC-LEX-CFM	TET	*mcr-1*
	Afqa River	**Afqa 1 (1)**	4	PEN	AMP	*mcr-1; bla$_{TEM}$; bla$_{CTX-M}$;* Class 1 Integron
		Afqa 1 (2) *	16	PEN-AMP-AMC-STR-TET-SXT	KAN	*mcr-1; mcr-2; mcr-8; bla$_{TEM}$*
		Afqa 1 (3) *	8	PEN AMP-AMC-STR-TET-SXT		*mcr-1; bla$_{TEM}$;* Class 1 Integron
		Afqa 2 (1) *	4	PEN-AMC-LEX	STR	*mcr-1; bla$_{TEM}$,* Class 1 Integron
		Afqa 2 (2) *	8	PEN-AMC	KAN-STR	*mcr-1*
		Afqa 3 (1)	16	PEN	KAN-STR	*mcr-1*
		Afqa 3 (2) *	4	PEN-AMC-KAN		*mcr-1*
		Afqa 3 (3) *	8	PEN-AMC-SXT	AMP-STR	*mcr-1;* Class 1 Integron
Beqaa	Bar Elias River	**BE 1 ***	8	PEN-AMP-AMC-CTX-LEX-CFM-GEN-STR-TET-SXT-CHL	FEP	*mcr-1; mcr-8; bla$_{ACT-14}$, fosA; mdfA; oqxA; oqxB*
		BE 3 (1) *	4	PEN-AMP-AMC-LEX-CFM-GEN	FEP-KAN-STR-CHL	*mcr-1;mcr-2; mcr-3; bla$_{TEM}$; bla$_{SHV}$;* Class 1 Integron
		BE 3 (2) *	8	PEN-AMP-AMC-FEP-CTX-LEX-CFM-STR-TET-SXT	GEN-KAN-CIP	*mcr-1; mcr-3; bla$_{SHV}$*

	Bejaji River	**Bejaji 1**	32	PEN-AMP-AMC-CTX-LEX-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	FEP-CFM-DOR-IPM	*mcr-1; mcr-2, mcr-3, mcr-8 mdfA*
	Ain El Zarqa River	**AZ 2 (1) ***	16	PEN-AMP-LEX		*mcr-1; mcr-3; bla$_{SHV}$; bla$_{CTX-M}$;* Class 1 Integron
		AZ 2 (2) *	32	PEN-AMC-FEP-CTX-LEX-CFM-MEM-GEN-KAN-STR-TET	AMP-DOR-MEM-CIP	*mcr-1; bla$_{CTX-M}$;* Class 1 Integron
		AZ 2 (3)	4	PEN	STR	*mcr-1; bla$_{CTX-M}$;* Class 1 Integron
		AZ 3 (1) *	4	PEN-AMP-AMC-GEN-KAN-STR-TET-NOR-SXT-CHL	CFM	*mcr-1; mcr-2; mcr-3; bla$_{TEM}$*
		AZ 3 (2) *	8	PEN-AMC-LEX-GEN-KAN-STR-TET	CFM-MEM-IPM	*mcr-1*
		AZ 3 (3) *	4	PEN-AMP-AMC-LEX-STR-TET-CHL	FEP-CFM-GEN-KAN	*mcr-1; bla$_{TEM}$*
North Lebanon	Naher El Bared	**Bared 1 (2) ***	32	PEN-AMP-AMC-LEX-CFM-GEN-KAN-STR-TET	FEP-IPM-CIP	*mcr-1; bla$_{TEM;}$ bla$_{NDM}$;* Class 1 Integron
		Bared 1 (3) *	8	PEN-AMP-AMC-CTX-LEX-TET	STR	*mcr-1; bla$_{TEM}$*
		Bared 3 (1) *	8	PEN-AMP-AMC-CTX-LEX-CFM-GEN-STR-TET-SXT-CHL	FEP	*mcr-1*
		Bared 3 (2) *	16	PEN-AMP-AMC-CTX-LEX-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	CFM	*mcr-1.1; bla$_{TEM-1B}$; bla$_{CTX-M-1}$; bla$_{SHV-101}$; aac3-IId; aph3'-Ia; aph6-Id; dfrA14; dfrA1; floR; fosA6; mdfA; mphA; oqxA; oqxB; qnrS1; sul2; tetA*
	Zgharta River	**Zgh 1 (1) ***	above 64	PEN-AMP-AMC-FEP-CTX-LEX-CFM-DOR-MEM-GEN-KAN-STR-TET	IPM	*mcr-1; mcr-2; bla$_{NDM}$; bla$_{IPM}$*
		Zgh 1 (2) *	32	PEN-AMC-LEX	CTX-CFM-GEN-KAN-STR	*mcr-1*
		Zgh 1 (3) *	above 64	PEN-AMC-FEP-CTX-LEX-CFM-DOR-MEM-GEN-KAN-STR	IPM-CIP	*mcr-1; mcr-2*

