SUDAN UNIVERSITY OF SCIENCE AND TECHNOLOGY

COLLAGE OF GRADUATE STUDIES

Molecular Detection of Colistin Resistance Genes Mcr-1, 2, 3, 4 and Mcr-5 of *Pseudomonas aeruginosa* Isolated from Selected Hospital in Khartoum State

الكشف الجزيئي عن جينات المقاومة للكوليستين ام سي ار ١، ٢،٣،٤ و ام سي ار ٥ في بكتريا الزائفة الزنجارية المعزول من العينات السريرسة في ولاية الخرطوم

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بسم الله الرحمن الرحيم

قال تعالى:

﴿ وَلَمَّا بَلَغَ أَشُدَّه وَاسْتَوَى آتَيْنَاه حُكْمًا وَعِلْمًا وَكذَلِكَ نَجْزِي الْمُحْسِنِينَ ﴾

صدق الله العظيم

سورة القصص، الآية (14)

DEDICATION

To my wonderful mother "Rama" who never stops giving in so many ways.

The symbol of love and giving to my sisters, brothers and family members.

To my friends who encourage and support me as well as to everyone in my life who has touched my heart

To Sudan University of Science and Technology and all their staff

I dedicate this research thesis

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In the name of ***ALLAH***, the most Merciful, the most compassionate all praise be to Allah, The Lord of the worlds; and prayers and peace be upon Mohammed his servant and messenger.

First and foremost, I must acknowledge my limitless thanks to ***ALLAH*** ever-magnificent, ever-thankful, for help and blessing.

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To anyone may I have forgotten, I apologize, thank you as well.

ABESTRACT

Antimicrobial resistance is a major threat to human health and *Pseudomonas* is one of non-fermenting Gram-negative bacilli with clinically wide range of infections with a multidrug resistance characteristic, so there was re-emergence of use of Colistin -a drug with toxicity effects- as a last drug option and now there were wild world reports of its resistance. In this investigation, 50 clinical isolates *Pseudomonas aerogenosa* were collected from different Hospitals in Khartoum State (Royal care, Fedial, Alzaytouna, Omer Sawi, Al-raqi, Khartoum ENT, and Khartoum South North hospital Teaching Hospital). Traditional microbiological Techniques were used to re-identify bacterial isolates. All of these isolates were tested for resistance to common using antibiotic disc using the Kirby-Bauer disk diffusion technique according to CLSI guidance and investigated for the presence of mcr-1,2,3,4 and mcr-5 as plasmid mediate resistance genes to Colistin using multiplex PCR. The present cross-sectional study was conducted from January 2022 to June 2022. Out of the 50 isolates, 18 were isolated from urine samples, 11 from wound swabs, 6 from blood, 2 from high vaginal swabs, 8 from sputum, 3 from ear swabs, and 2 from body fluids, after culturing most strains showed pigmentation 32 (74%) and 18 (36%) were non-pigmented. Demographic data show male to female ratio was 0.85:1. Ages ranged from 4 to 76 years with an average of 49. Approximately one-third of 18 (36%) isolates were resistant to Aztreonam, 10 (20%) were resistant to Ciprofloxacin and Colistin, 11 (22%) were resistant to Gentamicin, Amikacin and Piperacillin/Tazobactam combinations, only one isolate was resistant to Imipenem. From 18 urine samples, 3 (19%) isolates were resistant to Norfloxacin. Only 14 (28%) isolates were MDR, most isolates 18 (36%) were sensitive to all antibiotics used. Mcr genes were isolated from 35 of *P. aeruginosa* (70%), mostly 28 (56%) was mcr-3, followed by 6 (12%) positive for mcr-2 and mcr-1 was found in one isolate (2%), mcr-4 and mcr-5 were not detected. Most mcr genes were present in Non-MDR isolates 31 (62%) and there was moderate negative correlation (R= -0.52) and *p*-value = 0.00 between present of mcr genes and drug resistance of isolates. There were no association between present of these mcr genes, age and gender of patient (*p*-value = 0.74, 0.24 respectively). This study was the first nationwide surveillance report on the detection of the mcr-3 gene among *P. aeruginosa* isolates from clinical samples in Sudan, this result indicates the spread of mcr genes in different Sudanese hospitals. In addition, this finding is higher than previous reports of mcr genes that have been reported in Sudan.

المستخلص

مقاومة الادوية اصبحت من اكبر المخاطر التي يواجهها العالم يوميا كما ان البكتريا المعروفة بالزائفة الزنجارية مميزة جدا عن مثيلاتها من ذات التصنيف بانها بكتريا شديدة المقاومة للادويه مع تسببها باغلب الامراض وتواجدها في المستشفيات بكثرة ولذلك تم اعاده استخدام دواء الكولستين في علاج الاصابه بها كاخر خيار للعلاج اذا كانت البكتريا مقاومه لغيره, وهو دواء قديم تم ايقاف استخدامه لسميته. حاليا تم تسجيل مقاومته حول العالم، وذلك تهدف هذه الدراسه للتحقيق في وجود جينات ام سي ار 1,2,3,4 و5 وهي جينات تتواجد في البلازميد في البكتريا المقاومه للكولستين. أجريت الدراسة الحالية من يناير 2022 إلى يونيو 2022 وتم استخدام التقنيات البكتيريه لعزل البكتريا اولا ثم اجراء اختبار الحساسيه لها ثم الكشف عن وجود الجينات المقاومه باستخدام اختبار البرمله المتعدد. تم جمع ما مجموعه 50 عينه بكيريه من الزائفة الزنجارية تم عزلها من مستشفيات مختلفة (مستشفى رويال كير، فضيل، الزيتونة، مستشفى الشرطه بحري، الراقي، الخرطوم، والمستشفى التعليمي بشمال الخرطوم) . من بين 50 عينه بكتيريه، كانت هنالك 18 من العينات معزوله من عينات البول، و 11 من مسحات الجروح، و 6 من الدم، و 2 من المسحات المهبلية ، و 8 من البلغم، و 3 من مسحات الأذن، و 2 من سوائل الجسم، وبعد الزراعة معظم السلالات أظهرت امتلاكها للون الاخضر 32 (74٪) و 18 (36٪) كانت عديمه اللون. وتبين البيانات الديمغرافية أن نسبة الذكور إلى الإناث كانت 0.85 : 1. تراوحت الأعمار من 4 إلى 76 عامًا، بمتوسط 49 عامًا. كان ما يقارب ثلث العينات اي 18 عينه (36٪) مقاومة لـ الازترونيم، و 10 (20٪) مقاومة لـ السبروفلوكساسين و للكولستين، و 11 (22٪) كانت مقاومة لـ الجنتامايسين والاميكاسين و البراسلين/تازوباكتام ، كانت عينه واحدة فقط مقاومة لـ الامبيم . من بين 18 عينة بول، كانت 3 منهم (19٪) مقاومة للنورفلوكساسين. كان هناك م مجموعه 18 عينه (32%) غير مقاومه لاي دواء في حين كان هناك 14 (28%) من البكتريا مقاومه لاكثر من ثلاثه ادويه المستخدمه في الدراسه. و تم عزل جينات ام سي ار من 35 عينه (70%) معظمها 28 (56٪) كانت ام سي ار 3، تليها 6 (12٪) إيجابية لـ ام سي ار 2 ، و ام سي ار 1 وجد في عينه واحده (2٪). لم يتم ايجاد كل من ام سي ار 4 و 5. لم تكن هنالك علاقه بين وجود الجينات وعمر او جنس المريض كما ان اغلب الجينات كانت في القروبات البكتيريا الاقل مقاومه للادويه 31 (62%). كانت هذه الدراسة أول تقرير على مستوى البلاد من التقارير التي تم نشرها حول اكتشاف جين ام سي ار 3 من الزائفة الزنجاربه من العينات السريرية في السودان، وتشير هذه النتيجة إلى انتشار جينات ال ام سي ار في المستشفيات السودانية المختلفة. بالإضافة إلى ذلك، فإن هذا الاكتشاف أعلى من التقارير السابقة التي كانت تقوم بالبحث عن وجود جينات ام سي ار في السودان.

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CLED	Cysteine Lactose Electrolyte Deficient media
CLSI	Clinical and Laboratory Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
MCR	Mobile Colistin Resistant
MDR	Multi Drug Resistant
PDR	Pan-drug Resistant
SUST	Sudan University of Science and Technology
UTI	Urinary Tract Infections
VAP	Ventilator associate pneumonia
WHO	World Health Organization
WGS	Whole Genome Sequence
XDR	Extensively Drug Resistant
AMR	Antimicrobial Resistance

CHAPTER I

INTRODUCTION

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1. INTRODUCTION

1.1. Background

Antimicrobial resistance (AMR) is a major threat to human health globally; the distribution of antimicrobial resistant bacteria has had a significant impact on human health because they can cause death, spread to others and have economic consequences. In the review commissioned by the UK government argued that AMR could kill 10 million people per year by 2050, and in a study conducted in 2019 on 204 countries and territories to estimated deaths and disability-adjusted life years (DALYs) as the first comprehensive assessment of the global burden of AMR they found that; there were an estimated 4·95 million deaths associated with bacterial AMR in 2019 including 1·27 million deaths attributable to bacterial AMR and they found that *Pseudomonas aeruginosa* is the one of six leading pathogens for deaths associated with resistant (O'Neill, 2016; Fernandez, 2022).

Pseudomonas is one of top non fermenting Gram-negative bacilli with clinically wide range of infections nearly impossible to eradicate, continuous pressure in hospitalized and immune-compromised patients, also, *Pseudomonas* is tend to have many resistance mechanisms beside its intrinsic resistance, include adaptive resistance and acquired resistance which is unable to return to susceptibility. Besides that, development of newer antibiotics has slowed down and emergence of Multi drug resistance MDR isolates has led to the re-emergence of the 'old forgotten' antibiotic "Colistin" as the last drug option, whose use had almost stopped (after the 1970s) due to the high incidence of nephrotoxicity and neurotoxicity (Sousa and Pereira, 2014; Schwarz and Johnson, 2016).

