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Prevalence of Colistin Resistance Among Clinical Isolates of *Enterobacteriaceae* Family in Palestinian Hospitals

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Prevalence of Colistin Resistance Among Clinical Isolates of *Enterobacteriaceae* Family in Palestinian Hospitals

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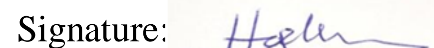
1- Head of Committee: Dr. Hatim Eideh Signature: 

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1442 / 2021

Declaration:

I certify that this thesis submitted for the degree of Master, is the result of my own research, except where otherwise acknowledged, and that this study (or any part of the same) has not been submitted for a higher degree to any other university or institution.

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Date: 24/8/2021

Dedication

I dedicate my thesis work to my family especially to my husband Bashaar Dudeen for his patience to take care of my duties for a long time, and for supporting me to be the best. A special feeling of gratitude to my parents, whose words of encouragement and push for tenacity ring in my ears. I also dedicate this thesis to my teachers, supervisors and friends at Al-Quds University, who have supported me throughout the master thesis work. I will always appreciate all they have done.

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Abstract

Background: Microbial resistance to antibiotic is an increasing global public health threats. Colistin are polycationic piptides antibiotics used as an effective traetment against multidrug resistant (MDR) gram negative bacteria mainly acting on lipopolysaccharide (LPS) and considred as one of the important antibiotics in human medicine despite its side effects and toxicity in some cases. Colistin resistance can be acquired by chromosomal mutations that affect the lipid A moiety of the lipopolysaccharide chain or by acquisition of MCR genes.

Aims: The general aim of this study was to investigate the prevalence of colistin resistance among clinical Enterobacteriaceae isolates from Palestinian hospitals. In addition to identify *Enterobacteriaceae* spp that are resistant to colistin. A third aim, is to compare colistin susceptibility using the Disc Diffusion Method (DDM) in relation to the reference method; Broth Micro-Dilution Method (BMD). A fourth aim is to detect the plasmid coded gene (*mcr-1*) responsible for colistin resistance as a marker for colistin resistance.

Methodology: A total of 80 bacterial isolates were collected from three hospitals in Palestine (AL-Makassed Hospital in Jerusalem, Al-Ahli Hospital and Alia Hospital in Hebron). The isolates were identified as *Enterobacteriaceae* and the speciation was done using Enterotube identification kit. The antimicrobial susceptibility testing for colistin was done by disc DD and BMD method. Furthermore, we used PCR reaction to detection of plasmid *mcr-1* gene, which code for colistin resistance.

Result: A total of 80 isolates were tested using DD and BMD method to determine the susceptibility of the clinical isolates to colistin. Only two isolates were colistin resistance one was *Serratia marcescens* and the other was *Proteus mirabilis* according to DDM results. However, when BMD method was used, ten (10) was identified; confirming the two isolates previously reported positive using DDM, the remaining were not detected by DDM. Escherichia coli was the most common organism among colistin resistance isolates (40%), followed by *Proteus* (20%), *Serratia* (20%), *Enterobacter cloacae* (10%) and *Klebsiella pneumoniae* (10%). Cifuroxime a beta lactum showed highest resistance (80%), followed by Ciprofloxacin a quinoone (55%). The most effective drug other than colistin, was Amikacin (80%) followed by

Gentamycin, Ertapenem, Merobenem and Tazobactum/Pipercillin (65%) respectively. All the colistin resistant isolates were tested negative for plasmid *mcr-1* gene after amplification by PCR.

Discussion: Colistin resistance is largely attributed to the *PmrA-PmrB* and *PhoP-PhoQ* regulatory systems and their responses to environmental changes. Resistance may also be Plasmid-mediated by the gene *mcr-1*. These limit and complicate treatment options of infection caused by *Enterobacteriaceae* in Palestinian Hospitals, which in turn calls for immediate actions to controlling and monitoring the use of antimicrobials in general and colistin in particular.

Conclusion: High resistant rate was reported for colistin (12.5%) using BMD. Although, DDM was inexpensive and easy to perform, it failed to detect most of the colistin resistant isolates. BMD method was very reliable and gave excellent results although it is expensive and not routinely performed in the clinical microbiology laboratories. For plasmid detection using PCR, all the colistin resistant isolates tested negative for the *mcr-1* gene, indicating that the cause of resistance could be physiologically or chromosomal mediated.

Keywords: Drugs resistance**,** colistin antibiotic, plasmid *mcr-1* gene, broth micro-dilution, *Enterobacteriaceae* family.

انتشار مقاومة الكوليستين بين عائلة البكتيريا المعوية في المستشفيات الفلسطينية

إعداد: تسنيم علي أبو سباع

إشراف: د. حاتم خالد عيده

ملخص

خلفية الدراسة: تشكل مقاومة المضادات الحيوية هي تهديدا متزايدا للمرضى وموظفي الرعاية الصحية في جميع أنحاء العالم. كوليستين هو مضاد حيوي متعدد الببتيدات يستخدم كدواء فعال ضد مقاومة الأدوية المتعددة للبكتيريا سالبة الجرام بشكل رئيسي على عديدات السكريات الدهنية. ويعتبر أحد المضادات الحيوية المهمة في الطب البشري على الرغم من آثاره الجانبية وسميته في بعض الحالات. يمكن اكتساب مقاومة الكوليستين عن طريق الطفرات الصبغية التي تؤثر على الدهون (أ) جزء من سلسلة عديدات السكريات الدهنية أو عن طريق اكتساب جينات MCR.

أهداف الدراسة: كان الهدف العام من هذه الدراسة هو التحقيق في انتشار مقاومة الكوليستين بين عزلات المعوية السريرية من المستشفيات الفلسطينية. بالإضافة إلى التعرف على البكتيريا المعوية المقاومة للكوليستين. الهدف الثالث، هو مقارنة قابلية كوليستين باستخدام طريقة انتشار القرص فيما يتعلق بالطريقة المرجعية؛ طريقة التخفيف الدقيق للسائل. الهدف الرابع هو الكشف عن الجين المشفر البلازميد (MCR-1) المسؤول عن مقاومة الكوليستين كعلامة لمقاومة الكوليستين.

منهجية البحث: تم جمع 80 عزلة بكتيرية من ثلاثة مستشفيات في فلسطين (مستشفى المقاصد بالقدس، والمستشفى الأهلي، ومستشفى عالية في الخليل). تم التعرف على العزلات على أنها مجموعة الباكتيريا المعوية وتم تحديد أنواعها باستخدام مجموعة تحديد .Enterotube بعد ذلك تم إجراء اختبار حساسية مضادات الميكروبات للكوليستين بطريقة القرص وطريقة انتشار السائل. علاوة على ذلك، استخدمنا تفاعل البلمرة المتسلسل للكشف عن جين البلازميدMCR-1 وهو رمز لمقاومة الكوليستين

النتائج: تم اختبار ما مجموعه 80 عزلة باستخدام طريقة انتشار القرص وانتشار السائل الدقيق لتحديد قابلية العزلات السريرية للكوليستين. اثنتان فقط من العزلات كانت مقاومة للكوليستين أحدهما كان السيراتية والآخر من الأمعائية المذرقية وفقًا لنتائج انتشار القرص. لم يتم اكتشاف الباقي بواسطة طريقة انتشار القرص. ومع ذلك، عند استخدام طريقة انتشار السائل الدقيق، تم تحديد عشرة عزلات، كانت الإشريكية القولونية أكثر الكائنات الحية شيوعًا بين عزلات مقاومة الكولستين (40٪)، تليها المتقلبة (20٪)، السراتيا (20٪)، الأمعائية المذرقية (10٪) والكلبسيلا الرئوية (10٪). أظهر سيفوروكسيم بيتا لاكتوم أعلى مقاومة (80٪)، يليه سيبروفلوكساسين كينون (55٪). أكثر الأدوية فاعلية غير الكوليستين هو أميكاسين (80٪) يليه جينتامايسين وإرتابينيم وميروبينيم وتازوباكتوم / ببيرسيلين (65٪) على التوالي. تم اختبار جميع العزلات المقاومة للكوليستين سلبية بالنسبة لجين البلازميد MCR1بعد التضخيم بواسطة تفاعل البلمرة المتسلسل.

الاستنتاج: تُعزى مقاومة الكوليستين إلى حد كبير إلى الأنظمة التنظيمية PmrA-PmrB وPhoP-PhoQ واستجاباتها للتغيرات البيئية. قد تكون المقاومة أيضًا بوساطة البلازميد بواسطة الجين mcr-1. تؤدي هذه العوامل إلى الحد من وتعقيد خيارات العلاج الخاصة بالعدوى التي تسببها البكتيريا المعوية في المستشفيات الفلسطينية، والتي تتطلب بدورها إجراءات فورية للتحكم في استخدام مضادات الميكروبات بشكل عام والكوليستين بشكل خاص ومراقبته.

الخلاصة: تم الإبلاغ عن ارتفاع معدل المقاومة للكوليستين (12.5٪) باستخدام طريقة انتشار السائل الدقيق. على الرغم من أن طريقة انتشار القرص كانت غير مكلفة وسهلة الأداء، إلا أنها فشلت في الكشف عن معظم العزلات المقاومة للكوليستين. كانت طريقة انتشار السائل الدقيق موثوقة للغاية وأعطت نتائج ممتازة على الرغم من أنها باهظة الثمن ولا يتم إجراؤها بشكل روتيني في مختبرات الأحياء الدقيقة السريرية. لاكتشاف البلازميد باستخدام تفاعل البلمرة المتسلسل، تم اختبار جميع العزلات المقاومة للكوليستين على أنها سلبية بالنسبة للجين mcr-1، مما يشير إلى أن سبب المقاومة يمكن أن يكون فسيولوجيًا أو كروموسوميًا.

الكلمات الدالة: مقاومة الأدوية، المضاد الحيوي كوليستين، جين البلازميد mcr-1، التخفيف الدقيق للسائل، عائلة البكتيريا المعوية.

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List of Abbreviations

Abbreviation	Term
ATCC	American Type Culture Collection
AKI	Acute Kidney Injury
BMD	Broth micro-dilution
CDC	Centre for Disease control and prevention
CAMPs	Cationic antimicrobial peptides
CMS	Colistimethate Sodium
COLR C^R	Colistin R
CLSI	Clinical and Laboratory Standard Institute
CFU	Colony forming unite
CAN	Colistin and nalidixic acid
DDM	Disc Diffusion Method
DNA	Deoxyribonucleic acid
ESBL	Extended spectrum B lactamase
ECDC	European Centre for disease control
EUCAST	European Committee for Antimicrobial susceptibility testing
FDA	Food and Drug administration
GNB	Gran Negative Bacilli
LAMP	Loop-mediated isothermal amplification
LPS	Lipopolysaccharide
MCR	Mobilized colistin resistance
MDR	Multi drug resistance
MH	Mueller Hinton
MIC	Minimum inhibitory concentrations
NADH	Nicotinamide adenine dinucleotide
NP	Nordmann\Poirel
OPrH	Outer membrane protein H
PEtN	Phosphoethanol amine

PDR	Pan Drug resistance
PCR	Polymerase Chain Reaction
R	Resistant
S	Sensitive
TCSs	Two component regulatory system
UTI	Urinary tract infection
VAP	Ventilator associated pneumonia
WHO	World health organization
XDR	Extensively drug resistance

Chapter One

The Introduction and Literature Review

1.1 Background

Antibiotic resistance is an increasing global public health threats. Medical treatment intervention depends on obtaining effective antibiotic treatment, and without it, many medical procedures, such as transplantations, major surgery and the care of prematurely born babies are at stake.

The European Centre for Disease Control (ECDC) together with the Centre for Disease Control & Prevention (CDC) have defined and classified different degrees of antimicrobial resistance as follow; Multidrug resistant (MDR) was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories. Extensively drug resistant (XDR) was defined as non-susceptibility to at least one agent in all antimicrobial categories. Pan-drug resistant (PDR) was defined as non-susceptibility to all agents in all antimicrobial categories. (Magiorakos et al., 2012)

Polymyxins are a group of polypeptide antibiotics that are composed of five chemically different compounds (polymyxins A–E), which was obtained from *Bacillus polymyxa* in 1947 (Ainsworth, Brown, & Brownlee, 1947; Stansly, 1949). polymyxin E, also known as colistin, was isolated from spore forming soil bacteria called *Bacillus polymyxa* subsp. *colistinus* by koyama and coworkers in the 1949. Only polymyxin B and polymyxin E have been used in clinical practice (Gupta et al., 2009) and considered as a last-line defense against the complicated Gram-negative MDR bacteria, especially

carbapenem-resistant *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* , which are categorized under Urgent or Serious threat level by CDC (CDC; 2013).

