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THE OCCURRENCE OF PLASMID MEDIATED COLISTIN RESISTANCE GENE, *MCR-1*, IN MULTI-DRUG RESISTANT *ESCHERICHIA COLI* IN FRESH AND READY TO EAT VEGETABLES IN LEBANESE WHOLESALE MARKETS

by
MONA WALID ZEIDAN

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Nutrition and Food Sciences
of the Faculty of Agricultural and Food Sciences
at the American University of Beirut

Beirut, Lebanon
December 2020

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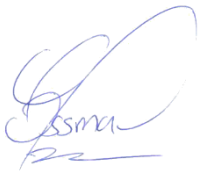
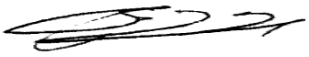


Dr. Issmat Kassem, Associate Professor — Advisor
Nutrition and Food Sciences



Dr. Samer Kharroubi, Associate Professor — Member of Committee
Nutrition and Food Sciences

Dr. Hadi Jaafar, Associate Professor — Member of Committee
Agriculture

Date of thesis defense: January 11, 2021.

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ACKNOWLEDGMENTS

"*Hard work pays off. This thesis is the fruit of hard work and dedication."*

First and foremost, I would like to thank God, for he gave me more than I deserved.

I would like to express my deepest appreciation to my advisor, **Dr. Issmat Kassem**, you taught me never to give up, to work hard and have confidence in what I am doing. This would not have been possible without your guidance and continuous support.

Thanks to my committee members **Dr. Samer Kharroubi** and **Dr. Hadi Jaafar**.

I thank Faculty of agriculture and Food sciences and **Dean Mohtar** for the persistent support throughout my graduate studies.

A special thanks to my lab mates and colleagues for helping me with this research. Mrs. Tsolaire Sourenian, you were beside me through it all and you taught me a lot. Ms. Jouman Hassan, Ms. Zeinab Hmade, and Mr. Abdallah Alhajj Sulaiman you supported me and helped me a lot.

Thank you Ms. Hanin Chafei, Ms. Sara Grazza, and Ms. Sara El Khechen, I am grateful to have worked with you.

My friends, you have always been beside me and supported me through the best and worst. You always managed to make me feel special. Thank you for always being there for me.

My sister and my brother, my life is nothing without you. I am extremely grateful for your support.

My Parents, you are my backbone.
Thank you to my father and mother for always being by my side. For the times you went with me for sample collection, for every time you heard me complaining, for every single thing you made to help me give the best of me, for the things you did to keep me and my siblings smiling, Thank you. This thesis is dedicated to you.

ABSTRACT

OF THE THESIS OF

Mona Walid Zeidan for Master of Science
Major: Food Safety

Title: The Occurrence of Plasmid Mediated Colistin Resistance Gene, *mcr-1*, in multi-drug Resistant *Escherichia coli* in fresh and Ready to eat vegetables in Lebanese Wholesale Markets.

Antimicrobial resistance is increasing worldwide. Colistin, also known as polymyxin E, is a last resort antibiotic that is used to treat complicated Gram-negative bacterial infections when other antibiotics fail. Colistin resistance has been considered to be chromosomally mediated, until the discovery of the mobile colistin resistance gene by *Liu et al*. in 2016. After that, the mobile colistin resistance gene, *mcr-1*, has been detected worldwide in more than 40 countries in different niches. Hence, the aim of this study is to determine the presence and dissemination of *mcr-1* in *Escherichia coli* in fresh vegetables in different Lebanese wholesale markets. Results of this study show that colistin resistant *E. coli* were detected in 36.2% of the 105 vegetable samples collected from different wholesale markets and 24 *mcr-1* positive *E. coli* were isolated. The minimum inhibitory concentration of colistin ranged between 4 and >64µg/ml. The majority (54.17%) of the isolates detected were multi-drug resistant, exhibiting resistance to penicillin (100%), ampicillin (58%), amoxicillin + clavulanic acid (96%), cefepime (4%), cefotaxime (25%), cephalexin (75%), cefixime (25%), doripenem (8%), meropenem (4%), imipenem (0%), gentamicin (0%), streptomycin (25%), tetracycline (33%), ciprofloxacin (4%), norfloxacin (8%), trimethoprim-sulfamethoxazole (29%), and chloramphenicol (17%). Various plasmid types have been detected in the isolates including IncI1α, IncF, IncI1γ, IncX1, IncFII, IncFIIS, IncW, IncA/C, and IncFIIK. BOX-PCR fingerprinting showed that the 24 *mcr-1* positive *E. coli* isolates belonged to 18 different genotypes. This study is the first in Lebanon to detect the presence of *mcr-1* in *E. coli* in fresh vegetables in wholesale markets. Our findings highlight an urgent need to devise immediate measures to control the proliferation of colistin resistance and *mcr-1* in Lebanon.

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ABBREVIATIONS

et al.	*et allii (and others*)
TBX	Tryptone Bile X-glucuronide
WHO	World Health Organization
CLSI	Clinical and Laboratory standards Institute
PCR	Polymerase Chain Reaction
E. coli	*Escherichia coli*
mcr-1	*Mobile colistin resistance gene*
AMR	Antimicrobial resistance
MIC	Minimum inhibitory concentration
mL	Milliliters
µL	Microliters
µg	Microgram
rpm	revolutions per minute
bp	Base pairs
V	Volts
MDR	Multidrug resistant
MENA	Middle East and North Africa

To
My Beloved Family

CHAPTER I

INTRODUCTION

A. Vegetable Contamination and Antimicrobial Resistance

Fresh fruits and vegetables are important components of a healthy and balanced diet (1). However, the risk of microbiological contamination of vegetables is a major concern and can occur across the food chain; from farm to fork (2). Fresh produce are often consumed raw, putting consumers at risk of infection by contaminating organisms (1). Contamination of fresh vegetables occur through various routes including the use of unsafe water as a source for irrigation, the inappropriate use of fertilizers or manure, and the intrusion of livestock or wild animals in field (3). Contamination can also occur due to transportation, improper storage conditions, or poor handling practices along the food chain (2). The differences in the contamination levels of vegetables can be affected by farm location, weather or climatic conditions, and types of vegetable crops (2). Among the most common pathogens that contaminate vegetables is *Escherichia coli* (3).