Polymixins are cationic cylic oligopeptides bind to LPS of Gram-negative bacteria outer membrane and lead to cell lysis, whereas it is not active against some Gram-negative aerobic bacilli, Gram-negative and Gram-positive aerobic cocci, Gram-positive aerobic bacilli, some anaerobes, fungi and parasites. It also used in veterinary beside human

treatment in food producing animal as prevention and treatment of infection caused by Gram-negative bacteria, only polymyxin E (Colistin) and polymyxin B are used clinically in humans. Increasingly use of Colistin in food producing animal which lead to increase the present of MDR *Pseudomonas* and contributing to the emergence of resistance to this drug (Liao *et al*., 2022 ; Okdah *et al*., 2018).

Acquired resistance to Colistin can be mutational result from modifications of the bacterial cell surface or transferable by plasmid-mediated genes, the first report of plasmid mediated Colistin resistance gene mcr-1 was reported in 2015 in China. Till now they are ten mcr genes with variants of them. There were two described surveillance methods to detect mcr1 to mcr 9 using Multiplex PCR, the detection of Colistin resistance is technically very complicated and WHO recommended to confirm Colistin resistance using standerd method other than disc diffusion and determine the possible presence of mcr genes (Bortolaia. *et al*., 2021).

1.2 Rationale

Pseudomonas is a high-resistance bacteria and in the clinical laboratories when the primary culture plates of organisms are resistant to a range of commonly used antibiotic discs this may be suggestive of *P.aeruginosa* and clinicians may face very limited treatment options as a result of the spread of Multi drug resistance strains. Nowadays, wild world distribution of Colistin resistance is reported (Vatansever *et al*., 2020; Barut Selver *et al*., 2019; Azimi and Lari, 2019). Most medical laboratories in Sudan use disc diffusion methods to report Colistin resistance, which may contribute to the unawareness of resistance; the testing is a challenge due to the cationic nature of Colistin, which causes it to adhere to the negatively charged polystyrene surfaces used in routine laboratory plates and high molecular weight of Colistin make it difficult to diffuse. Detection of Colistin resistance in Sudan, which is a country known for its high burden of infectious diseases and the presence of low or no restriction on the antimicrobial use in both veterinary and medicine, indicates the emergence of untreatable diseases in our area and the possibility of horizontal transferring Colistin resistance genes (mcr genes) by plasmid to highly resistant bacteria. Besides that, there is a limit published data are available on the prevalence of mcr genes in *Pseudomonas* in Sudan, so this study was conduct to fill this gab and the obtained results may spot light in such area.

1.3 Objectives

1.3.1 General objectives

To detect the presence of Colistin resistance genes (mcr-1,2,3,4 and mcr-5) genes of *Pseudomonas aerogenosa* isolated from different specimens in Khartoum State.

1.3.2 Specific objectives

1. To purified and re-identification the clinical isolates by conventional bacteriological methods.
2. To determine the antimicrobial susceptibility patterns of the commonly used antibiotics against *Pseudomonas aerogenosa* isolates from different clinical specimens.
3. To determine the occurrence of plasmid-mediated Colistin resistance genes (mcr-1,2,3,4 and mcr-5) of *Pseudomonas aerogenosa* clinical isolates using multiplex PCR.
4. To find out the possible association between mcr1 to mcr5 genes and multi drug resistance isolates of *Pseudomonas aerogenosa*.
5. To associate between the presences of mcr-1to mcr-5 and age, gender of patients and antimicrobial susceptibility testing.

CHAPTER II

LITERATURE REVIEW

CHAPTER II

LITERATURE REVIEW

2.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa belonged to *Pseudomonads* which are non-fermentative, Gram-negative rods that are widely distributed in nature as saprophytes, or as commensals and opportunistic pathogens of plants, animals and humans. In medical microbiology, of species that are opportunist pathogens in an immune-compromised patient, *Pseudomonas aeruginosa* is pre-eminent, followed by *P.fluorescens*, *P.putida*, *P.pickettii*, and *P.stutzeri*. All *Pseudomonads* are aerobic, motile, with mono- or mulitrichous polar flagella, oxidase, and catalase-positive. Many species produce characteristic water-soluble pigments. The fluorescent *Pseudomonads*, *P.aeruginosa*, *P.fluorescens*, and *P.putida*, produce pyoverdin (fluorescein), a yellow fluorescent pigment that acts as a bacterial siderophore. Also, *P.aeruginosa* produce phenazines pyocyanin (blue) and pyorubrin (red), demonstration that the organism produces pigment pyocyanin confirms the identification of a *Pseudomonads* as *P.aeruginosa*. Rare isolates of *P.aeruginosa* produce the dark brown pyomelanin pigment. *P.aeruginosa* growth readily on most common diagnostic media, on blood agar may produce diffuse haemolysis. In cultures after 24 h, six distinct colonial types may be observed, and type I is the most common (large eye shape colonies), may produce grape-like smell of aminoacetrophenone, and can grow in wide range of temperatures 4- 42 ℃ (Tille, 2015)

P.aeruginosa is a classic opportunist pathogen with innate resistance to many antibiotics and disinfectants, it's found in warm moist situations. Their isolation is significant only when there is a risk of transferring it to compromised patients as hospitalized patients. It belonged to ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species) that associated with MDR nosocomial infections such as pneumonia, urinary tract infections (UTIs), surgical site infections and bloodstream

infections, escaping the currently available antibiotic regimen (Yoo *et al*., 2022 and Lister *et al*., 2009).

2.2 Antimicrobial resistance

2.2.1 History of Antibiotics and resistances

For thousands of years, humans have been powerless victims of a variety of illnesses by bacteria, many of which have reached epidemic proportions and killed millions, the first antibiotic, salvarsan, was used to treat syphilis in 1910, but it quickly faded when it was discovered that the drug had potentially fatal side effects. Within a century, the continuous discovery of antibiotics changed modern medicine; the discovery of penicillin in 1928 introduced a golden age of natural antibiotic research that lasted until the mid-1950s, Since then, a gradual decline in antibiotic discovery, misuse, and abuse in human and veterinary medicine has led to the current antimicrobial resistance crisis, and antimicrobial resistance (AMR) has become a global threat to public health systems in the last two decades (Hutchings *et al*., 2019; Mohr, 2016).

Antimicrobial-resistant diseases are most commonly caused by Gram-negative bacteria in humans and animals, the most effective environmental organisms are found in this bacterial category. In Europe, Gram-negative infections are responsible for about two-thirds of antibiotic-resistant bacteria-related mortality. Gram-negative bacteria are also responsible for 45–70% of ventilator-associated pneumonia (VAP), 20–30% of catheter-related bloodstream infections, and other infections common in intensive care units, such as surgical site and urinary tract infections (UTIs) (Arzanlou *et al*., 2017). In 2017 WHO published a list of bacteria for which new antibiotics are urgently needed; *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacteriaceae* are at the top of priority (Tacconelli *et al*., 2018). Besides that, as the resistance increase and documents from the different studies around the world specially carbapenems resistance (Ayobami *et al*., 2022; Singhal *et al*., 2022; Alshaya *et al*., 2022), nosocomial infection with this bacteria is also an acritical issue as Gram-negative pathogens display high levels of antibiotic resistance in nosocomial infections and their ability to become more resistant to all existing antibiotics makes them a global hazard (Arzanlou *et al*., 2017 and Ferri *et al*., 2017).

Internationally, the terms multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) are all used. A multitude of definitions determining

multidrug resistance of Gram-negative organisms exist worldwide. The definitions differ depending on their purpose and on the issueing country or organization, MDR was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories, XDR as having non-susceptibility to at least one agent in all except one or two antimicrobial categories and PDR was non-susceptible to all agents in all antimicrobial categories (Zakaria *et al*., 2020 and Wolfensberger *et al.,* 2019).

2.2.2 Classification of antibiotic resistance

There are three categories of antibiotic resistance: intrinsic, acquired and adaptive. The bacterium's specific traits cause intrinsic resistance. There have been few new antibiotics approved for Gram-positive bacteria like *Staphylococcus aureus*. Gram-negative bacterial infections, on the other hand are significantly more difficult to treat and more resist because of their intrinsic resistance to antibiotics. Many Gram-negative bacteria are resistant to a wide range of drugs, as example, because they possess the low permeability outer membrane functions as a barrier to keep antibiotics out; In addition, many bacteria contain efflux systems that pump antibiotics out of the bacterial cell (Arzanlou *et al.,* 2017).

Acquired resistance occurs as a result of mutation or when bacteria obtain resistance genes from other bacteria through conjugation, transduction, or transformation. Antibiotic resistance genes can quickly propagate through a bacterial community. Antibiotic modification/inactivation, antibiotic target alteration, enhanced antibiotic efflux, and reduced antibiotic uptake are all examples of these. Once a bacterium acquired resistance to an antibiotic, it is unable to return susceptibility (Arzanlou *et al*., 2017).

Adaptive resistance is a drop in antibacterial activity against microorganisms that were previously susceptible to that antibiotic agent. It is characterized by a short increase in bacteria's ability to tolerate antibiotics as a result of changes in gene and/or protein expression levels triggered by environmental factors such as stress, nutritional factors, and antibiotic sub-inhibitory levels. Adaptive resistance, unlike intrinsic and acquired resistance mechanisms, which are stable and can be handed down through generations, is transient and usually fades once the antibiotic is removed. This type of resistance has been reported for aminoglycosides and polymyxins (polymyxin B and Colistin) in *Pseudomonas aeruginosa* and other Gram-negative bacilli (Arzanlou *et al*., 2017).