Antibiotic discovery was seen as one of medicine's greatest successes in the 1940s (van Hoek et al., 2011), saving millions of lives. Antimicrobial resistance has increased dramatically in bacteria over the last decade, reducing the efficacy of many clinically useful antibiotics. Gram-negative bacilli (GNB) are one of the most common infectious disease agents. Members of this family are ubiquitous, meaning they can be found not only in the intestinal microflora of humans and animals, but also in the environment. GNB are unique among resistant organisms because of their complex resistance mechanisms. Extended spectrum beta lactamases (ESBL), AmpC, and carbapenemases are the most common mechanisms. These hydrolyzing enzymes impart resistance to the most commonly used antibiotic class in clinical settings today. Furthermore, the resistance genes for these enzymes are often found on plasmids that also contain resistance determinants for other antibiotic groups, as a result, when infectious diseases do arise, therapeutic options are limited. GNB resistance to extended-spectrum cephalosporins and carbapenem necessitates the reintroduction of colistin (a polymyxin E antibiotic), which was previously discontinued due to its toxicity and side effects. Resistance has emerged as a result of the reintroduction of colistin in recent years, further complicating the clinical situation. Colistin resistance can be acquired by chromosomal mutations that affect the lipid A moiety of the lipopolysaccharide chain or by acquisition of mcr gene.

In a study carried out by (Olaitan, Diene, et al., 2014), they described a patient who had previously received colistin during hospitalization and was later found to have colistin-resistant *Klebsiella pneumoniae* in his stool, supporting the hypothesis that colistin resistance emerges as a result of antibiotic overuse in hospitals. In Addition, (Drozdinsky, Ben-Zvi, Kushnir, Leibovici, & Yahav, 2018) reported there has been no quantitative analysis of the dosages and/or durations of colistin administration that significantly increase the risk of colistin resistance developing in a strain or patient. In addition, (Hindiyeh et el., 2019), there is also research published in April 2019 about colistin-resistant *Klebsiella pneumoniae* in Palestinian hospitals. The finding was

attributed mainly to the premature termination of the mgrB transcript by nonsense mutations or insertion in 67% of the isolates or deletion of mgrB gene in 11% of the isolates.

There has been an intense usage of old antibiotics in medicine such as colistin due to the lack of new antobiotics over the last several years and the increase of bacterial resistance of available antibiotics. Colistin is used as an effective traetment against MDR gram negative bacteria and considred as one of the important antibiotics in human medicine despite its side effects and toxicity in some cases.

There has been an increase in colistin resistance of bacteria that were normally susceptible to it during the last decade **(fig 1.1)** (Rhouma, Beaudry, & Letellier, 2016) (Kempf et al., 2013; Olaitan, Morand, & Rolain, 2014)

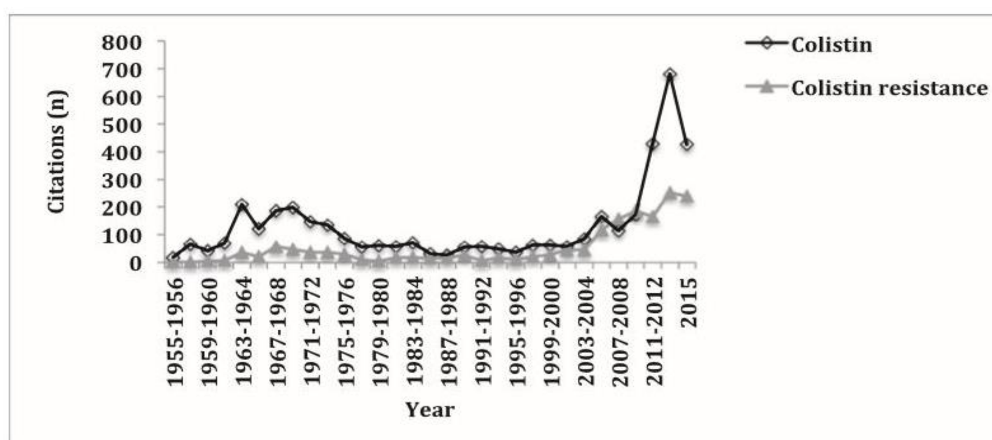

Fig 1.1: Number of citations found in the PubMed database from 1955 to 2015 using either the search phrase colistin or colistin resistance (Rhouma et al., 2016)

Unfortunately, the misuse and the overuse of colistin among animals and humans have led to global emergence of colistin-resistant pathogens. (**Table 1.1**) and **(figure 1.2)** illustrate the prevalence of resistant species in hospitals in livestock from different studies in the Middle East. (Dandachi, Chaddad, Hanna, Matta, & Daoud, 2019)

Colistin resistance mechanisms identified in the Middle East among Gram-negative bacteria (GNB) include: Direct manifestation of zoonotic transmission of mcr-1 from animal to human (Elnahriry et al., 2016), chromsomal mutation in PhoPQ and PmrAB

genes (Barrow & Kwon, 2009; Jayol et al., 2014), and Prior to administration of colistin during hospitalization, thus supporting the hypothesis of the development of colistin resistance as a result of hospital overuse of antibiotics (Lalaoui et al., 2019), Lack of quantitative investigation of the dosages and/or duration of administration of colistin,significantly increased the risk of developing colistin resistance in strains or patients (Drozdinsky et al., 2018).

Table 1.1: Mechanisms of colistin resistance described in GNB in the Middle East. (Dandachi et al., 2019)

Country	Origin	Species	Sequence type	Mechanism of colistin resistance
Iran	Clinical samples	*A. baumannii*		pmrB*
		A. baumannii		pmrA/B*
		K. pneumoniae		mgrB*
		P. aeruginosa		MexAB-OprM/MexXY-OprM**
Lebanon	Clinical samples	*K. pneumoniae*	ST268	mgrB*
		K. pneumoniae	ST2296	mgrB*, PhoQ*
		K. pneumoniae	ST348	pmrA/B*
	Poultry	*E. coli*	ST515	*mcr-1*
	Swine	*E. coli*		*mcr-1*
Palestine	Clinical samples	*K. pneumoniae*		mgrB*
		K. pneumoniae	ST512, ST76	mgrB*
Bahrain	Clinical samples	*E. coli*	ST648, ST224	*mcr-1* on IncI2
Qatar	Clinical samples	*E. coli*	ST95	*mcr-1* on IncHI2
United Arab Emirates	Clinical samples	*K. pneumoniae*	ST147	mgrB*
		E. coli	ST131	*mcr-1* on IncI2
Oman	Clinical samples	*E. coli*	ST10	*mcr-1* on IncI2
KSA	Clinical samples	*K. pneumoniae*	ST974, ST37, ST709, ST348, ST37	PhoP*
		K. pneumoniae	ST14, ST15, ST16, ST22, ST48, ST101, ST152, ST307	mgrB*
		K. pneumoniae	ST15	mgrB*, phoP*
		E. coli	ST68	*mcr-1* on IncHI2
Egypt	Clinical samples	*A. baumannii*		pmrCAB*
	Animal	*E. coli*	ST10	*mcr-1*
		E. coli O157		*mcr-1*
		E. coli O158		*mcr-1*

**Mutations. **Efflux pump over-expression.*

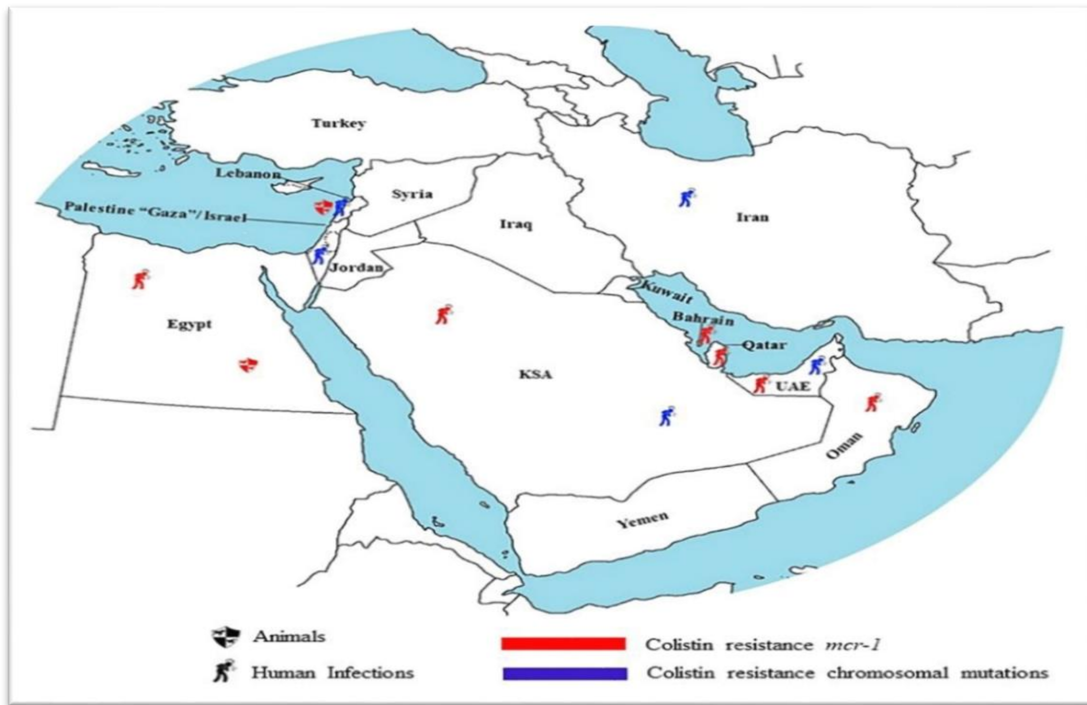

Fig 1.2: Geographical distribution of colistin resistance in humans and animals in the Middle East. (Dandachi et al., 2019)

1.2 History of Colistin

Over the last 50 years colistin is being used as the last line therapy for treatment of infections caused by multidrug resistance gram-negative bacteria.

Table 1.2: History of colistin from its discovery to today

Year	Event	Reference
1947	Polymyxins were acquired from a strain of *Bacillus polymyxa*	(Ainsworth et al., 1947)
1949	Colistin was isolate from *Bacillus polymyxa* subspecies *colistinus* by koyama and coworkers	(Stansly, 1949)
1959	Colistin is begin to be available in intravenous formulation as colistimethate sodium	(Gurjar, 2015)
1965	Colistin was originally thought to be different from polymyxins, but was later confirmed to be identical to polymyxin E	(Suzuki, Hayashi, Fujikawa, & Tsukamoto, 1965)

1970s -1980s	Polymyxins they were prevent in clinical practices because of their nephrotoxicity and neurotoxicity adverse events, In addition the availability of less toxic drugs, mostly like aminoglycosides	(Brown, Dorman, & Roy, 1970; Falagas & Kasiakou, 2006)
1990s-2000s	The increases of multi-drug resistant Gram negative bacilli (MDR) and also the lack of new effective antibiotics cause to the reemergence of polymyxins as a last-resort treatment selection	(Landman, Georgescu, Martin, & Quale, 2008; Li et al., 2006)
2000 onward	Now, colistin is considered as the last therapeutic option to confront life-threatening infections due to MDR gram negative bacilli	

1.3 Forms of colistin

There are two forms of colistin available commercially for clinical applications.

Table 1.3: Forms of colistin.