		Zgh 2 (1) *	8	PEN-LEX	GEN-KAN-STR	*mcr-1*
		Zgh 2 (2) *	8	PEN-AMP-SXT	IPM-TET	*mcr-1.1; mcr-4; mdfA*
		Zgh 2 (3) *	above 64	PEN-AMP-LEX	TET-IPM	*mcr-1; mcr-2; mcr-4; bla$_{SHV}$*
		Zgh 3 (3) *	above 64	PEN-AMC-LEX	AMP-KAN-STR	*mcr-1*
		Zgh 3 (2) *	64	PEN-AMP	KAN-STR	*mcr-1*
	Ain El Tine River	**ATM 1***	8	PEN-AMP-AMC-FEP-LEX-CFM-KAN-STR-TET-CIP-NOR-SXT-CHL	GEN	*mcr-1, bla$_{TEM}$; bla$_{SHV}$; bla$_{KPC}$;* Class 1 Integron
		ATM 1 (2) *	16	PEN-LEX-CHL	KAN-CIP	*mcr-1; bla$_{TEM}$; bla$_{SHV}$; bla$_{CTX-M}$;* Class 1 Integron
		ATM 1 (3) *	16	PEN-AMP-TET		*mcr-1;* Class 1 Integron
		ATM 2 (1) *	8	PEN-AMP-AMC-FEP-CTX-LEX-CFM-MEM-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	DOR	*mcr-1; bla$_{TEM-1B}$; bla$_{SHV}$; bla$_{CTX-M-15}$; bla$_{ACT-14}$; aadA8b; aph3''-Ib; aph3'-Ia; aph6-Id; dfrA14; floR; fosA; mdfA; mphA; oqxA; oqxB; qnrS1; sul2; tetA;* Class 1 Integron
		ATM 2 (2) *	16	PEN-AMC-FEP-LEX-CFM-KAN-STR-TET-CIP-SXT-CHL	GEN-NOR	*mcr-1.1; mcr-8; mdfA*
		ATM 2 (3) *	32	PEN-AMP-AMC-FEP-CTX-LEX-CFM-KAN-STR-TET-CIP-NOR-SXT-CHL	DOR	*mcr-1; mcr-2; mcr-8; bla$_{TEM}$; bla$_{SHV}$; bla$_{CTX-M}$*
		ATM 3 (1) *	16	PEN-AMP-FEP-CTX-LEX-KAN-STR-TET-CHL	CFM-CIP-NOR	*mcr-1; bla$_{TEM}$; bla$_{SHV}$; bla$_{CTX-M}$;* Class 1 Integron
		ATM 3 (2) *	16	PEN-AMP-AMC-CTX-LEX-CFM-DOR-MEM-KAN-STR-TET-SXT-CHL	IPM-CIP-NOR	*mcr-1; bla$_{TEM}$; bla$_{CTX-M}$;* Class 1 Integron
		ATM 3 (3) *	16	PEN-AMP-AMC-CTX-LEX-CFM-KAN-STR-TET-CIP-NOR-SXT-CHL	FEP-MEM	*mcr-1; bla$_{TEM}$;* Class 1 Integron