2.3 *A*ntibiotic sensitivity and resistance of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is intrinsically resistant to many antibiotics due to several mechanisms, the most important of which are the low permeability of their outer membrane, the production of AmpC beta-lactamase, and the production of several efflux systems from the resistance-nodulation-cell division family. Antibiotics likely to be most effective are aminoglycosides tobramycin and gentamycin with anti-pseudomonal penicillin or ureido-penicillins. Carbapenems, monobactam azetreonam, and non-toxic ceftazidime are also use. Quinolones, in particular ciprofloxacin have provided a major advance as the first highly active antipseudomonal agents effective by oral administration (Tille, 2015).

Due to their high levels of intrinsic and acquired resistance mechanisms, most drugs are known to be resistant to *P. aeruginosa* strains. In addition, *P. aeruginosa* adaptive antibiotic resistance is a novel strategy that combines biofilm-mediated resistance and the development of multidrug-tolerant persisting cells to cause infection resistance and recurrence. Because of the significant rise in the prevalence of *Pseudomonas* resistant to fluoroquinolones and aminoglycosides, as well as all B-lactam, this lead to the reconsideration of Colistin as a valid therapeutic option where no other less toxic or effective antibiotic is available (Bialvaei and Samadi Kafil, 2015; Pang *et al*., 2019).

2.4 Colistin

Colistin is frequently the sole effective antibiotic against multidrug-resistant bacteria, its a member of the polymyxin family of antimicrobials, which were first isolated from the spore-forming soil bacterium *Paenibacillus polymyxa*. Polymyxins A, B, C, D, and E are molecules in this group, however only polymyxin E (Colistin) and polymyxin B are used clinically in humans. Polymyxin B is made up of polymyxins B1, B1-I, B2, B3, and B6. Colistin is made up of polymyxin E1 and E2, two bactericidal pentacationic lipopeptides. Polymyxin B is a bactericidal agent that kills nearly all Gram-negative bacteria, targets the lipid A moiety of lipopolysaccharide (LPS), moving its cationic charges, by increase of cell permeability leading to cell wall lysis and bacterial death. Colistin has an excellent antibacterial activity mainly against Gram-negative bacteria such as *P. aeruginosa, Escherichia coli, Enterobacter spp., Salmonella spp., Shigella spp., Klebsiella spp.,* and *Acinetobacter baumannii,* but not against *Burkholderia, Serratia, Proteus spp., Providencia spp, Morganella morganii, Serratia spp. Brucella spp.,* and *Neisseria spp.* (Bialvaei and

Samadi, 2015 ; Bortolaia. *et al*., 2021). However, polymyxin B and Colistin suffer from major issues in safety (dose-limiting nephrotoxicity, neurotoxicity, and acute toxicity), pharmacokinetics (poor exposure in the lungs) and efficacy (negligible activity against pulmonary infections). It is commonly given as the Sodium salt of Colistin methanesulfonate, which is an inactive prodrug, via injection or inhalation (the latter for individuals with cystic fibrosis, for example). It is less toxic than Colistin sulfate, which is used orally (with extremely limited absorption) or topically in patients with hematological illnesses or in Intensive Care Units (ICU) for selective gut decontamination everely limited their clinical utility (Prasannan *et al*., 2021; Roberts *et al*., 2022).

2.5 Colistin resistance mechanisms reported in *Pseudomonas*

Colistin resistance mechanisms have not been completely understood, but there are several possible mechanisms can be adaptive or acquired including both plasmid-mediated and chromosomal mutation. Since Colistin activity against cell membrane most study suggested the resistant mechanism occur in LPS, this may include efflux pump system and modification of cell membrane (Bortolaia. *et al*., 2021)

2.5.1 Modification of Lipopolysaccharide duo to chromosomal modulation

The principal of polycationic antimicrobial peptides resistance mechanism in Gram-negative bacteria is lipopolysaccharide (LPS) modification, which reduces the electrostatic affinity of LPS for positively charged Colistin via cationic groups 4-amino-l-arabinose (L-Ara4N) and phosphoethanolamine (pEtN), a protein for the whole process are encoded by a large operon named arnBCADTEF-ugd (arn). Operon arn expression is upregulated in response to outer membrane damage or perturbations via at least four two-component systems (TCSs) PhoPQ, PmrAB, ParRS, and CprRS (Puja *et al*., 2020). In chromosomal mutation it has been reported that; bacterial surface modifications occur as a result of overexpression of chromosomally mediated two-component system genes (PmrAB and PhoPQ) and mutation in lipid Abiosynthesis genes, which results in loss of the ability to produce lipid A and thus LPS chain (Mohr, 2016).

To understand the mechanism, firstly must keep in mind some facts: the pmr CAB operon encodes three proteins: (a) PmrA, a pEtN response regulator, (b) PmrAB, a sensor kinase protein, and (c) PmrC, a phosphotransferase. Also, the synthesis of the L-aminoarabinose group (L-Ara4N) on LPS occurs by activation of pmrHFIJKLM and pmrE

gene expression. But, the PmrAB two-component regulatory system is encoded by PmrA and pmrB, which are activated by various environmental stimuli such as low pH (5.5), ferric (Fe3+) iron, macrophage phagosomes aluminum (Al3+), etc., and results in PmrB activation. In turn, pmrB activates PmrA. However, the PmrA activates transcription of the pmrCAB operon, which was described before, and the attached pmrE gene results in LPS modifications with the addition of cationic pEtN and L-Ara4N moieties. Another PhoPQ two-component system encoded by PhoP and PhoQ genes expresses two proteins: (a) regulator protein PhoP and (b) sensor protein kinase PhoQ. Moreover, transcription activation of the pmrHFIJKLM operon occurs by PhoP, resulting in L-Ara4N addition to the LPS membrane. The PmrA protein is also activated by the PhoP gene either directly or indirectly via a connector protein (PmrD), causing the addition of pEtN to the LPS. Acquired Colistin resistance was reported in *K. pneumonia* and *E. coli* (Gogry *et al*., 2021). In a previous study done in 2014 in Korea about mutations and expression of PmrAB and PhoPQ related to Colistin resistance in *P. aeruginosa*, they concluded amino acid alterations in PhoPQ and PmrAB are associated with polymyxin resistance in clinical *P. aeruginosa* isolates (Lee and Ko, 2014).

Over past years, it has become evident that higher levels of resistance to polymyxins can be reached by the pathogen when mutations in genes pmrB, phoQ, parS, parR, cprS, and/or colS stably activate the corresponding TCSs, thus causing constitutive overexpression of operon arn and subsequent LPS modification (Muller *et al*., 2011, Puja *et al*., 2020, Gutu *et al.*, 2013). PhoPQ and PmrAB mutations are not the only mechanisms, there are different previously described acquisition mechanisms for Gram-negative bacteria, including *Pseudomona*s but these studies need more research and still do not fully cover the acquired resistance of *Pseudomonas* trigger by environmental conditions (Lee and Ko, 2014).

2.5.2 Role of Efflux Pumps MexXY/OprM

ParRS is a key element in the tolerance of *P. aeruginosa* to the last-resort antibiotic. In study conducted by Puja *et al.,* in 2020 in efflux pump MexXY/OprM they say it contributes to the tolerance and acquired resistance of *Pseudomonas aeruginosa* to Colistin. The mexXY genes belong to the ParR regulon and are de facto overexpressed in polymyxin-exposed bacteria through activation of ParRS. Also, deletion of the mexXY genes in a

constitutively activated ParR mutant was associated with significantly increased expression of the genes arnA, and pmrA in the absence of Colistin exposure, thereby highlighting a functional link between the MexXY/OprM pump and Ara4Nbased modification of LPS. Furthermore, this study found that both stable upregulation of the arn operon and drug-induced ParRS-dependent overexpression of the mexXY genes accounted for the elevated resistance of pmrB mutants to Colistin (Puja *et al.*, 2020).

2.5.3 Plasmid-mediated Colistin resistances

Plasmid-borne mcr genes encoding phosphoethanolamine transferases protein, lead to a reduction of the negative charge of LPS upon structural alteration of lipid A have been described as an important mechanism conferring decreased susceptibility to Colistin (Wang *et al.*, 2020). Plasmid-mediated genes confirm the resistance of Colistin. Till now they are 10 mcr (mcr1 to mcr 10) (Fernandez, 2022).

Table (1): Mobile Colistin resistance genes mcr1 to mcr 10 and their discover

Gene	Publication
Mcr-1	**Liu Y, 2016**
Mcr-2	**Xavier BB, 2016**
Mcr-3	**Yin W, 2017**
Mcr-4	**Carattoli A, 2017**
Mcr-5	**Borowiak M, 2017**
Mcr-6	**AbuOun M, 2018**
Mcr-7	**Yang Y, 2018**
Mcr-8	**Wang X, 2018**
Mcr-9	**Kieffer N, 2019**
Mcr-10	**Wang C, 2020**

2.5.3.1 Mcr-1

Colistin is widely used in veterinary medicine, particularly in the management of diarrhoeal illnesses in poultry and pig production. Mcr-1 was initially discovered in late 2015 in *E. coli* isolates from food animals and their meat gathered in China between 2011-2014, as well as in *E. coli* and *K. pneumoniae* isolates collected from human patients in China in 2014. This was accomplished by sequencing the entire genomes of all *E. coli* isolates and then comparing the assembled sequences to sequence data from two databases to check for the availability of the mcr-1 gene (Liu *et al.*, 2016).