	Colistin sulfate	**Colistimethate sodium (CMS)**
Form	Sulfate salt	Sodium salt
Chemical formula	$C_{52}H_{98}N_{16}O_{13}$	$C_{58}H_{105}N_{16}O_{28}S_5$
Molecular weight	1155.4 g/mol	1649.8 g/mol
Synonyms	- Colistin Sulphate - Colistin sulfate - Colimycin sulfate Colymycin sulphate - - Polymyxin E sulfate -PolymyxinE sulphate	Colistin methanesulfate- Colistimethate sodium- - Pentasodium colistimethanesulfate Colistin sulfonyl methate-
Production	Isolated from *Bacillus polymyxa* subspecies *colistinus* by koyama and coworkers (Kasiakou et al., 2005)	Derived from colistin, which is reacted with formaldehyde and bisulphite sodium resulting in an additional component of –CH2SO3- on all of free amine components producing an anionic CMS molecule (Barnett, Bushby, & Wilkinson, 1964)
Charge	Cationic	Anionic

Activity	Active form	Inactive form (prodrug of colistin) - In a solution with physiological pH, colistimethate will immediately change into an active form after going through spontaneous hydrolysis reaction and releasing the –CH2SO3– component into methanesulphonate derivate and colistin in the plasma (Loho & Dharmayanti, 2015)
Toxicity	More toxic (neurotoxicity, nephrotoxicity)	Less toxic (Chen & Kaye, 2009)
Administration(18)	- Orally (taplets or syrup) for bowel decontamination - Topically (powder) for bacterial skin infection	Parenteral formulation (intravenously, intramuscularly) (Ledson, Gallagher, Cowperthwaite, Convery, & Walshaw, 1998) Nebulization (Chen & Kaye, 2009)
Absorption	Very poor absorption from gastrointestinal tract.	Very poor absorption from gastrointestinal tract.
Half-Life	5 hours	2-3 hours following either intravenous or intramuscular administration in adults and in the pediatric population

1.4 Chemical structure

Polymyxins are polycationic peptides made up of a cyclic heptapeptide, a linear tripeptide segment, and a fatty acid tail linked to the N-terminus of the tripeptide **(Fig. 1.3)** (Ezadi, Ardebili, & Mirnejad, 2019). It contains both hydrophilic and hydrophobic regions and therefore, it has amphipathic feature, it can give fine and stable reaction in polar (with electrostatic charge) or non-polar condition against the target, i.e. the lipopolysaccharide LPS membrane of the bacteria that are necessity for their antimicrobial activity(Loho & Dharmayanti, 2015). Two commercially available polymyxins, polymyxin B and polymyxin E (colistin) are identical structurally. They are different only by single amino acid in the heptapeptide ring, with a phenylalanine in polymyxin B and a leucine in colistin (A. Kwa, Kasiakou, Tam, & Falagas, 2007). In addition, at least four main components of polymyxin B, B1, B2, B3, and B4, and

two main types of colistin, A and B (also known as polymyxins E1 and E2, respectively), have been described.

At least 30 components have been isolated from colistin and 13 identified (Govaerts, Adams, Van Schepdael, & Hoogmartens, 2003; Orwa et al., 2001). They differ in the composition of amino acids and fatty acids. Two major important elements are colistin A (polymyxin E1) and colistin B (polymyxin E2). Minor elements involve polymyxin E3 and E4, norvaline-polymyxin E1, valine-polymyxin E1, valine-polymyxin E2, isoleucine-polymyxin E1, isoleucine-polymyxin E1 polymyxin E7 and isoleucine-polymyxin E8. (Elverdam, Larsen, & Lund, 1981; Ikai et al., 1998; Orwa et al., 2001)

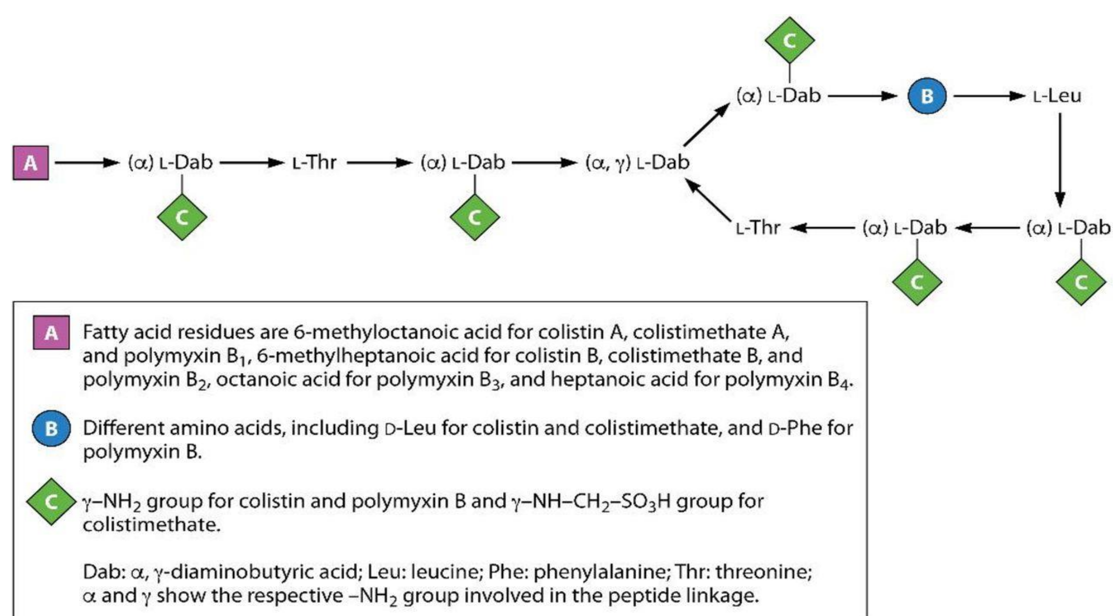

Fig 1.3: Structures of colistin, polymyxin B, and colistimethate (Ezadi et al., 2019)

1.5 Mechanism of actions

The similarities between the chemical structures of polymyxin B and colistin indicate that their mechanisms of action are exactly the same. (Nation, Velkov, & Li, 2014)

Mechanism of action of colistin against GNB is mainly on lipopolysaccharide (LPS) of the outer membrane **(Figure 1.4).** Due to its positive charge, colistin bind electrostatically with the negatively charged outer membrane of GNB and competitively replace calcium (Ca+2) and magnesium (Mg+2) ions from the phosphate groups of LPS (Gough, Hancock, & Kelly, 1996). Binding of colistin to the outer membrane is antagonised by divalent cations (Schindler & Osborn, 1979), resulting in

a reduce antibacterial activity, Destabilisation of LPS leads to the disruption and disarrangement of membrane, and the leak of periplasmic and cytoplasmic content causing bacterial death.

The endotoxin (lipid A) of GNB which is part of LPS, can be shed by bacteria during antimicrobial treatment and can be responsible for endotoxic shock. Colistin has an anti-endotoxin activity by binding to and neutralizing the LPS (Escartín et al., 1982)

Colistin furthermore acts by a number of other mechanisms, such as an inhibition of essential and vital respiratory enzymes (nicotinamide adenine dinucleotide [NADH]-quinone oxidoreductase) in the bacterial inner membrane (Deris et al., 2014)

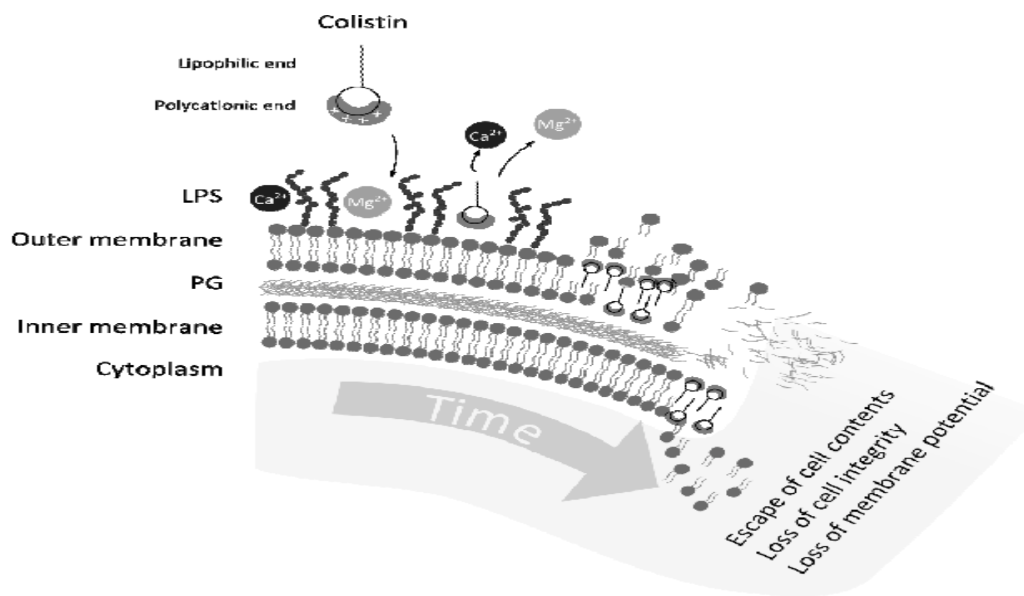

Fig 1.4: Mechanism of action of colistin (Loho & Dharmayanti, 2015)

1.6 Mechanism of resistance

Colistin must be administered as stated by its indication, based on the results of sensitivity test and should be closely monitored with appropriate dose since improper and irrational administration of colistin may induce cross resistance with polymyxin B resulting in hypervirulent new strain. (A. L. Kwa, Tam, & Falagas, 2008)

Intense use of colistin causes widespread of colistin resistance worldwide and despite its low rate which is less than 10%, the resistance rates are continuously increasing. Resistance to colistin has been described in many GNB species such as *Acinetobacter*

baumannii, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* (Bialvaei & Samadi Kafil, 2015)

Many theories have been proposed regarding the general mechanism of developing resistance of colistin among GNB as explained below;

1.6.1 Lipopolysaccharides modifications

Colistin resistance in GNB is most commonly associated to LPS modifications via various ways that involves the two-component regulatory systems (TCSs) (Bialvaei & Samadi Kafil, 2015). *PhoPQ* and *PmrAB* are two TCSs whose functions and regulations interfere. *PhoPQ* and *PmrAB* both contain a sensor kinase (*PhoQ* and *PmrB*, respectively), which senses environmental signals such as low Mg^{+2}, low pH or the presence of antimicrobial peptides. Exposure to colistin might alter the expression patterns of these TCSs. Activation of these sensor kinases lead to the phosphorylation of a response regulator *PhoP* and *PmrA*, respectively, which, once phosphorylated, typically promote their binding to promoters of regulated genes. Hence, phosphorylation of *PhoP* enhances the transcription of various genes, including *pmrD,* whose product binds to and stabilizes *PmrA* in its phosphorylated state. Phosphorylation of *PmrA* upregulates the transcription of enzymes that are essential for the addition of 4-aminoarabinose (Lara4N) and/or ethanolamine to the lipid A component of LPS. These additions participate to colistin resistance by reducing the negative charge of the bacterial membrane, and thereby decreasing the binding of positively charged colistin, these adaptive mechanisms of resistance were generally of moderate level. (Beceiro et al., 2011; McPhee et al., 2006)

Two-component systems (TCSs) **(Fig 1.5)**, mainly *PhoP-PhoQ* and *PmrA-PmrB*, regulate the expression of the majority of genes associated in LPS modification in Gram-negative bacilli. In *Salmonella spp., E. coli*, *K. pneumoniae*, and *P. aeruginosa*, activation of these TCSs under specific stress situation generally guide to LPS modification. The inner membrane sensor histidine kinase *PhoQ* and/or *PmrB* is activated under conditions of bacterial growth in the presence of low concentrations of Mg^{+2} and Ca^{+2} and high concentrations of Fe^{+2} or in the presence of cationic compounds including polymyxins. When sensor kinases (*PhoQ, PmrB*) are activated, they phosphorylate their relative cytoplasmic response regulator (*PhoP, PmrA*), which in turn modulates the expression of the target genes arnBCADTEF (also called

pmrHFIJKLM) and pmrCAB, responsible for LPS modifications by L-Ara4N and PEtN, respectively. In addition, PmrAB is activated by *PhoP-PhoQ* via the product of the pmrD gene, which in turn activates pmrA for activation of the arnBCADTEF operon. Inactivation of MgrB (a negative repressor of the *PhoP-PhoQ* system) by amino acid substitutions leads to overexpression of the *phoP-phoQ* operon as well, causing activation of the pmrHFIJKLM operon, thus leading to the production of L-Ara4N (Barrow & Kwon, 2009; Jayol et al., 2014)