Antibiotics are added to agricultural fields worldwide through wastewater irrigation or manure application, resulting in antibiotic contamination. This indicates that the higher the antibiotic concentration supplied by wastewater, irrigation water, or animal manure, the greater the quantities translocated to different crop tissues (4). Therefore, the development of resistance to most available antibiotics is increasing worldwide (5). Most studies report that resistance to different antibiotics, including carbapenems is increasing. When other antibiotics fail to treat the bacterial infection, colistin becomes one of the available last resort antibiotics against multidrug resistant

gram-negative bacteria (5). Colistin, also known as polymyxin E, was introduced into clinical use in the 1950s, and it has been used for decades in veterinary medicine to treat animal intestinal infections. However; due to its toxicity, the use of colistin has been limited to treating severe infections in humans caused by multi-drug resistant gram-negative bacteria, thus it was considered to be a last resort antibiotic (6). The use of colistin has also been challenged by the emergence of resistance, that is, plasmid-borne mobile colistin resistance or *mcr-1*. *mcr-1* gene is a major threat in the environment and certain studies reported on *mcr-1*, mainly in *E. coli* from livestock, meat, humans, river water or vegetables from Asia and Europe, and other continents (7). Hence, we aimed here to study the emergence colistin resistant *E. coli* in vegetables.

B. Mechanism of Colistin Resistance via mcr

The mechanism by which colistin exerts its effect and kills gram-negative bacteria relies on the disruption of the membrane permeability through polar, hydrophobic and electrostatic interactions. Electrostatic interaction takes place between the positively charged polymyxin (colistin) and the negatively charged phosphate group of lipid A on LPS (lipopolysaccharide) of the outer bacterial membrane. Therefore, colistin resistance is induced by chromosomally mediated modification of the lipid A portion of lipopolysaccharide (LPS) in the outer membrane, leading to reduced affinity for polymyxins (8). However recently, modification of the lipid A portion of lipopolysaccharide is thought to be mediated by the mobile colistin resistance gene, which encodes a phosphatidyl-ethanolamine transferase, allowing the modification of the lipid A component of the LPS, leading to resistance (9). *mcr-1* is usually carried on a conjugative plasmid which facilitates its transferability between bacteria.

C. Detection of *E. coli* in Vegetables in Different Countries

Several studies showed the presence of *E. coli* in vegetables. For example, a study performed in Iran included a total of 116 samples of fresh-cut vegetables, ready-to-eat salads, and mung bean and wheat sprouts, and *E. coli* was detected in 3 out of 32 fresh-cut vegetable samples (9.4%) and in 6 out of 20 ready-to-eat salad samples (30%) (10). Another study took place in Delhi, India where 150 fruits and vegetable samples were collected and *E. coli* was isolated from 26 (17.4%) of the samples. Cefotaxime (third generation cephalosporin) resistance was detected in 7 (26%) isolates of *E. coli.* Another study in India involved a total of 120 samples of salad vegetables, fruits and sprouts, collected from street vendors. *E. coli* was detected in 23 vegetables (31.9%) and 16 sprouts (66.7%) (12). However, studies from Korea, Germany, and United States have reported a lower rate of *E. coli* contamination ranging from 6% to 14% (11).

Similarly, *E. coli* was found in 16 samples out of 32 (50.0%) minimally processed vegetables from different supermarkets in the city of Brasilia, DF, Brazil (13). In addition, a total of 410 raw vegetable samples were collected and processed from various open air markets and supermarkets in Luzon, Philippines. Out of these 23 (5.61%) were positive for *E. coli* (14). Furthermore, a study in Jakarta involved 65 salad vegetables and 63 fruits. Among these, 76 suspected *E. coli* colonies were isolated (15). Most of *E. coli* isolates showed resistance to one or more antimicrobial agents, indicating that most of *E. coli* recovered from the salad vegetable and fruit samples in Jakarta were antibiotic resistant (15). In Italy, 5% of minimally processed vegetables investigated were contaminated by *E. coli*. Moreover, In Canada, Bohaychuk *et al*. assessed the prevalence of *E.coli* in fresh vegetables produced in Alberta and found this organism in 8.2% of lettuce, spinach and carrots samples (16).

In Mexico, some non-O157 STEC strains were identified in raw foods, such as

vegetable salads (17). Similarly, 91 samples of raw vegetables and sprouted seeds were collected from the retail market in the Czech Republic. *E. coli* was detected in 24 (26.4%) out of 91 samples. In this study, resistance to more than one group of antibiotics was found in three isolates (18). Furthermore, a study performed in Belgium involved 8 lettuce farms where 738 samples were collected, including lettuce seedlings, soil, irrigation water, and lettuce leaves. From these samples, 473 isolates of *E. coli* were isolated. 11.4% of 473 isolates were found to be resistant to one or more of the antimicrobial agents tested (19). In addition, in Zambia, a study included 160 samples. The analysis included different groups of exported fresh organic vegetables: mixed vegetables (baby corn, beans, carrots, chili Fresno, mangetout, peas and petty pan) and green beans. *E. coli* was detected in both mixed vegetables and green beans. The overall incidence for *E. coli* was 40% (20). Furthermore, vegetables in Canada were shown to contain generic *E. coli* that was more often found in leafy herbs (0.93%), followed by leafy vegetables (0.28%), cantaloupes (0.10%) and green onions (0.06%) (21).