Mcr-1 is a phosphoethanolamine transferase that modifies cell membrane lipid A head groups with a phosphoethanolamine residue, reducing affinity to Colistin. Another interesting fact is that the mcr-1 gene has been present for a long time, but undetected. In the 1980s Shen, identified a mcr-1-positive isolate from hens in China. Despite the fact that Colistin had been widely used in food-producing animals for many years, it was not available for human clinical use in China until 2017, signaling that veterinary usage of Colistin acted as a selection pressure for the expansion of mcr-1. Furthermore, bacterial isolates containing mcr-1 demonstrated complex resources such as human-linked settings and natural ecosystems, food, animals, and humans (Gogry *et al*., 2021). On other hand, some studies show that mcr-1-positive isolates frequently possess multiple resistance genes (Skov and Monnet, 2016).

The global spread of mcr-1 predated its identification as a Colistin resistance gene, and it was likely aided by human travel, as evidenced by the discovery of the gene in enteric bacteria from travelers returning to Europe after visiting countries with high mcr-1 prevalence in South America, Asia, and Africa (Liu *et al.*, 2016, Skov and Monnet, 2016, Boncompagni *et al.*, 2022).

2.5.3.2 Mcr-2 to Mcr-10

In 2016 mcr-2 was isolated from *E. coli* and *Salmonella* spp. from pigs, cattle, and chickens in Belgium from isolates that did not show the presence of mcr-1. The mcr-2 gene, as they termed, is 1,617 bp long, nine bases shorter than mcr-1 (1,626 bp), and shows 76.75% nt identity to mcr-1 (Xavier *et al*., 2016).

Mcr-3 was firstly discovered in human and animal *E. coli*, *Proteus* spp. and largely exists in aquatic animal-borne *Aeromonas* species. in countries in Asia, Europe, and South

America. They found this mobile Colistin resistance gene was already widely disseminated, therefore, it was recommended to screen the mcr-3 gene urgently in the surveillance of Colistin-resistant Gram-negative pathogens from animals, humans, and the environment (Yin *et al*., 2017). Mcr-3 expression also impairs phagocytosis efficiency both in vivo and in vitro, contributing to the increased persistence of mcr-3-positive bacteria in tissues compared with parental strains. Unlike mcr-4 which was discovered in the same year and distributed in Belgium, Italy, and Spain. It was isolated from *E. coli* and *Salmonella enterica serovar Typhimurium* from pigs and humans. mcr-5 isolated from poultry and poultry meat in of *S. enterica* serovar Paratyphi B d-tartrate fermenting and mcr-5-2 in porcine *E. coli* in Germany (Carattoli *et al*., 2017; Borowiak *et al*., 2017 ; Hammerl *et al*., 2018).

One year later, new three noval mcr was also documented; mcr-6 in *Moraxella* spp. isolates from pigs in the United Kingdom, mcr-7 in *K. pneumoniae* from chickens in China, mcr-8 in *K. pneumoniae* from humans and pigs in China (AbuOun *et al*., 2017; Yang *et al*., 2018 ; Wang *et al*., 2018).

In 2019 mcr-9 was isolated from a Multidrug-Resistant, Colistin-Susceptible *Salmonella enterica* Serotype Typhimurium Isolated from 2010, most closely resembled mcr-3. Pairwise comparisons of the predicted protein structures associated with all nine mcr homologues (Mcr-1 to -9) revealed that Mcr-9, Mcr-3, Mcr-4, and Mcr-7 share a high degree of similarity at the structural level (Carroll *et al.,* 2019). mcr-10 was first isolated from a clinical strain of *Enterobacter roggenkampii* in China (Wang *et al*., 2020).

2.6 Colistin resistance detection methods

2.6.1 Phenotypic detection:

Many challenges exist in phenotypic testing for Colistin susceptibility (false susceptible), including ability of polymyxin to diffusion in agar, which impairs the effectiveness of both disc diffusion and gradient diffusion procedures, and polymyxins' ability to adhere to the surface of plastics (e.g., the plastic of microtitre trays used for broth microdilution and of pipette tips) (Bortolaia. *et al*., 2021).

2.6.1.1 Broth microdilution method:

In 2015, both the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) joint working group recommended the use of broth microdilution (BMD) as the reference method (Group,

2018); Colistin should be examined using cation-adjusted Mueller-Hinton broth according to the International Organization for Standardization standard broth microdilution method (ISO 20776-1). No additives (including polysorbate-80 or other surfactants) may be used in any part of the testing, because polysorbate 80, for example, can act synergistically with polymyxins, artificially lowering the minimum inhibitory concentration (MIC). Trays must be manufactured of simple polystyrene, and polymyxin sulfate salts must be utilized (the methane sulfonate derivative of Colistin cannot be used as it is an inactive pro-drug that breaks down slowly in solution). Quality control is critical in detecting Colistin resistant phenotypes. Inclusion of a strain with low-level Colistin resistance is recommended to ensure that the procedure produces valid findings (Fernandez, 2022).

Colistin looks to be a poor mono-therapeutic option based on clinical outcomes, especially when alternative medications are available. Colistin dosage, in particular, is complicated since its pharmacokinetics vary greatly between patients. As a result, additional clinical outcome analysis is required to verify whether existing defined breakpoints and epidemiological cut-off values appropriately inform clinical decisions (Fernandez, 2022).

There are many commertionaly devise for microdilution method include UMIC Colistin (Biocentric, Bandol, France), MIC strip Colistin (MERLIN Diagnostika Bornheim-Hersel, Germany), sensitest Colistin (Liofilchem, Roseto degli Abruzzi, Italy), the sensititre System (Thermo Fisher Scientific, Waltham, MA, USA), microScan walkAway (Beckman Coulter, San Diego, CA, USA), vitek 2 (BioMérieux, Marcy l'Étoile, France), and BD Phoenix™ (Becton Dickinson, Le Pont de Claix, France) as described by (Bardet and Rolain, 2018).

2.6.1.2 Colistin Broth Disk Elution (CBDE)

The Colistin broth disk elution (CBDE) test involves placing 0, 1, 2, and 4 (10-g) Colistin disks in four distinct 10-ml cation-adjusted Mueller-Hinton broth tubes. These tubes are then incubated at room temperature to allow the Colistin from the disks to elute, resulting in supposed Colistin concentrations of 0, 1, 2, and 4 g/ml in these tubes, respectively, isolates of *P. aeruginosa* and *Acinetobacter baumannii* with Colistin MICs of ≥4 μg/ml are considered to be resistant to Colistin by the Clinical and Laboratory Standards Institute (CLSI) (Dalmolin *et al*., 2020).

2.6.1.3 Rapid Colistin NP test

This is a modification of polymyxin B NP test by Nordmann and Poirel, Colistin stock solution containing 0.2 mg/mL Colistin sulfate and rapid Colistin NP solution (pH = 6.7) with the necessary concentrations of CAMH broth, phenol red, distilled water, and D-glucose. As described by Nordmann and Poirel, the test was carried out in 96-well U-bottom sterile polystyrene microtiter plates with lids. Colistin resistance was derived from a change in the color of the NP solution (from orange to yellow) caused by a pH change due to bacterial growth in Colistin-containing wells (Nordmann *et al.*, 2016).

2.6.1.4. Selective agar (SuperPolymyxin, CHROMAgar COL-APSE, ChromID Colistin R agar)

Chromogenic media are widely used for screening because they allow bacteria to grow as properly colored colonies. The super polymyxin screening medium was the first agar medium for detecting intrinsic and acquired Colistin resistant Gram-negative rods. The commercial version of this medium is super polymyxin medium (ELITechGroup, Puteaux, France) for the identification of Colistin-resistant strains, including those with low MIC values (mg/L) that contain the mcr1 *(*Nordmann *et al.,* 2016).

CHROMagar COL-APSE is a sensitive and specific medium for Colistin-resistant bacterial pathogen growth. Because of the low detection limit (10^1 CFU), it may be useful as a primary isolation medium in the surveillance and recovery of Colistin-resistant bacteria from complex human, veterinary, and environmental samples, particularly those with plasmid-mediated mcr-1 or novel polymyxin resistance mechanisms (Momin *et al*., 2017).

In early 2018, a new chromogenic medium, CHROMID Colistin R agar, was introduced to the market, allowing for the screening of Colistin-resistant Enterobacteriaceae in clinical samples. The COLR medium employs borderline Colistin concentrations to classify strains as susceptible or resistant. This chromogenic medium is a qualitative detection method for Enterobacteriaceae and does not allow for the determination of Colistin MIC values against the test bacterial strains; thus, it should only be used as a screening test. On it, Colistin-resistant strains form colored colonies, with the color varying depending on the species. Colistin-resistant isolates, on the other hand, do not grow on the COLR plate (García-Fernández *et al*., 2019). The LBJMR medium is a new polyvalent culturemediumfor

the isolation and selection of Colistin-resistant bacteria and vancomycin-resistant bacteria (Bardet *et al*., 2017).

Agar dilution is used in the chromogenic method. Nonetheless, EUCAST does not recommend it for determining bacterial susceptibility to Colistin because the detectability threshold rises as the bacterial inoculums grow (Matuschek *et al*., 2018). There are specific phenotypic screening methods for the detection of mcr-1 includes matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) and inhibition of mcr-1 activity (Bardet and Rolain, 2018).

2.6.2 Genotypic detection

The reference test for detection bacterial mcr genes are polymerase chain reaction (PCR) (detect only known mcr genes), detection of mutation of genes can done after amplification by sequencing, and whole genome sequence (WGS) help in detect known or unknown Colistin resistance genes (Bardet and Rolain, 2018).