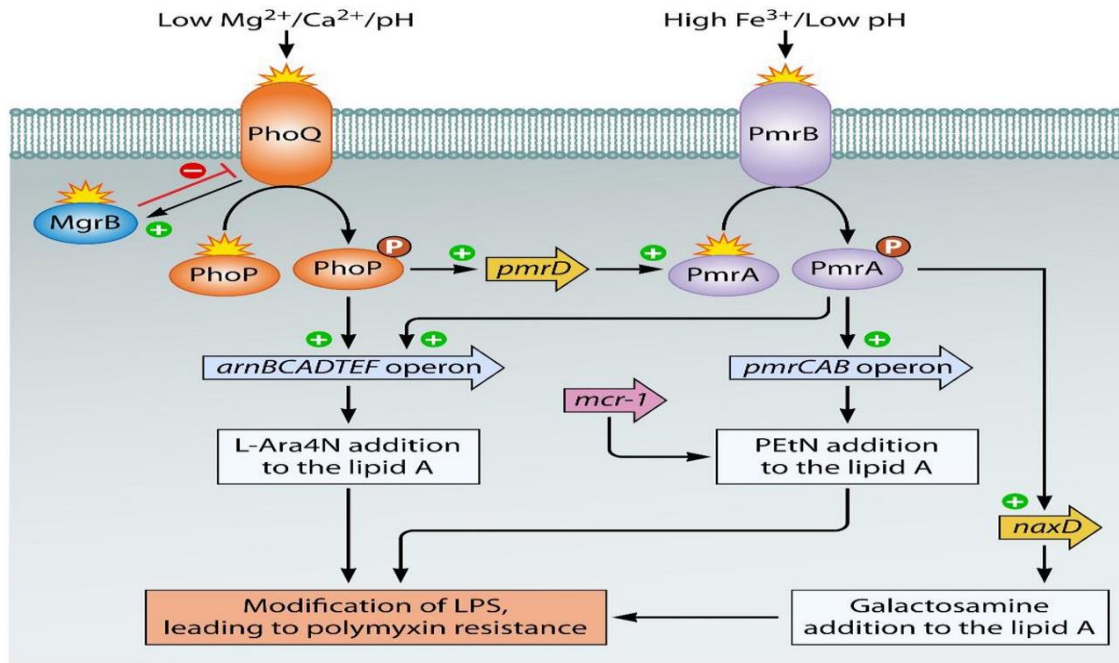

Fig 1.5: Mechanisms of LPS modification involved in polymyxin resistance in Gram-negative bacilli. (Barrow & Kwon, 2009; Jayol et al., 2014)

1.6.2 Efflux pumps

The mechanism of efflux pump may also have function in promote mechanism of bacterial resistance against polymyxins (Yahav, Farbman, Leibovici, & Paul, 2012). Most bacterial pathogens are resistant to cationic antimicrobial peptides (CAMPs), CAMPs resistance mechanism is dependent on an efflux pump/potassium antiporter system established by the RosA and RosB proteins. The RosA/ RosB system is activated by a temperature shift to 37C, but is also stimulated by the presence of the CAMPs, such as polymyxins. It is proposed that the RosA/ RosB system protects the bacteria by both acidifying the cytoplasm to prevent the CAMPs action and pumping the CAMPs out of the cell. (Bengoechea & Skurnik, 2000)

1.6.3 Heteroresistance

A different resistance phenomenon, that is called, heteroresistance, has become as a new challenge faced by antimicrobial therapy. This kind of resistance to polymyxins has been described in several Gram-negative pathogens, including *A. baumannii*, *K. pneumoniae*, *Salmonella* spp, *Enterobacter* spp, as well as *P. aeruginosa*. Heteroresistance is defined as resistance to specific antibiotics expressed by a subset of isolates of a microbe that are generally classified as susceptible based on in vitro susceptibility tests (Falagas, Makris, Dimopoulos, & Matthaiou, 2008)

Data propose that heteroresistance may happen as a result of mutations within the phoPQ or mgrB regulatory system (Bardet, Baron, et al., 2017), as well as the complete loss of LPS production due to a mutation in or the insertional inactivation of lipid A biosynthesis genes lpxA, lpxC, and lpxD (Moffatt et al., 2011).

1.6.4 Persisters

Persisters are mutant cells that neither die nor grow in the presence of an antibiotic, suggesting that they are in a state of dormancy (Lewis, 2007), and they grow only after removal of the antibiotic. Additionally, the progeny of persisters does not display increased resistance to the antibiotic but shows the same pattern of susceptibility to the antibiotic as the original bacterial population (Gefen & Balaban, 2009).

1.6.5 Plasmid-Mediated Mutations

Bacteria gain antibiotic resistance through two main routes: chromosomal mutation and the acquisition by horizontal gene transfer of mobile genetic elements, such as plasmids.

Plasmids are extrachromosomal DNA molecules, they are mostly circular dsDNA or linear dsDNA as in *Streptomyces spp* (Bao & Cohen, 2003). that replicate independently of the chromosome. And are able to transfer horizontally between bacteria by conjugation. In addition, plasmid DNA play an essential part in the dissemination of antibiotic resistance among clinically important pathogens by genes coding for various functions such as virulence factors and drug resistance.

The plasmids role in antibiotic resistance was first known in Japan when isolates which was sensitive or multidrug resistant during a single dysentery epidemic were isolated from the same patient. This indicate that sensitive strains became drug resistant, not by consecutive mutational steps, but by acquiring genetic elements in a single step. In 2014 Watanabe and Fukasawa reported that transfer of a plasmid that have the resistant genes triggered this process. Later on it was understood that plasmids are involved in the acquisition of antibiotic resistance genes and other determinants which contribute to the virulence of the recipient bacterial host (Ramirez, Traglia, Lin, Tran, & Tolmasky, 2014)

In November 2016, Liu et al. discovered for the first time transmissible polymyxin resistance in an *E. coli* isolate from food animals and raw meat in China (Y.-Y. Liu et al., 2016). The plasmid-borne gene *mcr-1,* which mediates the introduction of Phosphoethanol amine PEtN moiety to lipid A. has since been described in several species the family Enterobacteriaceae, such as *K. pneumoniae*, *Shigella sonnei*, *Salmonella* spp., and *Enterobacter* spp., from patients, animals, and environmental samples in different regions of the world (Baron, Hadjadj, Rolain, & Olaitan, 2016). In a short time, 13 *mcr-1* subgroups (*mcr-1.1* to mcr-*1.13*) were identified in several species of Enterobacteriaceae in different countries (Tanise V 2018). Until now, eight mcr variants (*mcr-1, mcr-2, mcr-3, mcr-4, mcr-5, mcr-6, mcr-7,* and *mcr-8*) showing nucleotide sequence identity to each other have been described in *Enterobacteriaceae* and other genera (Wang et al., 2018; Xavier et al., 2016). However, this is an essential global issue, where such transferable mechanisms are responsible for the rapid and ongoing dissemination of polymyxin resistance among clinical pathogens.

1.7 Clinical Applications and Uses

Colistin has become an alternative medication for bacteremia situations or sepsis by administering of CMS intravenous and in the form of CMS inhalation for ventilator associated pneumonia (VAP) cases caused by Gram negative MDRO. There are additional clinical applications of Colistin, such as for urinary tract infections by using colistin as intravesic CMS. In addition, oral administration of colistin (colistin sulfate) in the form of tablets or syrup can be prescribed for infections of the gastrointestinal tract. In the case of skin and soft tissue infections colistin may be given as the topical

preparation in the form of colistin sulfate. For infections of the eyes and ears, particularly those caused by Gram-negative MDRO bacteria, colistin sulfate was commonly used in the form of preparation for ointment or eye and ear drop.

Colistin should be performed based on the findings of the sensitivity test to limit the development of resistance due to prolonged use. Colistin contraindications are in patients with myasthenia gravis and polymyxin hypersensitivity. Colistin is not approved for women during the pregnancy and lactation period. Colistin can also be prescribed with dose modification and close supervision in patients with kidney failure.

1.8 Methods development to detect of colistin-resistance

Since resistance to colistin is a relatively new universal phenomenon, effective tools to detect and analyze pathogens causing infectious diseases in hospitalized patients which are resistant to colistin are needed.

1.8.1 Detection of colistin resistance using conventional methods

- Rapid NP Polymyxin Test for *Enterobacteriaceae* (Nordmann, Jayol, & Poirel, 2016a)**,** Is based on a pH examination, and resistance is detected by a change in color that occurs in two hours. A color change (orange to yellow) of the pH indicator (red phenol) evidences the production of acid metabolites consecutive to the glucose metabolism. The detection of *mcr 1* and *mcr 2* isolates was excellent when compared to the broth micro-dilution (BMD) susceptibility testing process (Poirel et al., 2018)

- Micromax Assay (Halotech DNA SL, Madrid, Spain)(Tamayo et al., 2013), In the presence of colistin, DNA fragmentation and cell wall damage may be detected. Bacteria are incubated with 0.5 g/ml colistin for 60 minutes before being trapped in a micro gel and lysed to remove damaged cell walls. After staining with SYBR Gold fluorochrome and observing with fluorescence microscope, the presence of DNA fragments is detected. The method is rapid and accurate in detecting resistance to colistin in the seventy *A. baumannii* isolates tested (20 resistant, 50 sensitive), but it is not precise in deciding the type of resistance.

1.8.2 Selective Culture Medium

Bacterial culture remains the gold standard method for isolation of pathogens in clinical specimen. The selective Colistin and Nalidixic acid (CAN) medium (agar containing colistin and nalidixic acid), containing 10 mg/L of colistin and 15mg/L of nalidixic acid detect mcr-1-positive isolates such as *K. pneumoniae*, and *E. coli* (Caspar et al., 2017; Payne et al., 2016). Furthermore, the SuperPolymyxin medium (Elitech Microbio, Signes, France) was created with the acute kidney injury (AKI) for detection of colistin-resistant isolates, including those with a low minimum inhibitory concentration (MIC) colistin and having the *mcr-1* gene. Since the SuperPolymyxin medium is made up of Eosin Methylene Blue (EMB) agar, which has greenish metallic sheen, it makes it easier to see *E. coli* strains (Nordmann, Jayol, & Poirel, 2016b).

In early 2018**,** a new chromogenic medium called CHROMID Colistin R agar (COLR) was introduced to the market, allowing for the detection of *Enterobacteriaceae* resistant to colistin in clinical samples. The COLR is a qualitative diagnostic medium that can be used to differentiate between colistin-resistant and susceptible isolates. Colistin-resistant strains on chromogenic media form colored colonies, the color of which varies by species. By contrast, colistin-susceptible isolates do not grow on the COLR plate. in accordance with the manufacturer's recommendations Escherichia coli colonies are pink to burgundy in color; Klebsiella pneumoniae and Enterobacter spp colonies are blue–green; and Salmonella spp colonies are white to colorless. The colonies of the Proteus tribe generated a beige–brown color. Most Gram positive bacteria, as well as yeast and molds, are inhibited by COLR. (García-Fernández et al., 2019).

Bardet et al in 2017 identified the medium LBJMR, a new multi-purpose culture medium to isolate and select colistin-resistant bacteria and vancomycin-resistant bacteria. This medium has been formed by adding colistin sulphate salt (4 μg/ml), vancomycin, and a substrate for fermentation of glucose to a Purple Agar Base, which show yellow colonies on a purple agar (Bardet, Le Page, Leangapichart, & Rolain, 2017)**.**

1.8.3 Specific Screening Methods for Detection of mrc-1

Since most medical microbiology laboratories equipped with fully automated systems to identify clinical isolates, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) identification of bacterial isolates resistant to

polymyxin is a promising and cost-effective process. It focuses on the detection of phosphoethanolamine on lipid A and detect lipid A modifications (Dortet et al., 2018; Osei Sekyere, Govinden, Bester, & Essack, 2016). In 15 minutes, the MALDIxin test detected polymyxin-resistant *E. coli* and distinguished between chromosome mediated and plasmid mediated resistance.