In Minnesota (USA), a total of 476 and 129 produce samples were collected from 32 organic and 8 conventional farms, respectively. *E. coli* was isolated from 8% of all fruits and vegetables analyzed. However, organic lettuce had the largest prevalence of *E. coli* (22.4%) compared with other produce types (22). Furthermore, in a study conducted in United States of America, results showed that 44 samples (10.65%) out of 414 were positive for *E. coli* (23). In Quito, Ecuador, a total of 90 samples were processed. Antibiotic-resistant *E. coli* was isolated only in leaf lettuce, alfalfa and parsley/cilantro (24). Furthermore, 33 *E. coli* positive samples (12.64%) were detected among 261 fresh vegetables and fruits analyzed in Turkey, with contamination being highest in green leaf lettuce (80%) (25). In addition, a study in Portugal included 144 products, including 74 vegetables and 70 fruits, 333 Gram negative antibiotic resistant

isolates were obtained; 3% of which was *E. coli.* Two *E. coli* isolates were considered to be multidrug resistant (26).

Another study was conducted in Saudi Arabia where A total of 150 lettuce samples were collected. Forty five lettuce head samples were analyzed for the contamination of inner and outer leaves. The *E. coli* was detected in 5 out of 45 samples tested for this bacterium (about 11%). Fifteen isolates of *E. coli* were retrieved from 5 positive samples (27). In Jordan, a total of 150 fresh leafy green vegetable samples were collected from different markets in Amman and Al-Zarqa. Results showed that 40.6% of fresh leafy green vegetable samples were found contaminated with *E. coli*. The *E. coli* isolates (27.8%) were multidrug resistant (28). In addition, results of a study performed in Morocco on 224 vegetable samples collected from restaurants in Fez city included 178 raw salads, 23 cooked salads and 23 fruits salads showed that *E. coli* was found in 93.33% of raw salads, 87.5% of cooked unsatisfactory salads and in all fruits salads (29). Furthermore, a study was done in Lebanon, where vegetables from the Beqaa region were collected. These vegetables were known to be irrigated by water from the Litani river. Specifically, a total of 33 vegetable samples collected from Barelias and irrigated from Litani River. About 36.36% of the samples were positive for *E. coli*. For samples from Jib-Janine, 6.66% were positive for *E. coli*. *E. coli* was not detected in vegetables collected from Kaaroun. The study revealed that the major cause for contamination of vegetables in Bekaa is the irrigation with wastewater where the transported pathogens from wastewater may survive in soil and crops, which will, in turn, be transported to consumers and potentially cause disease (30). Another study in Lebanon detected the presence of multidrug resistant *E. coli* in fresh vegetables, in which 32% of 60 isolates were MDR (32).

D. Colistin Resistance and *mcr-1* in *E. coli* in Vegetables

Few studies have been done on *mcr-1* in fresh vegetables and crops. However, *mcr-1* has been detected in vegetable samples from four countries, China and South Korea in Asia, and Switzerland and Portugal in Europe.

Several studies were done in China. In one study, a total of 538 fresh vegetable samples were collected from 53 supermarkets from 9 provinces in China. Twenty four *mcr-1* positive isolates were retrieved from 19 fresh vegetable samples and 23 were identified as *E. coli* (33). A similar study included 916 fresh vegetable samples that were collected from 41 farmers' markets and 12 supermarkets in Guangzhou, China. A total of 244 Extended Spectrum Beta-Lactamase (ESBL) producing Enterobacteriaceae were recovered from 216 samples. Six out of the 244 isolates were positive for *mcr-1* (4 of which were *E. coli*). Also 26 *E. coli* isolates were obtained from 916 samples and 3 of them were *mcr-1* positive (34). The contamination rate of vegetables with *mcr* positive Enterobacteriaceae in China might have been increasing in recent years. Recently, in 620 food samples surveyed in Shenzhen, vegetable products were shown to be contaminated with *mcr-1*-carrying bacteria, with a rate 7% (35). In addition, *E. coli* isolates were also detected among 712 vegetable samples collected from supermarkets from 10 provinces in China. The two isolates from leaf rape and spinach in two supermarkets of Shandong province, respectively, carried both blaNDM-5/9 and *mcr-1* (36). Similar study was conducted in South Korea; however, it involved vegetables (1324 vegetables and fruits) as well as fecal samples (150 fecal samples from healthy animals). The results of this study showed that seven colistin resistant strains were isolated from one lettuce (0.076%). All of the isolates were identified as *E. coli*. It was also noticed that colistin was widely used in food animals for infection control and that the manure of animals contaminated with *mcr-1* could have been reused as organic

fertilizer on agricultural farms in South Korea (37). Furthermore, a study was conducted in Switzerland on 60 ESBL-producing Enterobacteriaceae isolated from 42 imported vegetable samples. The results of this study showed the presence of the *mcr-1* gene in 2 out of the 60 vegetable strains (imported from Thailand and Vietnam). All these strains were *E. coli* (31). Similarly, a study was done in India where a total of 110 food samples were collected from 22 sources, including 63 vegetable samples and 3 fruits. The results obtained showed that 51/110 samples (46.4%) contained colistin resistant organisms (23 vegetables and 2 fruits). Among these 51 positive samples, 71 bacterial isolates were identified, 11 of these are *E. coli*. Three of these *E. coli* isolates harbored *mcr-1* gene (1 mutton and 2 poultry meat samples) and the vegetables and fruits didn't contain *mcr-1* positive *E. coli* (38). Another study reported the presence of *mcr-1* in an *E. coli* isolate recovered from lettuce produced and marketed in Portugal (39).