RT-qPCR or multiplex PCR can use to detect mcr genes as describe by previously protocols for screen and surveillance. The first multiplex for detection of mcr1-5 (Rebelo *et al*., 2018), and the second for mcr 6-9 (Borowiak *et al*., 2020). On the other hand, Microarray can use for mcr-1 and its variants (from mcr-1.2 to mcr-1.7 and mcr-2 genes), but it was not able to detect the other mcr genes (Bernasconi *et al.*, 2017).

2.7 previous studies

2.7. Previous studies of mcr genes in *Pseudomonas* around the world

There are many studies around the worlds to detect Colistin resistance mechanisms and mcr genes in *Pseudomonas* and others Gram-negative bacteria. In Pakistan, in 2019 there were the first report of mcr-1 in in *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, among 16 Colistin resistant isolates, the mcr-1 gene was detected in one *A. baumannii* ("1.61%" of total isolates; "16.6%" of Colistin resistant isolates) and one *P. aeruginosa* strain (1.19% of total isolates; 10% of Colistin resistant isolates) (Hameed *et al*., 2019). Another one in Iran in order to detect co-harboring of mcr-1 and β-lactamase genes, out of the 69 isolates of *P. aeruginosa*, mcr-1 gene were found in 10 isolates (14.49%), with low resistance to Colistin (14.49%) (Tahmasebi *et al*., 2020).

In Brazil there was a study for molecular detection of drug-resistance genes of blaOXA-23-blaOXA-51 and mcr-1 in *Pseudomonas aeruginosa*, they found one mcr-1 gene in Colistin resistance strain (Nitz *et al*., 2021).

In India, in first report of emergence of mcr-1 in *Pseudomonas*; out of the 5 Colistin in resistant *P. aeruginosa*, 4 isolates harbored mcr-1 as confirmed by PCR and sequencing (Pathak *et al*., 2020).

In Egyptian study that preformed in 2020, they found that, no isolates were positive for mcr-2 gene while 50% of Colistin-resistant isolates were positive for mcr-1. Efflux mechanisms were detected in 3 isolates (Abd El-Baky *et al*., 2020).

In 2018, large study on mcr-3 in china in Gram-negative isolates in 2016 and 2017, to detect the spread of mcr-3 within 13 provinces and provide a complete characterization of its evolution, structure and function, they found 9 samples (49/6497 = 0.75%) were *mcr-3* positive (Xu *et al.*, 2018).

2.7.2 Previous studies of mcr genes in Sudan

In Sudan, there are many studies on the detection of mcr genes in bacteria, the first report on mcr gene was done in 2016 to detect the mcr-1 gene in *Enterobacteriaceae*, and found mcr-1 gene in 7 of 50 isolates, mostly in *E. coli* (Mohammed, 2016). Another study was done to detect mcr-1 in Gram-negative isolates from a patient with urinary tract infection, 35 of 91 isolates were positive for the mcr-1 gene, 1 in *Citrobacter* species, 31 in *E.coli*, 1in *K.pneumoniae*, and 2 in *P.auroginosa* (Ali, 2017).

Another study also done in mcr-1 was carried out in 50 *K.pneumoniae* isolates and only one isolate showed positive for the mcr-1 gene (Babiker, 2020). In 2020, other study was done in Gram-negative bacteria to detect the mcr-1 gene also, and they found that 1 isolate of *Pseudomonas* carries the mcr-1 gene, isolated from 8 years old male and the isolate showed susceptibility to Colistin using the disc diffusion method (Zakaria *et al*., 2020).

The first detection of mcr-2 was done using a multiplex PCR surveillance protocol to detect mcr1 to mcr 5, from 165 isolates 11 were positive for mcr genes, they detected mcr-2 in one *Klebsiella* isolate and 5 were positive for mcr-1from another *Klebsiella* isolates, also one *Pseudomonas* isolate was positive for mcr-1 (Musa *et al*., 2021).

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

3.1 Study design

This study design is a descriptive cross-sectional laboratory based study.

3.2 Study area

This study was conducted in Khartoum State. *P.aeruginosa* isolates were collected from different hospitals (Royal Care, Fedial, Alzaytouna, Omer Sawi, Alraqi, Khartoum ENT, and Khartoum North Teaching Hospital). Laboratory tests were performed at the Sudan University of Science and Technology (SUST) and Exon lab for Molecular biology.

3.3 Study duration

This study was conducted during the period from January 2022 to August 2022.

3.4 Study subjects

The isolates of *P.aeruginosa* were obtained from different hospitals, and isolated from different clinical specimens (18 Urine, 11 Wound swabs, 6 Blood, 2 High vaginal swabs, 8 Sputum, 3 Ear swabs and 2 Body fluids).

3.5 Ethical consideration

Ethical approval to conduct this study was obtained from the Scientific Research Committee, Collage of Medical Laboratory Science, Sudan University of Science and Technology and from manges of selected hospitals.

3.6 Sample size

A total of fifty non-repetitive *P.aeruginosa* clinical isolates (n=50) were collected for this study.

3.7 Sampling technique

Non-probability convenience sampling technique.

3.8 Method of data collection

Data included sample type, gender and age of patients were collected from hospitals' Medical records.

3.9 Laboratory tests

3.9.1 Samples purification and identification

One colony from each isolate was sub-cultured on Cysteine Lactose Electrolyte Deficient media and Nutrient agar then incubated aerobically at 37°C overnight for further re-identification, purification and preservation.

3.9.2 Colonial characterization

Most *P. aeruginosa* showed large, low convex, smooth blue-green non-lactose fermenting, rough, and often oval shape colonies on CLED, some with blue-green diffusible pigments. (Appendix II-color plate (1)).

3.9.3 Gram stain

Smear was prepared from overnight culture on a dry slide, left to air dry, and fixed by pass of the slide three times rapidly through the flame of a Bunzen burner. Crystal violet stain was added to smear for 60 seconds and then washed with tap water. Lugol's iodine (mordant) was added for 60 seconds, then washed in tap water and decolorized rapidly with acetone alcohol and washed after 7 secs by tap water. Finally, the smear was covered with Safranin stain for 2 minutes and washed with tap water. The back of the slide was wiped clean and placed in a draining rack for air dry. A drop of oil was added to the dried smear and examined under the light microscope by oil lens 100X (Tille, 2015).

3.9.4 Oxidase test

Single and pure colony was taken by wooden stick and put in a commercially available paper disk with the substrate (1% tetramethyl-p phenylenediaminedihydrochloride), the purple color that appears within 60 seconds indicates positive results (Tille, 2015).

3.9.5 Indole test

A sterile loop was used to inoculate the tested organism into 1 ml peptone water, the tube was incubated at 37°Cfor 24 hrs. in the next day afew drops of Kovac's reagent (4 (p) – dimethylaminobenzaldehyde). *P.aeruginosa* is negative indole (no red ring appears) (Tille, 2015).

3.9.6 Urease test

The tested organism was inoculated into the slope surface of Christensen's urea medium with phenol red as an indicator using sterile straight loop, the medium was incubated at 37

°C for 24 hrs. Change in color of the indicator to purple-pink means a positive result (Tille, 2015).

3.9.7 Kligler Iron agar

The tested organism was inoculated in KIA medium, using a straight loop. Agar butt was stabbed, then the top slope was streaked (as a zigzag), the medium was incubated at 37°C for 24 hrs. Glucose fermentation, lactose fermentation, H_2Sproduction and gas production were observed *P.aeruginosa* give red butt and slope with no gas and no H_2S production (Tille, 2015).

3.9.8 Citrate Utilization test

The tested organism was inoculated of Simmon citrate medium with bromothymol blue as an indicator using sterile straight loop, then the medium was incubated at 37°Cfor 24 hrs, change in color of the indicator from light green to blue color or growth of streaking bacteria mean a positive result. *P.aeruginosa* is citrate positive (Tille, 2015).

3.9.9 Sample Preservation

The isolated organisms were stored in Research Laboratory in Sudan University of Science and Technology in 15% glycerol peptone water at 4°C.

3.10 Susceptibility test

All confirmed isolates were subculture from 15% glycerol in neutral agar getting overnight and pure growth. Kirby bauer disc diffusion tests were performed and results were interpreted according to the Clinical and Laboratory Standards Institute 2022. (Appendix II-color plate (3)). All isolates were tested on Muller-Hinton agar plates (pH 7.2-7.4), the surface was inoculated lightly and uniformly by a sterile cotton swab, the antimicrobial discs were placed and distributed evenly using sterile forceps on the inoculated plate. After aerobic incubation at 35˚C overnight inhibition zones were measured in millimeters (mm) using a ruler over the surface of the MH agar plate and the zones' diameters were recorded and interpreted according to CLSI guidelines 2022 as susceptible, intermediate, or resistant. *P.aeruginosa* ATCC 27853 was use as quality control. Antimicrobial disc (Bioanalyse company, Turkey) were applied on the surface of the agar (appendix (III)).

3.10 DNA extraction

Bacterial genomic DNA was extracted by boiling methods, in biosafety cabinet level II from fresh overnight incubated nutrient slope a loopful of culture was suspended in 200 µl of sterile DW in 1.5 Eppendorf tubes heating for 10 min at 95 °C in thermal block incubator (Chem-Tech).Appendix II- color plate(5), vortex, then cooling at -20 °C for 10 min following by centrifuged for 10 min at 12000×g, the supernatant was carefully collected and store at − 20 °C until use, all samples were checked for quantity using pre-PCR gel electrophoresis (Queipo-Ortuño *et al.*, 2008).