1.8.4 Reference method BMD antimicrobial susceptibility testing

The BMD procedure was conducted in compliance with the guidelines appended to to European Committee for Antimicrobial susceptibility testing / Clinical and Laboratory Standard Institute (EUCAST/CLSI). In brief, BMD panels were prepared in 96-well sterile polystyrene microplates in an extemporaneous way. Colistin dilutions ranging from 0.125 to 128 mg / l were performed in cation-adjusted Mueller Hinton (MH) broth, without addition of polysorbate 80 (Tween 80), and with a final concentration of 5x105 CFU / ml added in the form 100 µL per each well. This method was performed in triplicates in separate experiments, and the minimum inhibitory concentrations (MICs) were read at 35±2 ° C in ambient air after 16 to 20 h of incubation. Results were interpreted in accordance with EUCAST and CLSI recommendations on polymyxin breakpoint for MIC of *Enterobacteriaceae, Pseudomonas aeruginosa* and *Acinetobacter* spp, Strains with colistin MICs ≤ 2 mg/l were considered as susceptible (S) while those with MICs > 2 mg/l were resistant (R).

1.8.5 Devices Based on Broth Micro-Dilution Method

Many detection devices based on BMD methods have be created and are ready to use systems which eliminate many steps such as preparation of medium and antibiotic dilutions.

MIC Strip Colistin (MERLIN Diagnostika, Germany), The system has 12-well strips with dehydrated colistin concentrations ranging from 0.06 to 64 g/ml. Prepare a 0.5 McFarland suspension of the test bacteria in NaCl, transfer an aliquot into Mueller-Hinton II broth (CAMHB), inoculate the appropriate number of MIC-Strips, incubate for 18-24 hours at 35 37°C and read the results visually by looking for turbid growth or absence of turbidity (Matuschek, Åhman, Webster, & Kahlmeter, 2018).

The Sensititre System (Thermo Fisher Scientific, USA), uses 96-well trays with a customizable plate arrangement to present various antibiotics. It is possible to automate the steps of inoculation, incubation, and reading (based on fluorescence). A panel containing polymyxin B and colistin (0.25–4 mg/L) was recently evaluated and found to have a sensitivity of 95.2 percent and 100% specificity for the 21 *mcr-1* tested strains (Chew, La, Lin, & Teo, 2017).

1.8.6 Automated Systems

The automated systems designed in reduction of result time by increasing sensitivity and by avoid handling bias with incubation and real-time reading.

BD Phoenix automated system (Becton Dickinson, Le Pont de Claix, France), Colistin susceptibility testing was performed using an automated system called Phoenix, which performs an automated BMD process. The panel chosen to perform this evaluation was the Gram-negative panel NMIC-93 using the BMD method. The bacterial suspension and the inoculation of the panel were conducted according to the directives of the manufacturer. Under ambient air, the panels were incubated at 35±2°C for up to 16 h, and results were interpreted with the BD EpiCenter software. The system are able to detect all mcr-1carrying bacteria in the study by Jayol et al, including those with a colistin MIC of 4 g/ml (Jayol, Nordmann, Lehours, Poirel, & Dubois, 2018).

Vitek 2 (BioM´erieux, Marcy l'Etoile, France), It's a semi-automated device that uses 64-well reagent cards that carry dehydrated antibiotics and other reagents. It combines rapid identification and AST (Chew et al., 2017).

1.8.7 Genotyping and Molecular Screening

Amplification of nucleic acid by PCR and DNA Sequencing for detection antibiotic resistance genes are very sensitive and specific. These methods are supportive phenotypic techniques in confirming the resistant status of bacterial isolates. The majority of *Enterobacteriaceae* mutation are founds in the genes that code for the two element PmrA / PmrB and PhoP / PhoQ. The major resistance mechanism observed in *Klebsiella pneumoniae* strains appeared to be mutations in the mgrB gene, especially with the presence of insertional sequences. Amplification and sequencing are used to

screen for possible mutations on these chromosomal genes, a cumbersome and time-consuming process that takes three days and involves testing many target genes.

Multiplex PCR. In 1988, multiplex PCR was first identified as a method to detect deletions in the dystrophin gene (Chamberlain, Gibbs, Ranier, Nguyen, & Caskey, 1988). development of the multiplex PCR method have been designed to amplify several different DNA sequences simultaneously and to yield rapid results in less than 2 hours (amplification and gel electrophoresis). A well designed multiplex PCR can be reliable for fast screening of genes and internal control. This technique can be easily adapted to any laboratory that has a PCR amplification machine (Lescat, Poirel, & Nordmann, 2018).

Loop-Mediated Isothermal Amplification (LAMP) based assay for detecting the mcr-1 gene was recently developed and tested on 556 multidrug-resistant *Enterobacteriaceae* (Zou et al., 2017). Both the regular PCR and the LAMP-based assay found seven isolates to be positive (6 *E. coli* and 1 *K. pneumoniae*). LAMP is Chromogenic test that can be used to detect the presence or absence of a target gene. This test is a fast, accurate, and low cost method that has a better sensitivity than PCR (10-fold).

1.9 Problems Statement

The worldwide dissemination of colistin resistance has become a great health concern worldwide. This dramatic increase of resistance indicates its ability to transfer horizontally by conjugative plasmids or vertically by chromosomal mutation. Of note, since colistin one of the last lines of treatments to serious infections, it even adds more threats to the public health worldwide. In this study, we attempt to investigate the prevalence of colistin resistance in 3 Palestinian hospitals by comparing micro-dilution and Kirby Bauer detection methods within *Enterobacteriaceae* isolates from patients having a variety of infections such as urinary tract infection, respiratory tract infection and many more. Our finding will shed light on the efficacy of DDM used in our laboratories and the real status of colistin resistance and may help defining new strategies to controlling colistin resistance it in our hospitals and public health sector.

1.10 Study Objectives

The main objective of this study was to investigate the prevalence of colistin resistance among *Enterobacteriaceae* in Palestinian hospitals

The specific aims were:

- To identity the bacterial isolates obtained from the hospitals to species level
- To compare the efficacy of disc diffusion method in relation to the Broth micro-dilution Methods.
- To detect plasmid gene *mcr-1* responsible for colistin resistance

Chapter Two

Methodology

2.1 Materials

All materials used in this study are listed in **Table 2.1**. All reagents and chemicals were of analytical grade.

Table 2.1. Instruments and reagents used in the study.

Item	Components	Supplier/Company
Nutrients Broth	Peptone 5.00 g/L Sodium Chloride 5.00 g/L Meat Extract 1.50 g/L Yeast Extract 1.50 g/L Final PH (at 25 C) 7.4 ± 0.2	HIMEDIA Company
MacConkey Agar	Peptic digest of animal tissue 1.50 g/L Casein enzymic hydrolysate 1.50 g/L Pancreatic digest of gelatin 17.00 g/L Lactose 10.00 g/L Bile salts 1.50 g/L Sodium chloride 5.00 g/L Crystal violet 0.001 g/L Neutral red 0.03 g/L Agar 15.00 g/L Final PH (at 25 C) 7.1 ± 0.2	HIMEDIA Company
Colistin Sulphate Disc	Well of Discs	Al-Theqa Medical Supplies Company
ComASPTM Colistin	4 Systems (panels) of ComASP Colistin (panels individually packed in foil with silica gel desiccant) 16 Tubes of Mueller Hinton II Broth (3.6 ml)	Liofilchem Diagnostic Company

	Sealing Film Instructions Sheet and Test Results Form	
Controls	1. *Escherichia coli ATCC®* 25922 2. *Pseudomonas aeruginosa* ATCC® 27853	ATCC
Hy-Enterotest	Test kit Thermetically sealed in bags of 5 paddles Identification labels Indole Caps Chart for interpretation of results	Hylabs Company
Primers	MCR-1 Forward and Reverse	Hylabs Company
PCR Procedure	PCR tubes Escohealthcare Swift PCR thermocycler Device Green master mix Heat block 95C	Hylabs Company
Gel electrophoresis Procedure	DNA Ladder Ethidium Bromide solution Microwave UV-light documentation system TAE buffer 50X and 1X Agarose gel powder 100% Glacial acetic acid EDTA Ph = 8 Tris-Base Bio-Rad electrophoresis device	Hylabs Company

2.2 Methods

2.2.1 Study Population and Sample Collections

80 bacterial cultures were collected from patients having variety of infections from three hospitals in Palestine (AL-Makassed Hospital in Jerusalem, Al-Ahli Hospital and Alia Hospital in Hebron) during the period from August 22st till December 30th 2020. Samples were selected only if they belonged to the *Enterobacteriaceae* family.

2.2.2 Identification of *Enterobacteriaceae* by Hy-Enterotest system

The bacterial identification for samples were performed using Hy-Enterotube (Hylabs Company, Israel). This test is a breakthrough for the rapid, accurate, simple, convenient and economic identification of Enterobacteriaceae family. Seven biochemical properties will be demonstrated utilizing a method of stabbing butt and smearing a slant. The tubed media is

consisting of two layers and a paper cap for indole determination is impregnated with a solution of 4-dimethyl-aminobenzaldehyde. the lowest layer is a semi-solid media containing urea, sodium chloride, enrichments, agar, and phenol red as indicator, at Ph 6.8. The upper layer is a butt and a slant, containing dextrose, ONPG, enrichments, ferrous Sulphate, sodium thiosulfate, agar and phenol red as indicator at Ph 7.4 (**Table 2.2**).

Table 2.2: Differentiation of *Enterobacteriaceae* by Hy-Enterotest at 35±2 C for 18-24 h.

Reaction	Positive	Negative
Indole	Purple	No Change
ONPG	Orange	Pink
Glucose	Yellow	No Change
CO2	Bubbles	No Change
H2S	Black	Yellow
Motility	Fuzzy	Clear
Urease	Pink	Yellow

2.2.3 Broth micro-dilution BMD antimicrobial susceptibility testing method (AST)

ComASPTM Sensitest Colistin kit (Liofilchem, Roseto degli Abruzzi, Italy) were used in the study to test the clinical isolates**.** It contains compact panel of 4 tests containing seven twofold dilutions of dehydrated colistin ranging from Zero–16 µg/ml (**Table 2.3**)**.** Interpretative criteria to determine clinical resistance were based upon breakpoints described by the manufacturer by which any growth with MIC less or equal 2 µg/ml is considered sensitive and MIC more than 2 µg/ml is considered resistant. ComASP Colistin is not for use directly with clinical or other specimens. The microorganism to be tested must first be isolated on a suitable non-selective culture medium. In case of mixed culture, selected colonies should be purified by sub culturing. first, remove a panel from its envelope and set it aside for 10 minutes at room temperature; do not discard the envelope until all 4 tests have been completed. Using either the direct colony suspension or the growth method, prepare a suspension of the test organism. Set the density of the suspension to a McFarland 0.5 standard. Dilute the adjusted suspension 1:20 in saline, optimally within 15 minutes after production; this is Solution A. To make Solution B, mix 0.4 ml of Solution A with a tube of MH II Broth from the kit. dispense each well in a row with 100

l of Solution B. Cover the panel with the provided lid and incubate for 16-20 hours in ambient air at 36 2°C.

Table 2.3: A 4-test panel containing the dry antibiotic of seven two-fold dilutions (0.25 - 16 µg/ml). Growth indicates positive growth control; No antimicrobial agent in the well.

Test	Colistin Concentration (µg/ml)						
A Growth	0.25	0.5	1	2	4	8	16
B Growth	0.25	0.5	1	2	4	8	16
C Growth	0.25	0.5	1	2	4	8	16
D Growth	0.25	0.5	1	2	4	8	16

2.2.4 Disc Diffusion test of other antibiotics

Eight antibiotics that are routinely used in Patient treatment were applied in testing each isolate by disc diffusion test on Muller-Hinton agar as described for the DD test of colistin (Ciprofloxacin, Gentamicin, Tazobactam/Piperacillin, Ertapenem, Meropenem, Amikacin, Colistin Sulphate and Cefuroxime).

Table 2.4: MIC values for the eight antibiotics

Antibiotics	Sensitive (mm)	Intermediate (mm)	Resistant (mm)
Ciprofloxacin	≥ 21	16-20	≤ 15
Gentamicin	≥ 15	13-14	≤ 12
Tazobactam/Piperacillin	≥ 21	18-20	≤ 17
Ertapenem	≥ 22	19-21	≤ 18
Meropenem	≥ 23	20-22	≤ 19
Amikacin	≥ 17	15-16	≤ 14
Cefuroxime	≥ 23	15-22	≤ 14
Colistin Sulphate	≥ 14	12-13	≤ 11

2.2.4 DNA Extraction

A loopful of colonies from overnight bacterial growth was suspended in a test tube containing 1 ml of distilled water and subjected to boiling at 95°C in a heat block device for 10 min, cooled to ambient temperature, then centrifuged for five minutes at 10000 rpm.