A study performed on 1000 STEC isolates collected from a major produce-production area in California, USA showed that all the isolates were negative for *mcr* genes (6).

mcr-1 was also detected in vegetables in several countries. Therefore, appropriate measures are needed in-order to reduce the burden of antibiotic resistance in the environment. Those measures should include reducing the use of antibiotics mainly as growth promoters, improving the water status especially that used for drinking and irrigation, applying new strategies to treat wastewaters and others (40). Effective control measures should be implemented also in production facilities and subsequent processes to enhance the microbiological quality of fresh produce (41) and to ensure the production of safe, ready to eat vegetables to protect the health of consumers.

Lebanon has been suffering from wide-spread pollution due to the lack of

appropriate waste water treatment plants and due to a debilitated infrastructure. There is evidence contaminated irrigation water harbored *mcr-1*-positive *E. coli* in Lebanon. Given that raw vegetables are highly consumed in Lebanon, it became necessary to investigate the contamination of produce with *mcr-1*- carrying *E. coli*. The overall objective of this thesis was to investigate the occurrence and properties of colistin-resistant and mcr-1-positive *E. coli* on fresh produce.

CHAPTER II

MATERIALS AND METHODS

A. Sample Collection

Four types of fresh vegetable samples, Lettuce, Radish, Cucumber and Green onion, were collected from 24 wholesale markets from different regions across Lebanon, including Beirut, Mount Lebanon, South, Beqaa and North from November 2019 to November 2020. Specifically, a total of one hundred and five vegetable samples were collected in sterile bags and were transported to the laboratory in a cold box. Samples were processed within 18-24 hours of collection.

B. Isolation of Bacteria

Twenty-five grams of each sample were weighed and 225 ml of sterile Phosphate buffered saline (PBS) were added. After mixing, 100 ml of each mixture was filtered through 0.22-µm Millipore® membranes. The membranes were placed on an *Escherichia coli* selective medium (RAPID' *E. coli* 2 agar; Bio-Rad) supplemented with 4µg/ml of colistin (48).The samples were incubated at 44°C for 18-24 hours. Suspected (purple) *E. coli* colonies, 1 to 4 per sample, were purified. The purified colonies were stored in 1 ml Luria Bertani (LB) broth with 0.5 ml 80% glycerol and stored at -80°C for further analysis.

C. Polymerase Chain Reaction-Based Analysis

Genomic DNA was extracted from bacterial colonies using boil preparation. Bacterial colonies were suspended in 100 µl DNase free water and placed in a water

bath at 95°C for 15 minutes. The tubes were then centrifuged for 2 minutes at 13000 rpm. The supernatant was collected and transferred into sterile tubes. The tubes were then placed at -20°C for further analysis. The extracted genomic DNA was used as a template for the polymerase chain reaction analysis.

1. Detection of Mobile colistin Resistant Gene Using PCR

The extracted genomic DNA was used to screen for the presence of the mobile colistin resistant gene (*mcr-1*). The reaction was carried using two specific primers, the forward CLR5 primer (CLR5-F 5′-CGGTCAGTCCGTTTGTTC-3′) and the reverse CLR5-R primer (5′-CTTGGTCGGTCTGTA GGG-3'). The reaction mixture was prepared in sterile pcr tubes by adding 3 µl of genomic DNA to a mixture consisting of 12 µl of DNase free water, 4µl of Master Mix (5x FIREPol® Master Mix Ready to Load), and 0.5µl of each of the two primers, forward and reverse. The polymerase chain reaction consisted of 38 cycles, each cycle includes a denaturation step at 95°C for 1 minute, followed by annealing of the primers at 52°C for 45 seconds and finally an extension step at 72°C for 1 minute. A final extension step also took place at 72°C for 10 minutes. The expected size of the amplified gene product is 309 bp.

Following the polymerase chain reaction, detection of the *mcr-1* amplicon was done using gel electrophoresis. The amplified gene product was inoculated in 1% agarose gel, stained with ethidium bromide and subjected to electrophoresis for 35 minutes at 100V.

2. Box PCR-Fingerprinting

Box PCR was performed on all *mcr-1* positive *E. coli* isolates to determine their genetic profile and relatedness. The reaction was performed in 25 µl PCR tubes,

containing 3 µl DNA, 0.5 µl of the BOX-A1R primer, 4 µl Master Mix, and 17.5 µl of DNase free water (48). The PCR amplification reaction was carried in 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. Results were visualized and analyzed on 2% agarose gel stained with ethidium bromide and electrophoresed at 100V for 75 minutes.

3. Detection of other Antibiotic Resistance Genes

Screening for the presence of beta-lactamase encoding and carbapenem resistance genes was performed by PCR analysis. The genes included: bla_{TEM}, bla_{CTX-M}, bla_{SHV}, bla_{KPC}, bla_{IMP}, bla_{NDM}, and bla_{OXA-48}. Class 1 Integron genes were included in the analysis (Table 1) (48). 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. PCR reactions were placed in a thermocycler for 38 cycles. Then the corresponding gene amplicons were visualized by gel electrophoresis, in a 1% agarose gel stained with ethidium bromide and electrophoresed for 35 minutes at 100V.