3.12 Detection of mcr-1,2,3,4 and mcr-5 genes using Multiplex PCR

The extraction of genome was tested for detecetion of mcr-1,2,3,4 and mcr-5 using a set of primers as following

3.12.1 PCR Primers for identification of *mcr*-1 to *mcr*-5 genes

Table 3-1: Primers sequence used for amplification of Mcr-1 to Mcr-5 gene

Target gene	Sequence(5'- 3')	Amplicon size (bp)	Reference
***mcr*-1**	F-GTGTGGTACCGACGCTCGC	**460**	(Eiamphungporn *et al.*, 2018)
	R-CAAGCCCAATCGGCGCATC		
***mcr*-2**	F-CAAGTGTGTTGGTCGCAGTT	**715**	(Rebelo *et al*. 2018)
	R-AAATAAAAATTGTTCCGCTTATG		
***mcr*-3**	F-AAATAAAAATTGTTCCGCTTATG	**929**	
	R-AATGGAGATCCCCGTTTTT		
***mcr*-4**	F-TCACTTTCATCACTGCGTTG	**1,116**	
	R-TTGGTCCATGACTACCAATG		
***mcr*-5**	F-ATGCGGTTGTCTGCATTTATC	**1,644**	(Hammerl *et al.*, 2018)
	R- TCATTGTGGTTGTCCTTTTCTG		

3.12.2 Preparation of primers

The primers stock was prepared as 100 *p*mol/µl, from it 10 *p*mol was prepared by dissolved 5µl of each in 45µl sterile nuclease-free water in a new 1.5 Eppendorf tube, vortex, and stored at -20ºC till use.

3.12.3 Master Mix

Master Mix kits (INtRON Maxime PCR PreMix, Korea) containing all reagents for PCR except water, template and primers were used and storage at (-20˚C). PCR was carried out in a 25 µl volume for detection of *mcr*-1 to *mcr*-5 genes in the following volumes in 0.2 ml Eppendorf tube.

Table 3-2: Preparation of Reaction Mixture for *mcr*-1 to *mcr*-5 genes amplification.

Reagents	Volumes
premix	**5 µl**
Nuclease-free water	**13µl**
Primers mixture	**2 µl**
DNA template	**5 µl**
Total volume	**25 µl**

3.12.4 Protocol used for amplification of the *mcr*-1 to *mcr*-5 genes

The amplification was done by using 0.2 ml PCR Eppendorf tubes that were subjected to thermocycler PCR (Bio-Rad; C1000 touch, UK). (Appendix II- color plate (5)), running conditions were: 1 cycle of denaturation at 94 ℃ for 10 min, followed by 25 cycles of denaturation at 94 ℃ for 30 s, annealing at 58 ℃ for 90s and elongation at 72 ℃ for 60 s and a final cycle of elongation at 72 ℃ for 5 min (Rebelo *et al*., 2018).

3.12.5 Preparation of agarose gel

2% agarose gel was prepared for each run by weight of 2 g of agarose powder (iNtRON Biotechnology, Korea) and add a small amount of 1X TAE buffer (Thermo Scientific, Lithuania) mixed and then complete to 100 ml, the mixture was heated by

microwave for 1 min until a clear solution is produced, allowed to cool to 55ºC, then 2µl (0.2 for 10 µl) of Ethidium bromides (iNtRON Biotechnology, Korea) was added, mixed well and poured onto suitable gel tray that was equipped with combs to form wells for loading the PCR products. Any bubbles were removed and the gel was allowed to solidify at room temperature. After solidification, the comb was gently removed.

3.12.6 Electrophoresis of the PCR product

The gel casting tray was flooded by 1X TAE buffer near the gel cover surface, 5µl of PCR products of each sample was loaded into each well. (Appendix II- color plate (6)), then to the first and last wells of casting tray (3 µl) of (100bp) DNA ladder (marker) was injected for each run (Solis BioDyne, Europe). The gel electrophoresis apparatus was connected to the power supply (JY600; Beijing Junji-Dongfang, China). (Appendix II-color plate (7)). Then the electrophoresis was run at (120) V for (120 minutes).

3.12.7 Visualization of PCR product

The gel was visualized under the ultraviolet Trans- illuminator (Bio-Rad, UK), to detect the specific amplified products by comparing with 100 base pairs standard ladders.

3.13 Analysis of data

Data were introduced to Statistical Package for Social Sciences (SPSS) vision 20 software to estimate the *p* value of significance using Chi square test, means, frequencies and averages were also calculated

CHAPTER IV

RESULTS AND DISCUSSION

CHAPTER IV

RESULTS AND DESCUSSION

4.1 Results

The present cross sectional investigation includes 50 of *P. aeruginosa* from different hospitals (Royal care, Fedial, Alzaytouna, Omer Sawi, Alraqi, Khartoum ENT, and Khartoum North Teaching Hospital), Out of 50 isolates 18 (36%) were urine samples, 11 (22%) were wound swabs, 6 (12%) blood, 2 (4%) high vaginal swabs, 8 (16%) sputum, 3 (6%) ear swabs and 2 (4%) from body fluids. After culturing most strains were show pigmentation 32 (74%). The most pigmented strain was found in urine (n=13 (26%)) and wound samples (n= 10 (20%)) (Table 4.1). The male 23 were (46%) where female were 27 (54%) with ratio 0.85:1, ages ranged from 4 to 76 years with an average of 49, majority of the samples (n= 18 (36%)) were found in the age group (40 – 60) year (Table 4.2).

Approximately one-third 18 (36%) of isolates were resistant to Aztreonam, 10 (20%) were resistant to Ciprofloxacine, 11 (22%) resistant to Gentamicin, 10 (20%) to Colistin, 11 (22%) to Amikacin, 11(22%) to Pipracillin/tazobactam combinations. Only one isolate was resistant to Imipenem and from 18 urine samples 3 isolates (17%) were resistant to Norfloxacin (figure (4.1)).

Out of 50 *P. aeruginosa* isolates, only 14 (28%) were MDR, most isolates 18 (36%) were sensitive to all antibiotics, 12 (24%) isolates resist to one antibiotic while 6 (12%) isolates resist to two antibiotics. In MDR isolates we found two strains were resistant to ≥7 antibiotics, whereas just three isolates were found to be resistant to 6 antibiotics

Mcr genes were detected among 35 of *P. aeruginosa* (70%), mostly 28 (56%) were mcr-3, followed by 6 (12%) positive for mcr-2, and mcr-1 was found in one isolate (2%). Mcr-4 and mcr-5 were not detected (Figure (4.2)). There were no association between present of at least one mcr gene when compare with age and gender of patients with *p*-value (0.744) (0.244) respectively, (Table (4.3)).

Among 10 isolates resistant to Colistin by phenotypic method, 4 (8%) were carry mcr genes, 3 (6%) mcr-2 and 1 (2%) mcr-3, on the other hand 27 (54%) of mcr-3 were found in isolates that are sensitive to Colistin, whereas Mcr-2 in 3 (6%) of sensitive isolates. There was a significant association between the presence of mcr genes and the Colistin susceptibility profile using the disc diffusion method (*p-value* 0.04).

Most antimicrobial profiles show assosation with at least one mcr gene and isolates profile (*p*-value <0.05) un like of imepenim (*p*-value 0.109) and Ceftazidime (*p*-value 0.210) table (4.4).

Most Mcr gene were present in Non-MDR isolates 31 (62%), the mean of drugs resisted by *P. aeruginosa* in isolates with out any mcr gene was 3.56 (SD=1.11104) whereas the mean of them in isolates with at least one of mcr gene was 0.9 (SD=2.50250), there was a statistically significant association between the presence of at least one mcr gene and drug resistance with *p*-value = 0.000 (<0.05), which is a moderate negative correlation (R = -0.527) as illustrated in table (4.5).

Table (4.1): Frequency of pigmented strains among different clinical specimens

		Pigment appearance	
		Green Pigmention	**Non pigmented**
Specimens	**Urine sample**	13 (26%)	5 (10%)
	Sputum	7 (14%)	1 (2%)
	Wound	10 (20%)	1 (2%)
	Blood	4 (8%)	2 (4%)
	Ear swab	1 (2%)	2 (4%)
	High vaginal swab	2 (4%)	0 (0%)
	Body fluids	1 (2%)	1 2%)

Table (4.2): Distribution of gender among different age groups

		Gender		
	Age/years	**Male**	**Female**	**Total**
Age group	**<20**	1 (2%)	0 (0%)	1(2%)
	20-40	3 (6%)	10 (20%)	13 (26%)
	40-60	10 (20%)	10 (20%)	20 (40%)
	>60	9 (18%)	7 (14%)	16 (32%)
Total		23 (46%)	27 (64%)	50(100%)