2.2.5 Polymerase Chain Reaction PCR

The mcr-1 gene encodes phosphoethanolamine transferase enzyme (Liu et al, 2015). Expression of this gene in *Escherichia coli* results in the addition of phosphoethanolamine to lipid A and in this way confers resistance to colistin. Specific primer pair for amplification of *mcr-1* gene were used from (Y. Y. Liu et al., 2016) (**Table 2.3).**

A 5-µL aliquot of the supernatant of DNA extract was used per each polymerase chain reaction (PCR) following the protocol described in (Ali et al, 2009).

The final PCR reaction volume was 25µL it contained; 1 µL Forward Primer, 1 µL Reverse primer, 4 µL Dream Taq Green PCR Master Mix (5X) which already contains MgCl2 (Promega Company), 5 µL of Supernatant DNA extract and 9ul DDW. *Escherichia coli* ATCC-25922 and *Pseudomonas aeruginosa* ATCC-27853 were used as control. PCR program was carried out with the following conditions 94C 15 min; 25 cycles of 94C 30 sec; 58C 90 sec; 72C 60 sec with a final extension of 72 ^{0}C for 10 min. Finally, the PCR product size is 309 bp.

Table 2.3: Primer of Mcr-1 plasmid-mediated resistance gene

Target gene	Primers	Sequences (5' to 3')
mcr-1	mcr-1-F	5'-CGGTCAGTCCGTTTGTTC-3'
	mcr-1-R	5'-CTTGGTCGGTCTGTAGGG-3'

2.2.6 Gel electrophoresis

Agarose gel (2%) were used to separate PCR amplicons using100 volts for 2 hours 4g agarose powder were dissolved in 200 ml TAE buffer 1X and 1 drop of ethidium bromide by heating the solution for a few minutes until entirely dissolved. A 10 µL PCR sample was loaded into the respective wells of the gel next to 1 kbp DNA ladder.

Chapter Three

Results

3.1 Demographic Characteristics of the study

In this study, a total of 80 bacterial culture samples were collected from three major hospital laboratories in Jerusalem and Hebron, AL-Ahli Hospital/Hebron, Hebron Governmental Hospital and Al-Maqassed Islamic Hospital/ Jerusalem, all samples were identified as *Enterobacteriaceae* spp.

The male to female ratio among sample subjects was 1.6:1, the median age at recruitment was 45 years **(Table 3.1).** The majority of samples were confirmed as *E. coli* followed by *Klebsiella pneumoniae*. Isolates were collected from different clinical sources, the majority was from wounds samples were 21(26%), rectal swabs samples were 21(26%) followed by mid-stream urine samples were 18(23%), Body fluids samples were 9(11%), Urine Catheter samples were 6(8%), Blood samples were 4(5%) and cervical swab sample was 1(1%). **(Fig 3.1).**

Table 3.1: Distribution of 80 patients by age and sex.

Age (Years)	Sex		Mid-Point of interval
	Males	Females	
0-1	20 (40.81%)	11 (35.48%)	5
10	2 (4.08%)	2 (6.45%)	15
20	0 (0.0%)	0 (0.0%)	25
30	2 (4.08%)	2 (6.45%)	35
40	1 (2.04%)	2 (6.45%)	45
50	13 (26.53%)	7 (22.58%)	55
60	6 (12.24%)	4 (12.90%)	65
70	4 (8.16%)	3 (9.67%)	75
≥ 80	1 (2.04%)	0 (0.0%)	85
Total	49 (100%)	31 (100%)	

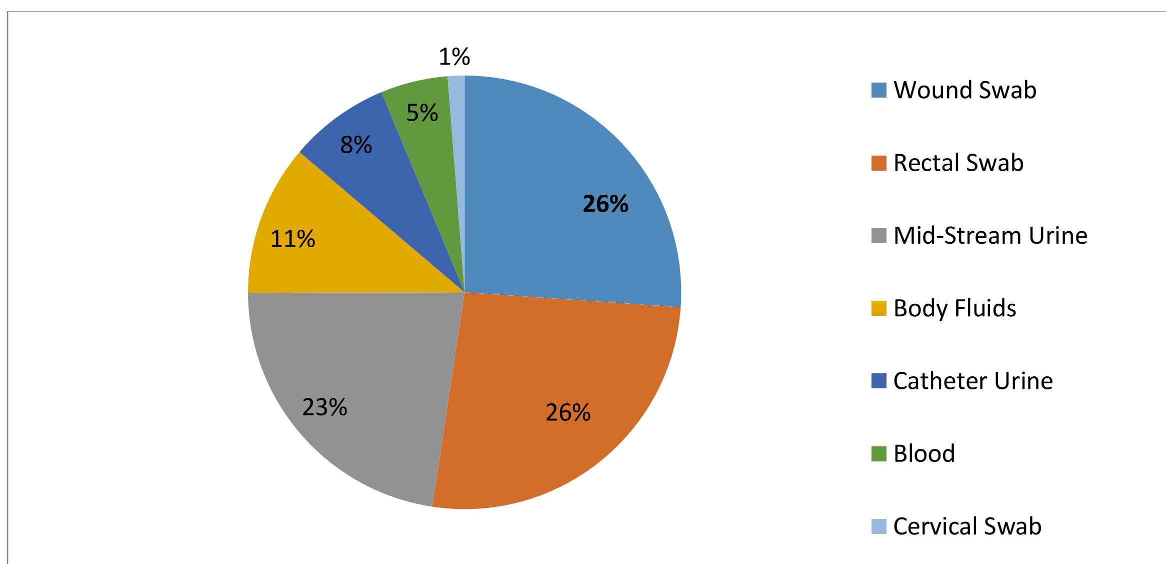

Figure 3.1: Distribution of sample based on clinical sources of isolates

All culture samples were subjected to Bacterial identification, antimicrobial sensitivity and colistin sensitivity using disc diffusion method on Muller Hinton agar. Only two isolates showed a positive result for colistin resistance; one from Al-Makassed Clinical Laboratory (*Serratia marcescens*) and the other from Hebron Governmental Hospital (*Proteus mirabilis*), both bacteria reported to have intrinsic chromosomal-mediated resistance for Colistin antibiotic **(Table 3.2).** According to DDM for *Enterobacteriaceae* family, all isolates with a zone diameter ≥ 14 mm were susceptible, whilst all isolates with a zone diameter ≤ 11 mm were resistant (Galani et al., 2008).

Table 3.2: Demographic characteristics of study sample (Colistin Sulphate MIC diameter ≥ 14 mm were S; diameter ≤ 11 mm were R; diameter 12-13 mm were I).

No	Date of Birth	*Enterobacteriaceae Spp*	Sample	Colistin Disc result	Hospital
1	1991	*E. coli ESBL*	Wound Swab	Sensitive	Al-Ahli
2	1963	*E. coli ESBL*	Wound Swab	Sensitive	Al-Ahli
3	2016	*E. coli ESBL*	MSU	Sensitive	Al-Ahli
4	1943	*E. coli ESBL*	MSU	Sensitive	Al-Ahli
5	2020	*E. coli*	Wound Swab	Sensitive	Al-Ahli
6	1973	*Klebsiella pneumoniae*	MSU	Sensitive	Al-Ahli
7	1947	*E. coli ESBL*	MSU	Sensitive	Al-Ahli
8	1942	*Klebsiella pneumoniae ESBL*	Wound Swab	Sensitive	Hebron
9	1955	*E. coli ESBL*	MSU	Sensitive	Hebron

10	2020	*E. coli ESBL*	MSU	Sensitive	Hebron
11	1965	*E. coli ESBL*	Wound Swab	Sensitive	Hebron
12	2020	*Serratia Marcescens*	Wound Swab	Sensitive	Hebron
13	2009	*E. coli ESBL*	MSU	Sensitive	Hebron
14	1937	*E. coli ESBL*	MSU	Sensitive	Hebron
15	2010	*E. coli ESBL*	MSU	Sensitive	Hebron
16	1970	*E. coli ESBL*	MSU	Sensitive	Hebron
17	2005	*E. coli ESBL*	Wound Swab	Sensitive	Hebron
18	1961	*Klebsiella pneumoniae ESBL*	MSU	Sensitive	Hebron
19	1961	*Klebsiella pneumoniae ESBL*	Body Fluids	Sensitive	Al-Ahli
20	2020	*E. coli ESBL*	MSU	Sensitive	Al-Ahli
21	2020	*E. coli CRE*	Rectal Swab	Sensitive	Al-Makassed
22	1946	*Klebsiella pneumoniae*	Blood	Sensitive	Al-Makassed
23	1970	*E. coli ESBL*	Wound Swab	Sensitive	Al-Makassed
24	2020	*E. coli ESBL*	Catheter Urine	Sensitive	Al-Makassed
25	2020	*E. coli CRE*	Rectal Swab	Sensitive	Al-Makassed
26	1946	*E. coli*	Catheter Urine	Sensitive	Al-Makassed
27	2020	*Enterobacter aerogenes*	Catheter Urine	Sensitive	Al-Makassed
28	1966	*Klebsiella pneumoniae CRE*	Blood	Sensitive	Al-Makassed
29	2020	*Klebsiella pneumoniae CRE*	Wound Swab	Sensitive	Al-Makassed
30	2019	*E.coli CRE*	Rectal Swab	Sensitive	Al-Makassed
31	1973	*Enterobacter aerogenes CRE*	Blood	Sensitive	Al-Makassed
32	2019	*E. coli CRE*	Rectal Swab	Sensitive	Al-Makassed
33	2020	*Klebsiella pneumoniae CRE*	Rectal Swab	Sensitive	Al-Makassed
34	1956	*E. coli CRE*	Rectal Swab	Sensitive	Al-Makassed
35	2020	*Klebsiella pneumoniae CRE*	Rectal Swab	Sensitive	Al-Makassed
36	1965	*Proteus mirabilis CRE*	Body Fluids	Sensitive	Al-Makassed
37	2020	*Klebsiella pneumoniae CRE*	Rectal Swab	Sensitive	Al-Makassed
38	2020	*Klebsiella pneumoniae CRE*	Rectal Swab	Sensitive	Al-Makassed
39	2020	*Klebsiella pneumoniae CRE*	Rectal Swab	Sensitive	Al-Makassed
40	1963	*Klebsiella pneumoniae CRE*	Rectal Swab	Sensitive	Al-Makassed
41	2020	*Serratia marcescens CRE*	Rectal Swab	Sensitive	Al-Makassed
42	1971	*Klebsiella pneumoniae CRE*	Rectal Swab	Sensitive	Al-Makassed
43	2019	*Enterobacter aerogenes CRE*	Rectal Swab	Sensitive	Al-Makassed
44	2020	*Klebsiella pneumoniae CRE*	Rectal Swab	Sensitive	Al-Makassed
45	1979	*E. coli CRE*	Rectal Swab	Sensitive	Al-Makassed
46	2020	*Klebsiella pneumoniae CRE*	Rectal Swab	Sensitive	Al-Makassed
47	1957	*E. coli CRE*	Rectal Swab	Sensitive	Al-Makassed
48	2020	*E. coli CRE*	Rectal Swab	Sensitive	Al-Makassed
49	2020	*E. coli ESBL*	Wound Swab	Sensitive	Al-Makassed
50	1959	*E. coli ESBL*	MSU	Sensitive	Al-Makassed
51	2020	*Klebsiella pneumoniae ESBL*	Catheter Urine	Sensitive	Al-Makassed
52	1983	*Klebsiella pneumoniae*	Wound Swab	Sensitive	Al-Makassed
53	1985	*Enterobacter aerogenes*	Body Fluids	Sensitive	Al-Makassed
54	1962	*Enterobacter cloacae*	Body Fluids	Sensitive	Al-Makassed