Table 1. A list of the tested genes and the PCR conditions

Gene and Primers	**Denaturation**	**Annealing**	**Extension**	**Size**
mcr-1 gene CLR5-F (5′-CGGTCAGTCCGTTTGTTC-3′) and CLR5-R (5′-CTTGGTCGGTCTGTA GGG-3’)	95°C for 1min	56°C for 45 sec	72°C for 1 min	309 bp
bla-$_{CTX-M}$ Forward: ATGTGCAGYACCAGTAARGTKATGGC Reverse: TGGGTRAARTARGTSACCAGAAYCAGCGG	95°C for 1min	56°C for 45 sec	68°C for 1 min	529 bp

"Table 1 - *Continued*"

Gene and Primers	Denaturation	Annealing	Extension	Size
bla-$_{IMP}$ gene Forward (TGAGCAAGTTATCTGTATTC) Reverse: (TTAGTTGCTTGGTTTTGATG)	95°C for 1min	56°C for 45 sec	68°C for 1 min	232 bp
bla-$_{OXA-48}$ Forward: (GCTTGATCGCCCTCGATT) Reverse: (GATTTGCTCCGTGGCCGAAA)	95°C for 1min	56°C for 45 sec	68°C for 1 min	238 bp
bla-$_{NDM}$ Forward: GGTTTGGCGATCTGGTTTTC Reverse: CGGAATGGCTCATCACGATC	95°C for 1min	56°C for 45 sec	68°C for 1 min	521 bp
Intl1 gene Forward: CCTCCCGCACGATGATC Reverse: TCCACGCATCGTCAGGC	95°C for 30 sec	55°C for 45 sec	65°C for 1 min	250 bp
bla-$_{TEM}$ gene Forward: ACCAATGCTTAATCAGTGAG Reverse: GCGGAACCCCTATTTG	95°C for 1min	56°C for 45 sec	68°C for 1 min	963 bp
bla-$_{KPC}$ gene Forward: (CATTCAAGGGCTTTCTTGCTGC) Reverse: (ACGACGGCATAGTCATTTGC)	95°C for 1min	56°C for 45 sec	68°C for 1 min	498 bp
bla-$_{SHV}$ Forward: CACTCAAGGATGTATTGTG Reverse: TTAGCGTTGCCAGTGCTCG	95°C for 1min	56°C for 45 sec	68°C for 1 min	822 bp

D. Antibiotic Resistance Using the Disc Diffusion Assay

Disc Diffusion Sensitivity method was used to assess antibiotic resistance phenotypes of the *mcr-1 positive E. coli* isolates. Using Muller Hinton broth, the optical density of the samples was adjusted using a 0.5 McFarland standard. Using a sterile cotton swab, the samples were spread on Muller Hinton agar plates. Nineteen antibiotics were used including 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), and Erythromycin. Four antibiotic discs were added to each plate, and then they were incubated at 37°C for 18-24 hours. Erythromycin was used as a control due to the natural intrinsic resistance of *E. coli* to this antibiotic. Antibiotic resistance was

determined by measuring the diameter of zone of inhibition around each antibiotic disk and compared to Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility (EUCAST) standards (43, 44).

E. Colistin Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) was done in-order to determine the minimum concentration of colistin that inhibited the growth of the bacteria. MIC for colistin was determined for all *mcr-1* positive isolates. The wells of a 96-microtiter plate were inoculated with 100 µl of bacterial suspension adjusted at an optical density of 0.05 at OD600 and challenged with of prediluted colistin of different concentrations (1, 2, 4, 8, 16, 32, 64 µg/ml). The plates were the incubated for 18-24 hours at 37°C and analyzed with the micro-plate reader at $\lambda = 600$ nm.

F. Plasmid Transformation

Plasmid Extraction was done on nine *mcr-1*-positive-*E. coli* isolates using the QIAGEN® Plasmid Mini Kit 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. 50µl of competent cells were mixed with 10µl 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 90-second incubation on ice. After that, 940µl 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 removed. The pellet was re-suspended in the remaining 0.1 ml LB and then spread on a LB agar plates supplemented with 2

μg/ml of colistin. The Plates were incubated at 37° C for 18-24 hours. The transformants were harvested and further analyzed for *mcr-1* using PCR as well as checked for their MIC and resistance phenotypes (46, 48).

G. Commercial Sequencing

Commercial sequencing was performed on 6 *mcr-1* positive isolates to confirm that *mcr-1* was detected. The amplified *mcr-1* fragments were purified using the QIAquick® Gel Extraction Kit and QIAquick® PCR Purification Kit and sent out to be sequenced commercially.

H. Plasmid Typing

The PCR Based Replicon Typing Kit 2.0, PBRT kit, was used on 16 *mcr-1*-positive-*E. coli* isolates to determine the incompatibility plasmid types as per manufacturer's recommendations (47). The visualization of the amplified DNA product was done using a 2.5% agarose gel stained with ethidium bromide and electrophoresed for 45 minutes at 100 V.

CHAPTER III

RESULTS

A. Detection of:

1. mcr-1

E. coli was successfully recovered from 25 vegetable samples from different geographic locations; with a 65.79% retrieval. A total of thirty colistin-resistant *E. coli* colonies were collected (1-4 colonies per sample). These isolates were screened for the detection of the *mcr-1* using PCR.

The *mcr-1* was detected in 24 of the colistin-resistant *E. coli* strains. These twenty four *mcr-1* positive *E. coli* isolates belonged to different types of vegetables; 62.5% of these isolates are form lettuce, 20.84% from radish, 8.33% from green onion, and 8.33% from cucumber.

Table 2. Percentages of vegetable types positive for *mcr-1*-carrying *E. coli*

Type	**Counts Positive for colistin resistant *E. coli***	**Samples with isolates positive for *mcr-1***	**Isolates positive for *mcr-1***	**Percentage of isolates positive for *mcr-1* (%)**
Lettuce	15	11	15	62.5
Green Onion	7	2	2	8.33
Radish	11	4	5	20.84
Cucumber	5	2	2	8.33
Total	**38**	**19**	**24**	**100**

Six of the *mcr-1* positive colistin resistant isolates were purified and sent for commercial sequencing. All isolates showed 100% homology to previously reported *mcr-1* gene.