	Oustoune River	**Ost 2 (1) ***	8	PEN-AMP-AMC	FEP-DOR-MEM-KAN-STR	*mcr-1*
		Ost 2 (2) *	8	PEN-AMP	CTX-DOR-MEM-KAN-STR-CHL	*mcr-1.1; bla_{TEM-1B}; $bla_{CTX-M-14}$;aac3-IId; aadA1; aadA2; aph3"-Ib; aph3'-Ia; aph6-Id; dfrA14; dfrA1; ermB; floR; fosA3; mdfA; mphA; sul1; sul3; tetA*
		Ost 2 (3) *	8	PEN-AMP-AMC-STR	FEP-CFM-KAN-CIP	*mcr-1*
		Ost 3 (1) *	8	PEN-LEX	STR	*mcr-1;* Class 1 Integron
	Heker El Daher River	**HD 2 (2) ***	8	PEN-AMP		*mcr-1; bla_{SHV}; bla_{CTX-M}*
		HD 2 (3)	4	PEN		*mcr-1; bla_{SHV};* Class 1 Integron
		HD 3 (1) *	4	PEN-AMP-AMC-FEP-CTX-LEX-CFM-STR-TET-SXT		*mcr-1; bla_{TEM}; bla_{SHV}; bla_{CTX-M};* Class 1 Integron
		HD 3 (2) *	4	PEN-AMP-AMC-FEP-CTX-LEX-CFM-STR-TET-SXT	IPM	*mcr-1;mcr-2; bla_{TEM}; $bla_{CTX-M-15}$; bla_{DHA-1}; aph3')-Ib; aph6-Id; dfrA14; dfrA17; mdfA; mphA; qnrB4; qnrS1; sul1; sul2; tetA;* Class 1 Integron
		HD 3 (3) *	4	PEN-AMP-AMC-CTX-LEX-CFM-STR-TET-NOR-SXT	FEP-NOR	*mcr-1; bla_{TEM}; bla_{SHV}; bla_{CTX-M};* Class 1 Integron
	Bcharre River	**Bch 3 ***	8	PEN-AMP-AMC-FEP-CTX-LEX-CFM-KAN-STR-TET-CIP-NOR-SXT-CHL	MEM	*mcr-1.1; mcr-2; bla_{TEM-1B}; bla_{SHV}; bla_{CTX-M} ;aac3-IId; aadA1; aph3"-Ib; aph3'-Ia; aph6-Id; dfrA14; dfrA1; erm42; floR; fosA3; mdfA; mphA; sul1; tetA*

	Tannouri ne River	**Tan 2 (2) ***	8	PEN-AMP-AMC-CTX-LEX-CFM-GEN-KAN-STR-TET-SXT-CHL	IPM	*mcr-1; bla$_{TEM}$; bla$_{CTX-M}$*, Class 1 Integron
	Wadi Khalid River	**WK 1 (2) ***	4	PEN-AMP-AMC-CTX-LEX-CFM-STR-TET-SXT-CHL	GEN	*mcr-1;mcr-2; mcr-6; bla$_{TEM}$; bla$_{SHV}$; bla$_{CTX-M}$;* Class 1 Integron
		WK 1 (3) *	4	PEN-AMP-AMC-CTX-LEX-CFM-GEN-STR-TET-CIP-SXT-CHL	IPM	*mcr-1; bla$_{CTX-M}$*
	Oyoun Al Samak River	**OS 2 (3) ***	8	PEN-AMP-AMC-FEP-LEX-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL	DOR-IPM	*mcr-1*
Beirut	Beirut Port	**BP 1 (1) ***	8	PEN-AMP	DOR	*mcr-1;* Class 1 Integron
		BP 1 (2)	4	PEN	STR	*mcr-1;* Class 1 Integron
		BP 3 (1) *	16	PEN-AMC-LEX-STR-TET	AMP	*mcr-1;* Class 1 Integron
		BP 3 (2) *	8	PEN-AMP-KAN-TET-CHL		*mcr-1; bla$_{SHV}$; bla$_{CTX-M}$;* Class 1 Integron
		BP 3 (3) *	8	PEN-AMP-KAN-TET-CHL		*mcr-1; bla$_{TEM}$;* Class 1 Integron
	Beirut River	**Beirut 1 (1) ***	4	PEN-AMP-AMC-FEP-LEX--GEN-KAN-STR-TET-SXT-CHL	CFM-CIP	*mcr-1; bla$_{TEM}$; bla$_{SHV}$;* Class 1 Integron
		Beirut 3 (2) *	8	PEN-AMP-AMC-FEP-CTX-LEX-CFM-GEN-KAN-STR-TET-SXT-CHL	IPM	*mcr-1; bla$_{TEM}$;* Class 1 Integron

CHAPTER V

DISCUSSION

Antimicrobial resistance is an urgent global crisis that affects the lives of millions in different parts of the world. The problem of AMR does not only pose a threat to humans but also has significant ramifications on food safety and social development. Humanity is being pushed back to the pre-antibiotic era, causing an endless cycle of suffering and morbidity [182]. With the exponential increase of multidrug-resistant pathogens, colistin, a last-resort antibiotic, was reintroduced to the clinical practice [41]. Nevertheless, colistin was also extensively utilized in animal farming; this abuse and misuse of colistin led to the emergence of colistin-resistant organisms. Resistance to colistin was thought to be chromosomal [21]; however, recently, a mobile plasmid-borne mobile colistin-resistance gene, *mcr-1*, was shown to be horizontally transmitted between bacterial progeny [30].