55	1962	*Serratia Marcescens*	Body Fluids	Sensitive	Al-Makassed
56	1946	*Klebsiella pneumoniae ESBL*	MSU	Sensitive	Al-Makassed
57	1958	*proteus mirabilis*	Wound Swab	Resistance	Hebron
58	1957	*Klebsiella pneumoniae*	Wound Swab	Sensitive	Al-Makassed
59	2006	*Enterobacter aerogenes*	MSU	Sensitive	Al-Makassed
60	1970	*E. coli ESBL*	Wound Swab	Sensitive	Al-Makassed
61	1970	*E. coli ESBL*	Wound Swab	Sensitive	Al-Makassed
62	2020	*E. coli ESBL*	Catheter Urine	Sensitive	Al-Makassed
63	1966	*E. coli ESBL*	Wound Swab	Sensitive	Al-Makassed
64	1966	*Klebsiella pneumoniae ESBL*	Wound Swab	Sensitive	Al-Makassed
65	2020	*E. coli ESBL*	Blood	Sensitive	Al-Ahli
66	2020	*Klebsiella pneumoniae ESBL*	Body Fluids	Sensitive	Al-Makassed
67	1952	*Klebsiella pneumoniae ESBL*	Body Fluids	Sensitive	Al-Makassed
68	1991	*E. coli*	Cervical Swab	Sensitive	Al-Makassed
69	1966	*E. coli ESBL*	Wound Swab	Sensitive	Al-Makassed
70	1966	*Enterobacter cloacae ESBL*	Wound Swab	Sensitive	Al-Makassed
71	1964	*E. coli*	MSU	Sensitive	Al-Makassed
72	2020	*Enterobacter cloacae ESBL*	Wound Swab	Sensitive	Al-Makassed
73	2020	*Klebsiella pneumoniae*	Wound Swab	Sensitive	Al-Makassed
74	1968	*E. coli*	MSU	Sensitive	Al-Makassed
75	1968	*E. coli*	MSU	Sensitive	Al-Makassed
76	1944	*Enterobacter cloacae ESBL*	Catheter Urine	Sensitive	Al-Makassed
77	1962	*Serratia Marcescens*	Body Fluids	Resistance	Al-Makassed
78	2020	*Klebsiella pneumoniae*	Rectal Swab	Sensitive	Al-Makassed
79	1955	*Klebsiella pneumoniae CRE*	Body Fluids	Sensitive	Al-Makassed
80	2020	*Enterobacter aerogenes CRE*	Rectal Swab	Sensitive	Al-Makassed

Antimicrobial susceptibility testing was performed for all collected samples, eight antibiotics were commonly used in the three hospitals; Ciprofloxacin, Cefuroxime, Meropenem, Gentamicin, Piperacillin-Tazobactam, Amikacin and Colistin-Sulphate **(Fig 3.2).** However, there was additional antibiotics used in some samples such as amoxicillin-clavulanic acid, cotrimoxazole, cephazoline and ceftriaxone from Al-Makassed Clinical laboratory, Cefepime, Nitrofurantoin and Trimethoprim-Sulfamethoxazole from Alia laboratory and Fosfomycin, Levofloxacin and Augmentin from Al-Ahli Clinical laboratory. Different degrees of susceptibility were reported for the isolates against the tested antimicrobials. Cifuroxime showed highest resistance (81.25%), followed by Ciprofloxacin (56.25%). The most effective drug apart from

colistin was Amikacin (80.0%) followed by Gentamycin, Ertapenem, Merobenem and Tazobactum/Pipercillin respectively **(Table 3.2).**

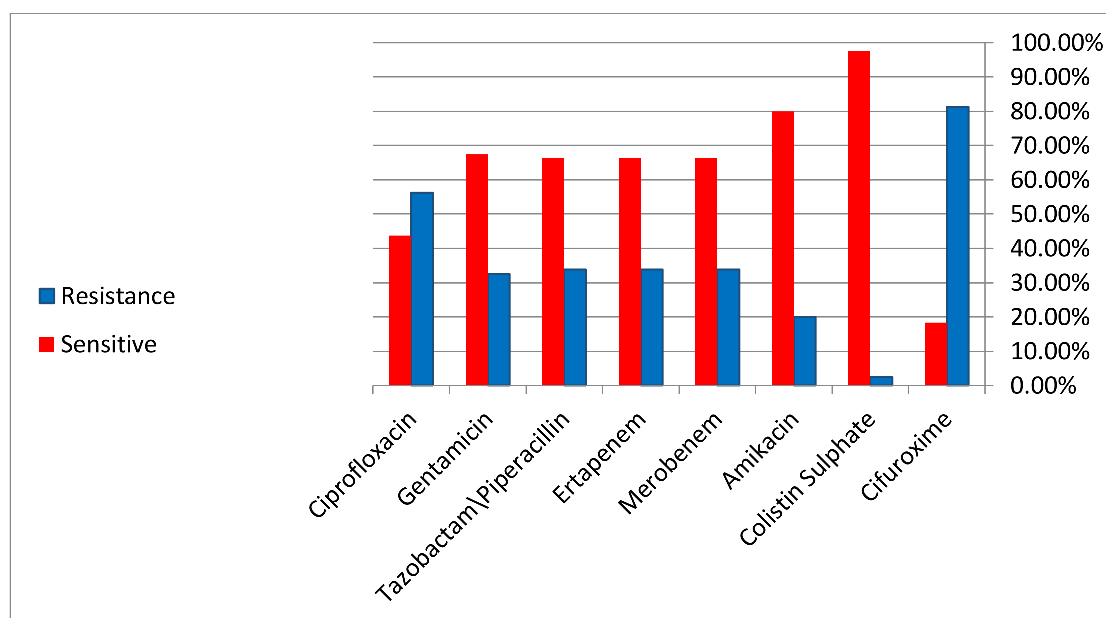

Fig 3.2: Percentage of antibiotic resistance pattern of all isolates

Table 3.2: Antimicrobial susceptibility testing of Eight antibiotics by using DDM

Antibiotics	Resistant	Sensitive
Ciprofloxacin	56.25%	34.75%
Gentamicin	32.50%	67.50%
Tazobactam\Piperacillin	33.75%	66.25%
Ertapenem	33.75%	66.25%
Meropenem	33.75%	66.25%
Amikacin	20.00%	80.00%
Colistin Sulphate	2.50%	97.50%
Cefuroxime	81.25%	18.25%

3.2 Confirmation of bacterial identity

All bacterial samples were tested and confirmed using Enterotube test in order to identify genus and species for all isolates, the results were consistent with the Hospital identifications **(Table 3.3).**

Table 3.3: Frequency of bacterial Species

Bacteria Species	Frequency	%
Escherichia coli	38	47.50%
Klebsiella pneumoniae	26	32.50%
Enterobacter aerogenes	6	7.50%
Enterobacter cloacae	4	5%
Serratia Marcescens	4	5%
Proteus mirabilis	2	2.50%
Total	80	100%

3.3 Standard antimicrobial susceptibility testing AST method for colistin

A total of 80 isolates were tested for colistin resistance using broth micro-dilution method. Our results were interpreted according to CLSI 2020 and EUCAST2020 by which any growth with MIC less or equal 2 µg/ml is considered sensitive and MIC more than 2 µg/ml is considered resistant. The MIC of all samples ranged from 0 to 16 MIC (0.25, 0.50, 1, 2, 4, 8 and 16). 8 out of 10 colistin sensitive turned out to be positive when the test was performed using BMD method. The most common organism among colistin resistance bacteria was *E. coli* (40%) followed by *Proteus* (20%) and *Serratia* (20%) **(Table 3.4).** The control that were used in this method was Positive control of *Proteus mirabilis* and Negative control of *E. coli* ATCC-25922 and *Pseudomonas aeruginosa* ATCC-27853. **(Fig 3.3).**

Table 3.4: Positive results of Broth Micro-Dilution (BMD) method for colistin

NO	Bacterial species	Colistin Sulphate Disc Result	BMD result µg/ml
7	*E.coli ESBL*	Sensitive	4
12	*Serratia Marcescens*	Sensitive	16
36	*Proteus mirabilis CRE*	Sensitive	8
47	*E.coli CRE*	Sensitive	4
57	*proteus mirabilis*	Resistant	16
60	*E.coli ESBL*	Sensitive	16
68	*E. coli*	Sensitive	4
72	*Enterobacter cloacae ESBL*	Sensitive	8
77	*Serratia Marcescens*	Resistant	16
78	*Klebsiella pneumoniae*	Sensitive	16

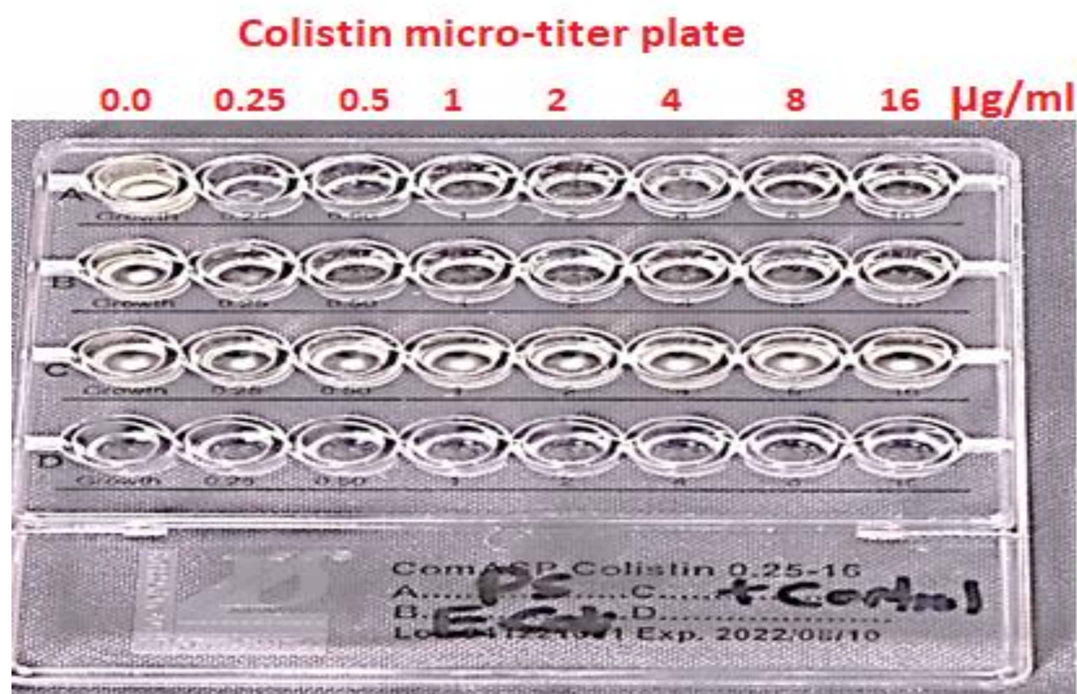

Figure 3.3: Positive (*Proteus mirabilis*) lane C and *E. coli* ATCC-25922 lane B and *Pseudomonas aeruginosa* ATCC-27853 lane A as Negative control

3.4 Polymerase Chain Reaction for plasmid *mcr-1* gene

PCR was performed for a total of 23 bacterial isolates, 10 of which were resistant based on MIC value of more than 2 µg/ml, while the remaining 13 isolates were considered based on MIC value of 2 µg/ml. All samples were negative for plasmid *mcr-1* gene.

Chapter Four

Discussion, Conclusion and Recommendation

4.1 Discussion

Colistin is an antibiotic that is effective against most multidrug-resistant Gram-negative bacteria. It is currently used as a last-resort treatment of Gram-negative bacterial infections which has become resistant to other antibiotics. Due to a lack of other antibiotics, colistin resistance is considered a severe problem. *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and members of the *Enterobacteriaceae*, such as *Escherichia coli, Salmonella spp., and Klebsiella spp,* have developed resistance to colistin. *Serratia spp., Proteus spp., and Burkholderia spp.,* on the other hand, are intrinsically resistant to this antibiotic (El-Sayed Ahmed et al., 2020). Clinicians should also be aware of the risk of colistin resistance among multidrug-resistant bacteria, which could emerge through mutation or adaption mechanisms.

This study was conducted to investigate and describe the prevalence of colistin resistant isolates and additional antibiotics among *Enterobacteriaceae* in three Palestinian hospitals. The findings of this study provide an estimate for the prevalence of colistin resistance among *Enterobacteriaceae*. Furthermore, this study confirmed the unreliability of DDM for colistin sensitivity testing method that is routinely by clinical microbiology laboratories. The poor and slow diffusion of polymyxins lead the results of disc diffusion test non reliable, yielding small zones of inhibition (Matuschek et al., 2017). This chapter highlights the major findings, conclusion and recommendations of this study.