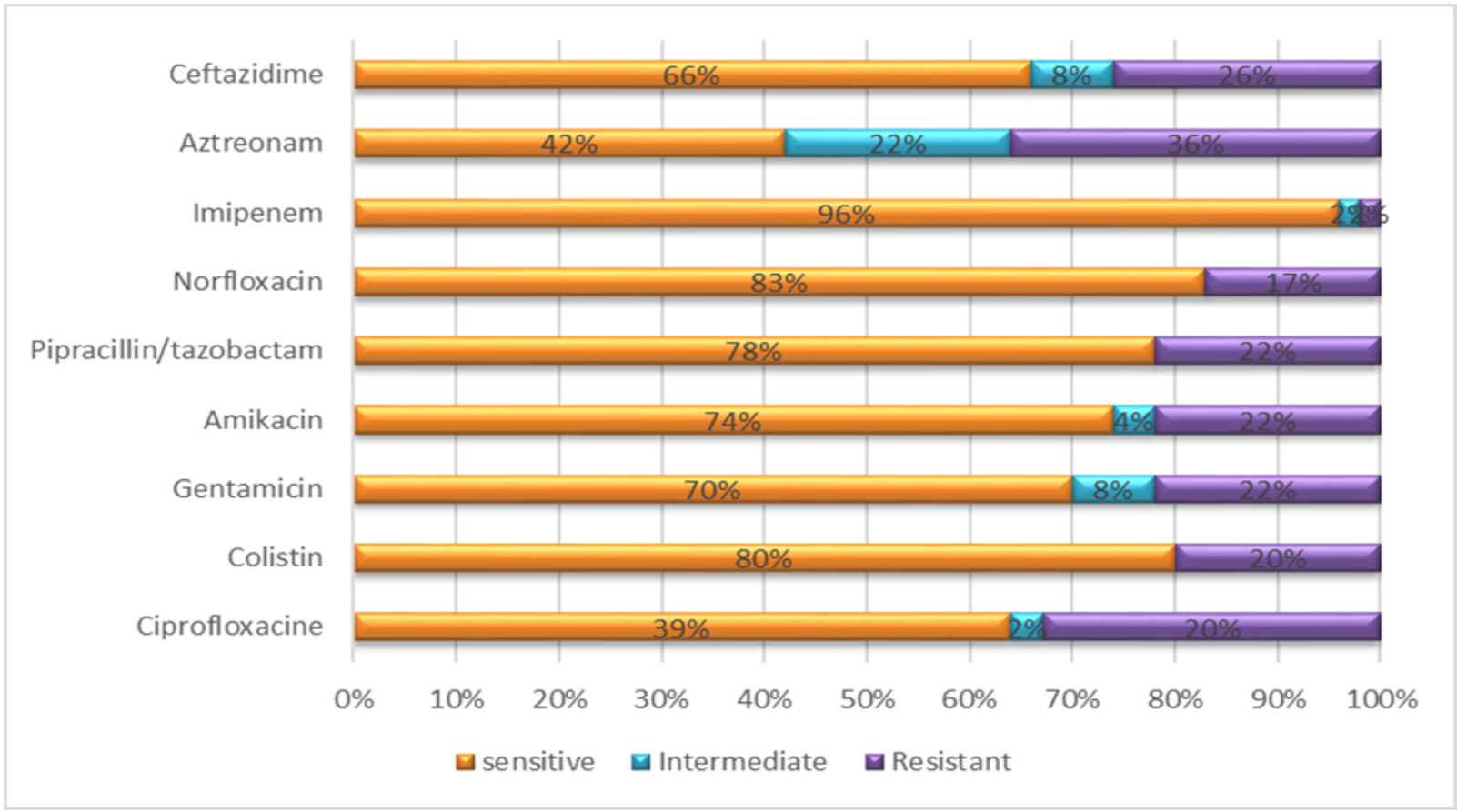


Figure (4.1) Antimicrobial susceptibility patterns of commonly used antibiotics against *Pseudomonas aeruginosa* isolates.

Table (4.3) Demographic characteristics of patients associated with the present of mcr-1 to mcr-3 genes of *Pseudomonas aeruginosa.*

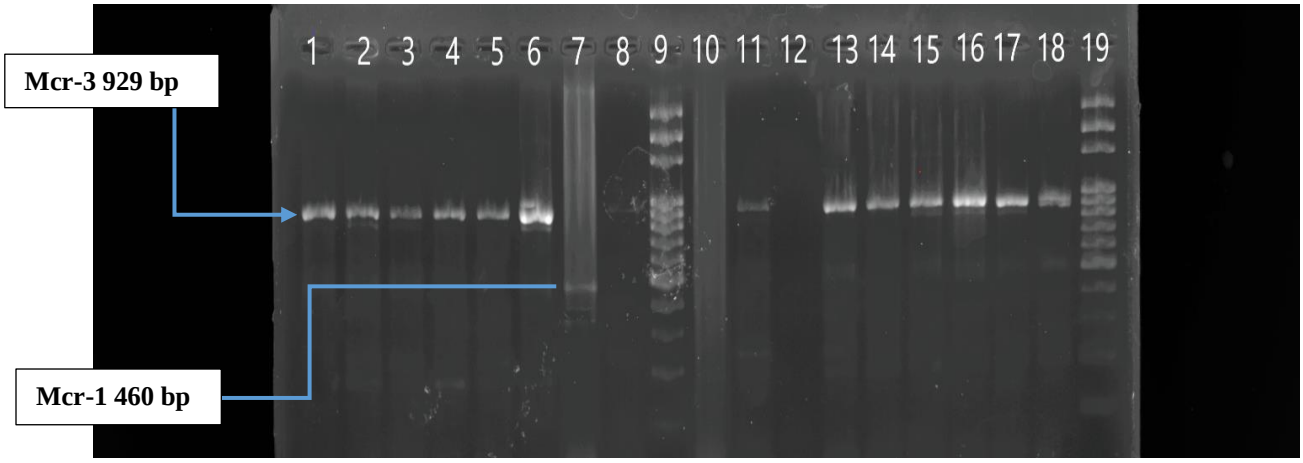
Variables		Present of mcr gene			*p*-value
		Mcr-1	**Mcr-2**	**Mcr-3**	
Age (Years)	<20	0 (0%)	1 (2%)	0 (0%)	0.744
	20 - < 40	0 (0%)	2 (4%)	7 (14%)	
	40 - < 60	0 (0%)	1 (2%)	11 (22%)	
	>60	1 (2%)	2 (4%)	10 (20%)	
Gender	Female	0 (0%)	2 (4%)	18 (36%)	0.244
	Male	1 (2%)	4 (8%)	10 (20%)	

Table (4.4): Correlation between presence of at least one mcr genes and drugs

	Colistin		ceftazidime			Aztreonam			Imipenem			Norofloxacin			Pipracilllin/ Tazobactam		Amikacin			Gentamicin			Ciprofloxacin		
	R	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**R**	**S**	**I**	**R**	**S**	**I**	**S**	**R**	**I**	**S**	R
MCR-1	0	1	0	1	0	1	0	0	0	0	1	0	0	0	0	1	0	1	0	0	1	0	0	1	**0**
MCR2	3	3	1	0	5	1	1	4	0	0	6	0	0	2	2	4	0	4	2	0	4	2	1	3	**2**
MCR3	1	27	3	6	19	8	7	13	0	0	28	0	10	10	1	27	1	25	2	0	27	1	0	28	**0**
P- value	0.044		0.210			0.019			0.109			0.025			0.002		0.030			0.002			0.000		

Table(4.5): Mcr genes amonge MDR and Non-MDR *Pseudomonas aeruginosa* isolates

	MDR		Non-MDR		*P- value*
Mcr gene	present	Absent	Present	Absent	
Mcr-1	0	14	1	35	
Mcr-2	2	12	7	29	*p*-value = 0.000
Mcr-3	2	12	23	13	
Present of at least one mcr gene	4	-	31	-	



Figure(4.2): PCR result of mcr genes:
Lane: 9 and 19 ladder 100 bp.
Lane: 7 positive for mcr-1 (460bp).
Lane: 8, 10, and 12 show negive results for mcr genes.
Lane: 1-6, 11, snd 13-18 positive for mcr-3 (929bp).

4.2 Discusstion

Colistin is a last-resort antibiotic used to treat multidrug-resistant (MDR) lactose non-fermenting Gram-negative bacteria like *Pseudomonas aeruginosa*, when essentially no other options are available (Jung *et al*., 2021) This study was the first report documented the present of mcr-3 gene among *Pseudomonas* isolates from clinical samples in Sudan.

In 2020 one of Sudanese studies was done in 185 Gram-negative bacteria to detect the mcr-1 gene and they found 1 isolate of *P. aeruginosa* carries the mcr-1 gene from 21 isolates (Musa *et al*., 2021), this match with our study that found only mcr-1 gene, this may give suggestion that mcr-1 is not the common mcr gene in *Pseudomonas* because it founded in only one isolates, but due to their purpose of study they only detect mcr-1 and no data available about other mcr genes in their study.

There were many studies that conducted on Gram-negative bacteria including *P. aeruginosa* around the world to detect mcr-1 and many of them detect mcr-1 in few isolates as in the first report of mcr-1 gene in MDR *P. aeruginosa* in Pakistan (n=250), 85 of isolates were *P. aeruginosa*, 15 of them were Colistin resistance and only one isolate carry mcr-1 gene (Hameed *et al*., 2019), similar result was reported in Brazil when they study the present of OXA-23, OXA-51 and mcr-1 in *P. aeruginosa* (Nitz *et al*., 2021). On the other hand, another two studies detect high mcr-1 carried isolates, in the first one mcr-1 gene detected in 10 isolates (14.49%) out of 69 and another one reported 4 isolates out of the 5 Colistin resistant *P. aeruginosa* (Tahmasebi *et al*., 2020, Pathak *et al*., 2020), in the same year an Egyptian study found 50% of Colistin-resistant isolates were positive for mcr-1(Abd El-Baky *et al*., 2020) this variation may be duo to geographical different and countery policity in Colistin use in human and animal.

The first study conducted in Sudan was done to detect mcr-1 gene in *Enterobacteriaceae* and found mcr-1 gene in 7 of 50 isolates mostly in *E. coli* (Mohammed, 2016). Another study also done to detect mcr-1 in 50 *K.pneumoniae* isolates and only one isolate showed positive for the mcr-1 gene (Babiker, 2020), all these research was done on mcr-1 only and show low level of acquiring gene.

At National Ribat University another study was conducted to detect mcr-1 to mcr-5 in Gram-negative bacteria, only mcr-1 was detected in *Pseudomonas* and it's the first report of mcr-2 in Sudan from *K.pneumoniae* (Musa *et al*., 2021). On the other hand, in this study

mcr-3 (56%) is the most common mcr gene detected and it's the first reported mcr-3 in *Pseudomonas* in Sudan followed by mcr-2 (12%) which is not detected in previously Sudanese study using the same multiplex PCR surveillance protocol that describes by. Rebelo in 2018, and this indicate introduction of new mcr resistant gene in sudan than previously detected. In an Egyptian study that conducts to detect mcr-1 and mcr-2 in *Pseudomonas* they found that mcr-1is the most common resistant gene than mcr-2 un like our finding, this may be due to the different in study area (Abd El-Baky *et al*., 2020).