Growing evidence suggests that environmental samples, mainly the aquatic environment, acts as a reservoir for antibiotic-resistant organisms and genes [167]. Water resources are ubiquitous with bacteriophages, plasmids, and integrons, which play a pivotal role in transmitting resistance genes through horizontal gene transfer[163]. Consequently, the United Nations Environment Program (UNEP) recognized environmental AMR as one of the top six emerging issues of concern today [183]. The prevalence of antibiotic-resistant bacteria in water resources is mainly due to anthropogenic wastes from hospitals and municipal effluents and agricultural pollutants [166]. The release of antibiotics or their metabolites from wastewater into the aquatic environment selectively allows the

development of antibiotic-resistant microbes' generations [162, 163]. Recently, the mobile colistin resistance gene (*mcr*) has been detected in river samples collected from China, Switzerland, and Italy. Given the significant role, water plays in the food chain, natural circulation, agricultural practices, and recreational actives, the detection of such genes poses an essential risk for the direct and indirect transmission of this gene to humans [166].

Lebanon, a small middle eastern country, with plentiful water resources, suffers drastically from post-civil war feeble infrastructure and wastewater management issues. Above that, Lebanon's underdeveloped policies regarding the use of antibiotics in animals [124] and humans have threatened freshwater resources with antibiotic-resistance gene contamination and antibiotic residues. All these factors amalgamate and lead to the high dissemination of the *mcr-1* gene in different niches in Lebanon; irrigation and seawater, pre-harvest poultry and livestock farms, and in Syrian refugee camps [144, 156-159]. Therefore, it became a necessity to investigate the prevalence of the *mcr-1* gene in river water in Lebanon. Our study focused on the detection of the *mcr-1* gene in 14 perennial rivers and a water spring across five districts in Lebanon, which are usually affected by agricultural and anthropogenic wastes, making them a suitable environment for the detection of the *mcr* gene. This study concentrated on the detection of *mcr-1* in *E. coli*, which is an indicator of fecal contamination in water resources [184].

Samples were collected from three sites in triplicates along the rivers yielding one hundred thirty-five freshwater samples, 98.3% showed growth of colistin-resistant *E. coli* when filtered and enriched on *E. coli* selective media supplemented with colistin. The highest counts of colistin-resistant *E. coli* were reported in Beirut river, 10^4 CFU/100, which was not surprising given the magnitude of sewage water discharge into the river in

this region. A total of 116 colistin-resistant *E. coli* were recovered (retrieval rate 60%) and screened for the detection of *mcr-1* to *mcr-8*, extended-spectrum *β*-lactamase genes (ESBLs), carbapenem resistance genes, and Class 1 Integrons by PCR analysis. Also, the isolates were subjected to colistin minimum inhibitory concentration (MIC) assay, and phenotypic antibiotic resistance profiling as per the Clinical and Laboratory Standards Institute (CLSI) guidelines [172, 173]. All the colistin-resistant isolates were positive for the *mcr-1* gene, highlighting the wide geographical dissemination of this genetic marker in different districts in Lebanon. The amplified *mcr-1* gene products were further purified for 15.5% of the samples and confirmed by commercial sequencing, which showed 100% homology to the reported *mcr-1* in the literature. Other *mcr* gene variants were also detected in 16%, 9%,2%,1% and 8% of the isolates for *mcr-2, mcr-3, mcr-4, mcr*-6 and *mcr*-8, respectively. However, these genes were not confirmed by sequencing.

Antimicrobial analysis of the *mcr-1* positive isolates indicated a colistin's MIC ≥ 4 μg/ml, ranging between 4 μg/ml to 64 μg/ml. Most samples had a MIC of 8 μg/ml, which is higher than the daily recommended dosage of colistin. Also, the antimicrobial properties of the 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. It is worth mentioning that some isolates were colistin and carbapenem-resistant at the same time. These findings are highly problematic since colistin is given as salvage therapy when cephalosporins and carbapenems fail [131]. Also, 94% of the isolates were multidrug-resistant. This high prevalence of MDR *E. coli*, harboring the *mcr* genes, emphasizes the magnitude of