2. bla and Class I Integron *Genes*

Three of the *mcr-1* positive isolates were positive for bla_{CTX} which and one was positive for bla_{TEM} (4.34%). These genes are associated with cephalosporin resistance. None of the carbapenem resistance genes, bla_{OXA-48}, bla_{NDM}, bla_{IMP} *and* bla_{KPC}, was detected in the isolates. Class I Integron genes were also detected in two isolates (8.69%).

3. Box PCR Fingerprimting

All *mcr-1* positive *E. coli* isolates were subjected to BOX PCR analysis in-order to assess genetic diversity of the isolates. The isolates were highly diverse, belonging to 18 different genotypes.

B. Colistin Minimum Inhibitory Concentration

All *mcr-1* positive isolates had colistin MIC value greater than or equal to 4μg/ml (Fig. 1), while 37.5% and 12.5% of the isolates had an MIC ≥ 8 μg/ml and ≥ 32 μg/ml, respectively.

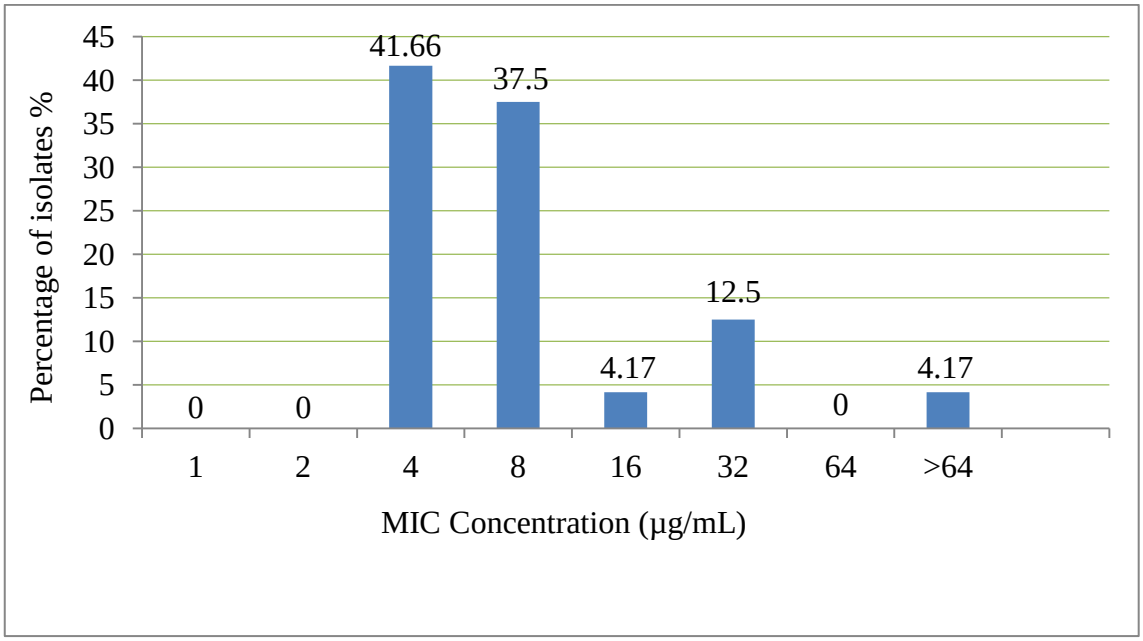

Fig. 1. The percentage of isolates with different colistin minimum inhibitory concentration

C. Antimicrobial Resistance

Thirteen isolates (54.17%) are considered multidrug resistant (resistance to ≥ 3 classes of antibiotics). Furthermore, 100% of the isolates were resistant to penicillin (PEN), 58% to ampicillin (AMP), 96% amoxicillin + clavulanic acid (AMC), 4% cefepime (FEP), 25% cefotaxime (CTX), 75% cephalexin (LEX), 25% cefixime (CFM), 8% doripenem (DOR), 4% meropenem (MEM), 25% streptomycin (STR), 33% tetracycline (TET), 4% ciprofloxacin (CIP), 8% norfloxacin (NOR), 29% trimethoprim-sulfamethoxazole (SXT), and 17% chloramphenicol (CHL) (Fig. 2; Table 4)..