In certain bacterial species, the disk diffusion test does not appear to be a valid approach for detecting colistin resistance. As a result, a reliable, quick, and inexpensive test to assess colistin susceptibility of pathogenic isolates in routine diagnostics is required. The pre-diffusion test is a reliable alternative to the agar-based colistin susceptibility test. Is capable of detecting both traditional (chromosomic) and transferable plasmid

(*mcr*) mechanisms of colistin resistance. For the disk diffusion procedure with 10-µg colistin paper disk on a noninoculated Mueller Hinton agar plate, Remove the disk by knocking the plate on the table after 2 hours of incubation at 35-37°C. Keep the plate at room temperature for another 18–22 hours before using it. The plate can be used right away or kept in the refrigerator (4°C) for one week after incubation at room temperature (Boyen et al., 2010). As many laboratories still rely on the cheaper disk diffusion test, the emergence of colistin resistance may be missed, it is clear that this type of resistance needs to be monitored closely, using the appropriate test methods.

4.1.1 Intrinsic resistance mechanisms to colistin

Polymyxin resistance arises intrinsically in *Proteus mirabilis* and *Serratia marcescens* due to cationic substitution of the LPS. Colistin affinity for binding to LPS decreases as a result of the modification of LPS and the increase in its charge. As a result, these species have developed intrinsic resistance. (Lin et al., 2014; Sidorczyk, Zähringer, & Rietschel, 1983). The lipid A of the LPSs of wild-type *Proteus mirabilis* are known to contain L-Ara4N (Sidorczyk et al., 1983), Polymyxin-susceptible mutants, on the other hand, frequently lack L-Ara4N in their LPSs, while those with lower resistance have fewer L-Ara4N substitutions. (Kaca, Radziejewska-Lebrecht, & Bhat, 1990). The eptC gene, which is involved in the modification of core LPSs with PEtN, has also been discovered in the genome of *P. mirabilis* (Aquilini, Merino, Knirel, Regué, & Tomás, 2014).

In Serratia marcescens mutants, inactivation of the arnB and arnC genes, which are part of the arnBCADTEF operon, results in polymyxin sensitivity (Lin et al., 2014). Thus, these findings reveal that the arnBCADTEF operon-mediated alterations of S. marcescens LPSs are partly or entirely responsible for the bacterium's intrinsic resistance to polymyxins. In contrast to non-intrinsically resistant bacteria, the arnBCADTEF operon appears to be constitutively expressed in intrinsically resistant bacteria. Why is this true for these two types of bacteria is the main question that remains unanswered, understanding this mechanism would help researchers better understand how polymyxin resistance evolved in Gram-negative bacteria.

4.1.2 Acquired resistance mechanisms to colistin

Antibiotic resistance to polymyxin antibiotics is based on a complex mechanism involving numerous genes involved in bacterial cell membrane remodeling. Given the current importance of polymyxins in clinical practice and the rise in bacterial resistance to these medications. Until 2015 chromosomal gene mutation was the only known mechanism of acquired resistance to colistin, later on a plasmid mediated *mcr-1* gene which encode phosphoethanol amine PEtN transferase that confer reduced sensitivity to colistin by catalyzing the addition of PEtN to lipid A moiety (Aghapour et al., 2019).

4.2 Comparison of colistin susceptibility testing using disc diffusion method (DDM) and Broth Micro-dilution (BMD) Method

In July 2016, a joint EUCAST and CLSI subcommittee provided recommendations indicating that broth micro-dilution using untreated polystyrene wells is the only viable approach for the evaluation of colistin resistance. Whereas, and that disk diffusion is ineffective due to the relatively high molecular weight of colistin molecule's causing its slow diffusion. The adhesive properties of colistin, which are related to its cationic nature, were highlighted in this document, suggesting that the BMD test should be performed with cation-adjusted Mueller-Hinton Broth without additives (in particular, no polysorbate-80 or other surfactants), and untreated polystyrene wells.

SensiTest Colistin kit (Liofilchem, Roseto degli Abruzzi, Italy) which have been used in our study evaluated on 353 isolates, including 259 *Enterobacteriaceae*, it exhibited an excellent association with other BMD methods, with 137 isolates resistant to colistin (19 intrinsically, 83 harboring the *mcr-1* gene)(Carretto et al., 2018). The SensiTestTM Colistin appears to be a simple but effective test for determining colistin susceptibility. The preparation process as well as results evaluation are simple, quick and uncomplicated, but is expensive. (**Table 3.4)** summarizes the findings of the investigation. All BMD found to give accurate yielding identical results, all the tests performed were considered valid (that is, growth was present in the growth control well) with using positive and negative control depending on ATCC.

The 80 clinical isolates of *Enterobacteriaceae* which were tested for Colistin resistance using both disk diffusion method and disc diffusion method, showed a clear variation

between aforementioned methods as eight samples indicated to be Sensitive for colistin using DDM while the same eight were resistant when tested by the BMD method. However, Disk diffusion method is considered to be a screening test and should not be used for colistin sensitivity evaluation.

The findings showed a total of 10 out of 80 (12.5%) isolates were confirmed to be colistin resistant using BMD method, the rate was higher in *E. coli* (40%) followed by *proteus* and *Serratia* (20%) respectively, in addition to the presence of a sample of each of these bacteria *Klebsiella pneumoniae* and *Enterobacter cloacae* from the 10 samples were resistant **(Table 4.1).**

Table 4.1: MIC Values for various *Enterobacteriaceae*

MIC in µg\ml	*Escherichia coli*	*Enterobacter*	*Proteus mirabilis*	*Serratia marcescens*	*Klebsiella pneumoniae*	Total
16	1 20.0%	0 0.0%	1 20.0%	2 40.0%	1 20.0%	5 100%
8	0 0.0%	1 50.0%	1 50.0%	0 0.0%	0 0.0%	2 100%
4	3 100%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	3 100%
2	6 46.1%	1 7.69%	0 0.0%	1 7.69%	5 38.4%	13 100%
1	0 0.0%	1 50.0%	0 0.0%	0 0.0%	1 50.0%	2 100%
0.50	3 27.2 %	1 9.09%	0 0.0%	1 9.09%	6 54.5%	11 100%
0.25	4 45.4%	1 9.09%	0 0.0%	0 0.0%	6 54.5%	11 100%
0	21 63.6%	5 51.1%	0 0.0%	0 0.0%	7 21.2%	33 100%
Total	38 (47.5%)	10 (12.5%)	2 (2.5%)	4 (5.0%)	26 (32.5%)	80 (100%)

Resistance to other antibiotics that are usually used in treatment, showed highest resistance Cefuroxime (81.25%), followed by Ciprofloxacin (56.25%). The most effective drug apart from colistin was Amikacin (80.0%) followed by Gentamycin (67.50%), Ertapenem (66.25%), Meropenem (66.25%) and Tazobactam/Piperacillin (66.25%) respectively.

Our colistin resistance rate was lower than the rate reported in Gaza strip. In a study carried out in Gaza a total of 41.4% of *Enterobacteriaceae* are reported to be colistin resistant. The highest colistin resistance was that of *Proteus* spp. (63.2%) for intrinsically resistance., followed by *Serratia* spp. (57.1%). meaning those two genera were not 100% naturally C^R as reported in (Qadi, Alhato, Khayyat, & Elmanama, 2021).

Highest resistance was recorded against trimethoprim (85%) and tetracycline (83%). The most effective drug was Amikacin (74%) was in agreement with our study (Qadi et al., 2021). This percentage is higher than that reported years before, when amikacin resistance was 3.9 percent among GNB responsible for nosocomial bacteremia and 1.8 percent among community-acquired isolates. (Harbarth et al., 1998). In a study performed by (Tayh et al., 2019) of ESBL-producing *Enterobacteriaceae* isolates, however, it was found to be lower than amikacin resistance which was 33.3%

In another study from Gaza on *E. coli* tested for colistin isolated from Chicken fecal materials, phenotypic resistance to colistin sulfate was found in 16 (14.5%) isolates (Elmanama, 2018). In addition, percent of our study is lower than the percent reported in (Bardet, Okdah, Le Page, Baron, & Rolain, 2019), a study which revealed a percent of 63.4% of colistin resistance. Furthermore, according to a study performed by BMD on Colistin MIC testing on 107 clinical MDR GNB isolates, 19 (17.8%) of the 107 clinical isolates tested resistant to colistin. This includes 9 (8.4%) isolates of *K. pneumoniae* (Hindler & Humphries, 2013).

On the contrary to this and the Gaza studies, in a four year cross sectional study on 13579 *Enterobacteriaceae* isolates from Spain, 91 were resistant to colistin, with a colistin resistance prevalence of 0.67%. The rates higher in *Enterobacter cloacae* (4.2%) than *Escherichia coli* (0.5%) and *Klebsiella pneumoniae* (0.4%). This contradiction could be attributed to the overuse and misuse of antibiotics in general and to colistin in particular (Prim et al., 2017). It is obvious that we are reporting a higher percent of colistin resistance in comparison to the published data in Kuwait in 2018 by

(Alfouzan, Dhar, & Nicolau, 2018) where the team reported resistance of 4.3% for *E. coli* and 7.7% for *Klebsiella.*

Using broth micro-dilution, 16/143 (11%) isolates were nonesusceptible to colistin and With E test, 4/143 (3%) isolates were nonsusceptible to colistin on *Enterobacteriaceae* family (Simar, Sibley, Ashcraft, & Pankey, 2017). These data strongly support other published findings that any colistin MIC should not be determined by E test because of major problems with false susceptibilities as the large cationic peptides of polymyxins diffuse poorly in agar and results should not be reported for clinical use. If colistin susceptibility is requested, testing by broth micro-dilution should be performed.

In our study there was no record of previous exposure to colistin and colistin resistance was not restricted to multidrug resistant isolates since antibiotic resistant isolates were sensitive to colistin and vice versa which was in agreement with the above mentioned study. This could be attributed to the intrinsic nature of resistance in some *Enterobacteriaceae* species such as *Proteus* and *Serratia* in addition to plasmid mediated acquisition (Prim et al., 2017).

4.3 Polymerase chain reaction and plasmid mcr-1 gene

Horizontal gene transfers by plasmid mediated colistin resistance to susceptible strains are considered a very significant challenge and global concern. These plasmids which carry mcr genes were reported in November 2016, *E. coli* isolated from pigs and meat from China (Y.-Y. Liu et al., 2016). Isolates containing *mcr-1* gene are sufficient for colistin to be resistant. In our study all the colistin resistant isolates which were tested by PCR for detection of mcr-1 gene were negative and the reason could be due to the DNA isolation method which have yielded very low copies of plasmids or due to chromosomal mediated resistant since majority of the genes involved in LPS modification are regulated by two-component systems, mainly *PhoP-PhoQ* and *PmrA-PmrB* located on the chromosome, or by other *mcr* variants or subgroups plasmid mediated resistance

In Palestine, (Hindiyeh *et al.*, 2019), there is also research published in April 2019 about colistin-resistant *Klebsiella pneumoniae* in Palestinian hospitals colistin resistance was

determined by micro-broth dilution assay and investigated by Polymerase Chain Reaction (PCR) amplification and sequencing the mgrB and *mcr-1*& 2 genes, The finding was attributed mainly to the premature termination of the mgrB transcript by nonsense mutations or insertion in 67% of the isolates or deletion of mgrB gene in 11% of the isolates. However, there is no prevalence of *mcr-1* and 2 genes.

4.4 Conclusion

High resistant rate was reported for colistin (12.5%) using BMD. Although, DDM was inexpensive and easy to perform, it failed to detect most of the colistin resistant isolates. BMD method was very reliable and gave excellent results although it is expensive and not routinely performed in the clinical microbiology laboratories. For plasmid detection using PCR, all the colistin resistant isolates tested negative for the *mcr-1* gene, indicating that the cause of resistance could be physiologically or chromosomal mediated.

4.5 Recommendation

Although studies have begun to reveal the processes underlying colistin resistance, further research is needed to completely understand the impact of the two regulatory systems on resistance, as well as the colistin dosages required to inhibit and overcome these emerging patterns. Although BMD is very liable technique for detection of colistin resistance, simpler inexpensive, reliable, sensitive, method should be developed for routine use such as Pre-diffusion method (Boyen et al., 2010). Further studies should be carried out in Palestine with larger scale of isolates to determine the exact prevalence rate of resistance to colistin. Although mcr-1 presence was not found in any of the 80 isolates, other mcr-variants may have existed.

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