environmental breaches and extensive usage of antibiotics in humans and animals in Lebanon. Similarly, it highlights that these organisms might be transmitted to other niches since rivers are used for irrigation and other recreational activities in Lebanon. Molecular analysis for the antimicrobial-resistant genes determined that the majority of the samples co-harbored the extended-spectrum beta-lactamase genes (ESBL genes), bla_{TEM}, bla_{SHV}, $_{blaCTX\text{-}M}$, eliciting resistance against beta-lactam antibiotics. The most prevalent gene was bla_{TEM}, 48%. These findings corroborate previous results, showing the high prevalence of the bla_{TEM}, in ESBL-producing Gram-negative bacilli in Lebanon [143]. bla_{SHV} and $bla_{CTX\text{-}M}$ were also detected in 42%, 36% of the isolates, respectively. Additionally, 10 % of the isolates were carbapenem-resistant, harboring bla_{IMP}, bla_{KPC} bla_{NDM}, or $bla_{OXA\text{-}48}$. The carbapenem-resistant genes were previously documented in Lebanon in irrigation water[156] and camp drinking and sewage water [158]. Consequently, this stresses the extent of contamination of rivers with antibiotic resistance genes and the urgent need for intervention. The Class 1 Integron gene was reported in 62% of the isolates, which accentuates the ability of these bacterial isolates to evolve, acquire, and express different resistance genes [185, 186].

The *mcr-1* gene was confirmed to be plasmid-borne, whereby *mcr-1* positive isolates from different geographic locations were successfully transformed into chemically competent *E. coli* JM109 (QIAGEN) by the heat-shock method. All the harvested transformants were *mcr-1* positive and had a colistin's MIC ≥ 2 μg/mL. None of the transformants were resistant to carbapenems indicating that these genes were not plasmid-borne. Further analysis of the plasmid types determined the detection of a variety of plasmid types IncI1 α, IncI2, IncX1, IncX4-FIBM, IncFIBM, IncFIB, IncFII, IncX4,

IncFIIK-FII, IncFIIK, IncFIBKN, IncFII IncX4-FII illustrating the genetic diversity of the plasmids. It is worth mentioning that IncI2, IncI1 α, and IncX4 plasmid are responsible for the worldwide dissemination of the *mcr-1* gene globally [40]

Box PCR fingerprint relatedness analysis demonstrated that the 116 *mcr-1*-positive-*E. coli* isolates belonged to 63 genotypes. Approximately 54% of the isolates were genotypically different from each other, highlighting the diversity of the isolates detected. Three *mcr-1* positive isolates recovered from Zgharta, Faraya, and Bcharre river showed a low fitness cost and persisted in the water matrix for more than 127 days without losing the *mcr-1* gene. The persistence of this gene for 127 days is problematic since it indicates the potential to be transmitted to different geographic locations until it reaches the sea and, in turn, affects neighboring countries[156, 157].

This is the first report of *mcr-1*-positive-*E.coli* in Lebanese river water as well as the MENA region. The prevalence of the *mcr-1* gene reported is among the highest worldwide, urging us to implement policies to restrict the unhindered use of colistin. The detection of the *mcr-1* in environmental water samples poses a significant risk to Lebanon and neighboring countries. Adopting a one health approach, strengthening antimicrobial stewardship, and banning colistin's utilization in animal farming becomes a necessity, to curtail the unfathomable effects of antibiotic resistance on humanity.

CHAPTER VI

CONCLUSION

Colistin, a last resort antibiotic, was reconsidered in the clinical context for its efficacy in treating complicated infections; however, the dissemination of the *mcr-1* gene globally jeopardized its effectiveness. Nevertheless, countries with economic distress and deliberated infrastructure fail to comprehensively address the dissemination of the *mcr* genes in humans, animals, and environmental samples, like what is happening in countries of the MENA region and especially Lebanon. The lack of initiative to study the dissemination of the *mcr* genes in these counties can make them a vital reservoir for transmitting this gene to different parts of the world. In Lebanon, the *mcr-1* gene was detected in humans, animals, and environmental samples, highlighting the high prevalence of this gene in a country with weak antimicrobial stewardship and unrestricted use of colistin in the agricultural sector. This study corroborates the previous findings and documents the high prevalence of *mcr-1* and other antibiotic-resistant genes in *E. coli* (n=116) in freshwater resources in Lebanon. Our data suggest that anthropogenic and industrial actives near these water resources play a significant role in contaminating these rich aquatic environments with antibiotic-resistant organisms and genes. Therefore, immediate intervention is needed to curtail the unfathomable threat of disseminating *mcr-1* in the aquatic environment. Thus, tackling colistin resistance requires the adoption of a one health approach, along with utilizing and implementing policies that restrict the use of colistin in animal farming for growth promotion.

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