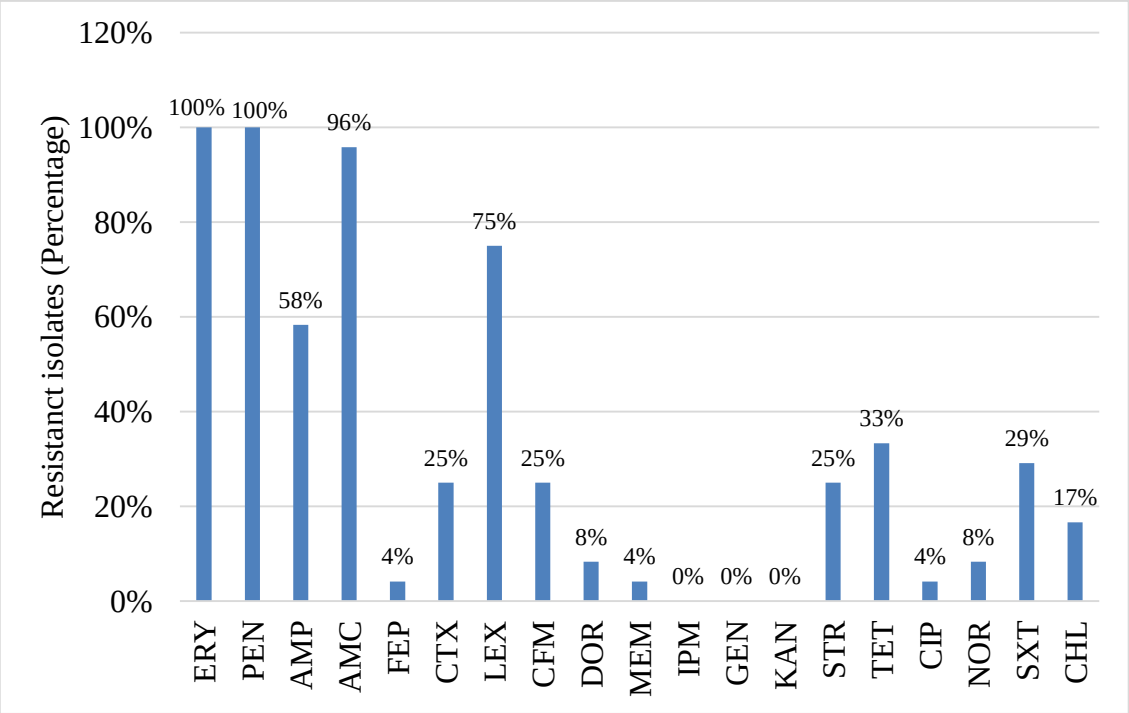

Fig. 2. The percentage of isolates resistant to different antibiotic

D. Plasmid Typing

Plasmid typing was done on 16 of 20 *E. coli* isolates. Several plasmid types were detected, including IncI1α, IncF, IncI1γ, IncX1, IncFII, IncFIIS, IncW, IncA/C, and IncFIIK (Table 3). The results indicated that the most prevalent plasmid among these isolates was IncFII followed by IncF.

Table 3. Plasmid Types Detected in Different *mcr-1* positive Isolates.

Location	**Isolate ID**	**Plasmid Type**
Al Jeyyeh	LJ4	IncFIIS
Madini Reyadeye	LB MR	IncFII, IncFIIS
Ferzol	LF YH	IncFIIS
Ferzol	RF YH	IncF, IncFII
Taalabaya	LT	IncI1α, IncI1γ, IncF, IncX1, IncFII
Taalabaya	GOT	Not assigned
Saida Souk Khodra	LS SK	IncFIIK

Madini Reyadeye	RMR	IncF, IncFIIS, IncA/C, IncW, IncFIIK
Saida Hesbi	RSH	IncFIIK, IncX1
Aley Souk Al Khodra	LA2 NA c (III)	IncF, IncFII
Aley Souk Al Khodra	CA SK1(III) a(1)	IncFII
Sin El Fil George Samaha	CSF/GS/res (III)	IncFII
Tripoli SK2	LTr SK2 (I)	untypable
Bhamdoun Dayaa Jemli Market	RBH (III)	IncF, IncFII
Kabrshmoun Market	LK	IncF, IncFIIK, IncFIIS
Hermel Ali	LHA (II)	IncF, IncFII

Table 4. Antimicrobial Resistance Profile and Colistin MIC of *mcr-1* positive *E. coli* isolates

Isolate Source	**Isolate Label**	**Colistin MIC (µg/ml)**	**Resistance Profile**	**Intermediate Resistance Profile**	**Genes Detected**
Al Jeyyeh	LJ1	32	PEN-AMC	STR	*mcr-1*
Al Jeyyeh	LJ3	8	PEN-AMC	STR	*mcr-1*
Al Jeyyeh	LJ4	8	PEN-AMC	STR	*mcr-1*
Al Jeyyeh	LJ5	32	PEN-AMC	STR	*mcr-1*
Madini Reyadeye	LB MR	8	PEN-AMP-AMC-LEX	STR	*mcr-1*
Ferzol	LF YH	8	PEN-AMP-AMC-LEX-TET	STR	*mcr-1*
Ferzol	RF YH	4	PEN-AMP-AMC-LEX-NOR-SXT	KAN-STR	*mcr-1*
Taalabaya	LT	16	PEN-AMP-AMC-LEX-STR-TET-CIP-NOR-SXT-CHL		*mcr-1*, bla_{T}, bla_{CT}, Class 1 integron
Taalabaya	GOT	8	PEN-AMP-AMC-LEX-TET	KAN-STR	*mcr-1*, Class 1 integron
Saida Souk Khodra	LS SK	4	PEN-AMP-AMC-LEX		*mcr-1*
Madini Reyadeye	RMR	4	PEN-AMP-AMC-LEX-DOR-STR-TET-SXT-CHL		*mcr-1*
Saida Hesbi	RSH	8	PEN-AMP-CTX-LEX-CFM-STR-TET-SXT-CHL	FEP	*mcr-1*, bla_{CT}
Aley Souk Al Khodra	LA2 NA c (III)	8	Pen-AMC		*mcr-1*
Aley Souk Al Khodra	CA SK1 (III) a(1)	4	PEN-AMC	KAN	*mcr-1*
Sin El Fil	CSF/GS/res (III)	32	PEN-AMP-AMC-CTX-LEX-CFM-CHL	FEP-MEM-KAN-STR	*mcr-1*

"Table 4 – *Continued*"

Isolate Source	**Isolate Label**	**Colistin MIC (µg/ml)**	**Resistance Profile**	**Intermediate Resistance Profile**	**Genes Detected**
Tripoli SK2	LTr SK2 (I)	4	PEN-AMC-LEX	AMP-KAN-STR	*mcr-1*
Bhamdoun Market	RBH (III)	4	PEN-AMC-LEX	IPM-KAN-STR	*mcr-1*, bla_C,
Kabrshmoun Market	LK	4	PEN-AMP-AMC-CTX-LEX-CFM-STR-TET-SXT	FEP	*mcr-1*, bla_C
Bhamdoun Market	RBH (II) A	4	PEN-AMC-LEX	KAN-STR	*mcr-1*
Hermel	LHA (II)	8	PEN-AMP-AMC-CTX-LEX-CFM-SXT	IPM-KAN-STR	*mcr-1*
Hermel	LHA (I)	4	PEN-AMP-AMC-CTX-LEX-CFM-SXT		*mcr-1*
Dbayeh	GOD 2B	>64	PEN-AMP-AMC-LEX-TET	KAN-STR	*mcr-1*
Dbayeh	LD	8	PEN-AMP-AMC-LEX-STR-TET	GEN-KAN	*mcr-1*
Bhamdoun Market	LBH I	4	PEN-AMC-FEP-CTX-LEX-CFM-DOR-MEM-STR	AMP-GEN-KAN	*mcr-1*

E. Assessment of the Transmissibility of the *mcr-1*-carrying Plasmids

mcr-1 was successfully transformed into chemically competent *E. coli* JM109 recipient cells using the heat-shock method for all the tested samples (n=9). All the transformants harbored the *mcr-1*, as shown by PCR analysis, confirming that the gene was transmissible via plasmids.

CHAPTER IV

DISCUSSION

Agricultural production and practices can lead to the contamination of freshly produced vegetables, including with antibiotic resistant bacteria (19). The misuse of antibiotics lead to the emergence of resistance in bacteria (19). Antibiotic-resistant strains can reach the environment through human excretions and manure of animals or can emerge in the environment by selective pressure. Thus, resistant bacteria have been isolated from soil, sewage, surface water, rural groundwater supplies and municipal drinking water (45). Antimicrobial resistance is considered to be a major food safety issue.

Colistin, a last resort antibiotic was recognized as a highest priority critically important drug for humans in 2012 (WHO 2012). Colistin is extensively used in animal farming (8), which led to the emergence of colistin resistance through the mobile colistin resistance gene, *mcr-1* (9), which was discovered by Liu et al. in China in November 2015. This gene has then been detected in more than 40 countries in different niches. It has also been detected in vegetable samples from four countries, China and South Korea in Asia, and Switzerland and Portugal in Europe.

In this study, 107 fresh vegetable samples of four different types, Lettuce, Radish, Cucumber and Green onion, were collected from 24 wholesale markets from different geographical locations in Lebanon. Colistin resistant *E. coli* were detected in 35.5% of the samples, from these 24 *mcr-1* positive *E. coli* were isolated.

The results of the following studies can be compared to the results obtained in our study in Lebanon. One of the studies was done in China and showed that vegetable

products were contaminated with *mcr-1* carrying bacteria (44). However, a study done in South Korea showed lower contamination rates with colistin resistant *E. coli,* where 7 colistin resistant *E. coli* strains were isolated from one Lettuce (0.076%) (37). Furthermore, the results of a study done in Switzerland showed the presence of the *mcr-1* in 2 out of the 60 ESBL-producing Enterobacteriaceae isolated from vegetables (imported from Thailand and Vietnam) (31). The rates detected in Lebanon were much higher. The prevalence of colistin resistant *E. coli* in vegetables collected from Lebanon reached 35.5%. In addition, *mcr-1* was detected in 24 *E. coli* isolates from 19 samples. Therefore, Lebanon suffers from higher contamination levels as compared to other countries. Comparing the MIC levels detected in our study with those in other countries, we noticed that the results are similar, in which the MIC values range between 4μg/ml to >64μg/ml, with the majority of the isolates having MIC value 4μg/ml [31,33,34, 35,36,37,38,39]. Only thirteen (54.17%) of the isolates in Lebanon were multidrug resistant.

The plasmid types detected showed a large diversity including IncI1α, IncF, IncI1γ, IncX1, IncFII, IncFIIS, IncW, IncA/C, and IncFIIK. It is worth mentioning that IncI1α is one of the plasmids responsible for the worldwide dissemination of *mcr-1*(42).

Our study is the first to report the presence of *mcr-1* positive *E. coli* in Lebanese vegetables as well as the MENA region. The level of *mcr-1* detected in fresh vegetables in Lebanon is considered to be among the highest. Therefore, several control measures should be taken to restrict the problem of antimicrobial resistance and to control the dissemination of colistin resistance. A One Health approach along with several policies should be implemented to ban the excessive use of colistin in agriculture. Colistin resistance is a public health issue that should be tackled urgently to prevent serious implications associated with untreatable/ or hard-to-treat infections.

CHAPTER V

CONCLUSION

The issue of antimicrobial resistance is increasing worldwide and is becoming one of the majors concerns, as we are heading to the post antibiotic era, where different antibiotics will no longer be able to treat bacterial infections. Colistin, also known as polymyxin E, which is a last resort antibiotic, is the only effective antibiotic to treat carbapenem and multidrug gram negative bacterial infections. However, the use of colistin has been challenged by the emergence of resistance, and the dissemination of mobile colistin resistance gene, *mcr-1*. This gene has been detected in different environmental samples in different countries as well as in Lebanon. It was detected in food, animal and water samples. In this study, high prevalence of *mcr-1* was detected in vegetable samples, knowing that vegetables constitute a major threat to human health as they are consumed raw. Lebanon lacks antimicrobial stewardship and the necessary policies that ban the excessive use of antibiotics. Colistin is available in Lebanon and there are no bans on the use of colistin especially in the agricultural sector. In this study, *mcr-1* was detected in 23 *E. coli* isolates form Lebanese vegetables; this level is high as compared to previous findings in other countries. This high level can be attributed to the use of contaminated and sewage water for irrigation, the excessive use of animal manures as fertilizers and the access to livestock to fields. A one health approach is needed to tackle the problem of colistin resistance and to avoid the worldwide dissemination of *mcr-1*. Policies should be implemented for instance to ban the use of colistin in agricultural sector, hence to help in controlling the issue of *mcr-1* dissemination.

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