In the present study most of mcr carries isolates were found in non MDR group this similar to A Pakistan study that found Colistin resistant *Pseudomonas aeruginosa* (n=3) was completely (100%) sensitive to most antibiotics (Arif *et al*., 2022) and incompatible with some studies (Abd El-Baky *et al*., 2020, Hameed *et al*., 2019). There was meta-analysis that conduct in china in 2018 to study mcr-3 they found most isolates were sensitive to commonly used antibiotics matching with our study as we found mcr3 mostly distributed in non –MDR isolates, but all Xu and their colleague's found most of their isolates were Colistin resistance (Xu *et al*., 2018) comparing with our isolates which are mostly sensitive to Colistin disc and this may be duo to different in phenotypical technique that we use to detect the resistant. In another study conducted in China they say "MCR-3 generally mediates low-level of Colistin resistance among *Enterobacteriaceae*, but occasionally confers high-level of resistance in *Aeromonads* (Yin *et al*., 2021).

In this research we found some plasmid-mediated Colistin resistance mcr genes in Colistin-sensitive *Pseudomonas* by disc diffusion method and this indicate "silent dissemination" which is carriage of unexpressed genes, silencing can occur in a variety of ways including the absence of an effective promoter, low levels of expression and changes or mutations to the gene, it's an important and very rare phenomenon in antibiotic resistance, although the mechanism of resistant in un expression gene is unknown (Enne *et al*., 2006).

Some of Colistin resistance strains by disc diffusion method in this study were negative for mcr-1,2,3,4 and mcr-5; this may be duo to use of disc diffusion method or indicates presents of other mcr genes or other resistance mechanisms as mutation (Bortolaia V. *et al*., 2021).

CHAPTER V

CONCLUTIONS AND RECOMMENDATIONS

CHAPTER V

CONCLUSION AND RECOMMENDATION

5.1 Conclusions

Our study findings confirmed the presence of Colistin resistant *Pseudomonas aeruginosa* in Khartoum, Sudan with high prevalence of mcr genes especially mcr-3.

5.2 Recommendations

1. Large sample size should be performed in other studies for more information about Colistin resistance.
2. Surveillance studies for mcr genes mainly mcr-3 and Colistin was recommended and susceptibility test must annually preform to control resistance as possible to the last report antibiotic (Colistin) against MDR Gram-negative bacteria in Sudan.
3. Gene sequencing and phylogenetic studies must be performing after detection of resistance genes for determination of novel mutation.
4. The government must take some steps to stop spread of resistance bacteria, especially which is positive to mcr genes by design and instructed policies.
5. Culturing and sensitivity testing of pathogenic bacteria should be taken before treatment begins.
6. Disc diffusion method for Colistin susceptibility should not use and must replace by other WHO recommended method.
7. Preform of molecular technique to detect mcr genes to resistance and sensitive strains to Colistin is recommended to detect “silent dissemination”.

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APPENDIX I

Data check list

Molecular Detection of Colistin Resistance Genes Mcr-1, 2, 3, 4 and Mcr-5 of *Pseudomonas aeruginosa* Isolated from Selected Hospital in Khartoum State

Patient ID:	Hospital name:
Patient age:	Gender:
Type of sample:	Isolate:

Culture result

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Sensitivity result

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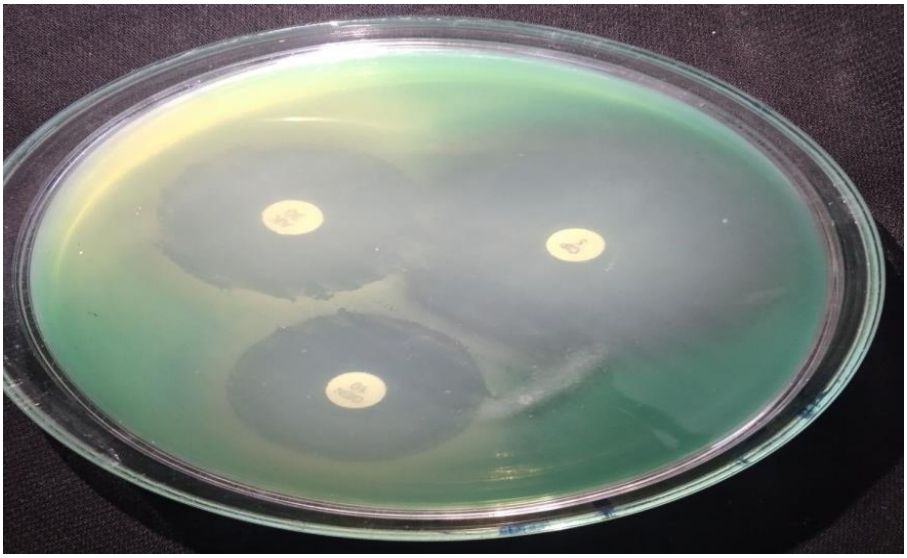
APPENDIX II

Color plates and instruments

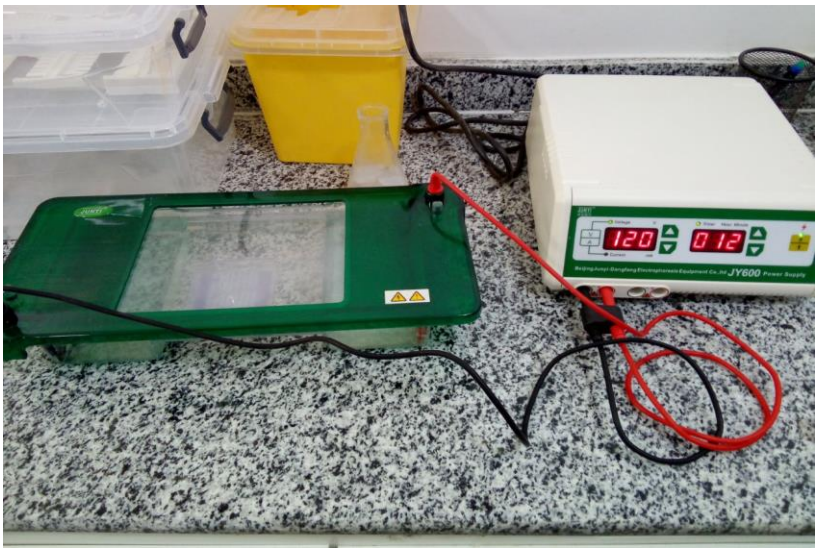
Color plate (1): *P. aeruginosa* colonial morphology on CLED



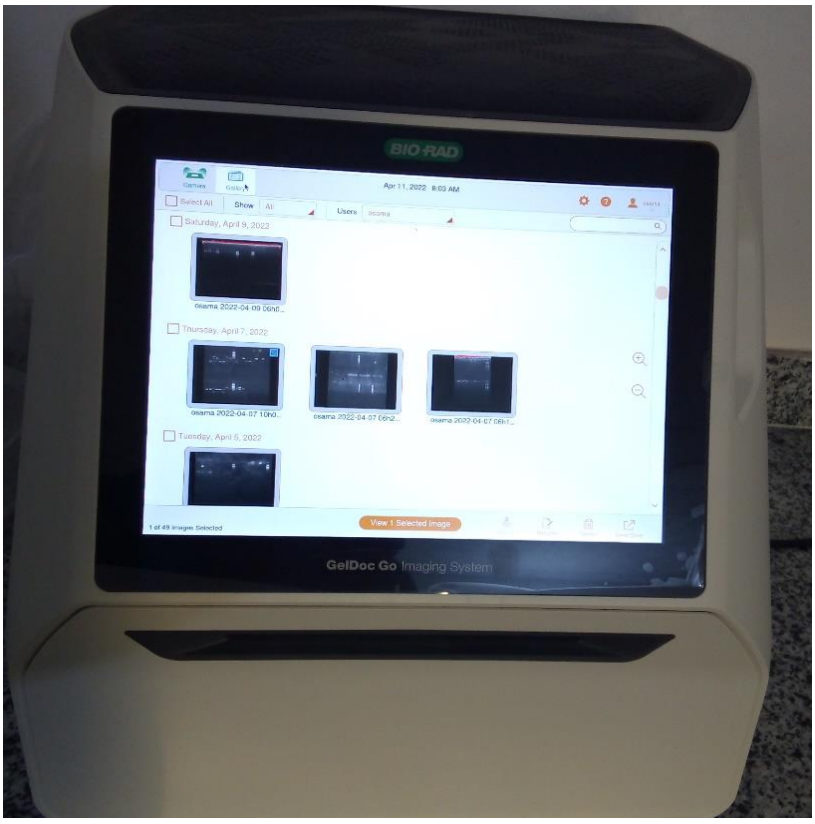
Color plate (2): Antimicrobials susceptibility testing using Kirby Bauer disc diffusion method

Color plate (3): Thermal block incubator (Chem-Tech)

Color plate (4): Thermocycler PCR (Bio-Rad; C1000 touch, UK)



Color plate (5): The gel electrophoresis apparatus was connected to the power supply (JY600; Beijing Junji-Dongfang, China



Color plate (6): Ultraviolet Trans- illuminator (Bio-Rad, UK)

APPENDIX III

Table (3.1): Antimicrobial drugs, abbreviations, concentration, and zone size interpretation[1]

Name	Abbreviation	Concentration (mcg)	Interpretive Standards (mm)		
			R (Resistant)	I (Intermediate)	S (Susceptible)
Amikacin	AK-30	30µg	≤14	15-16	≥17
Aztreonam	ATM-30	30µg	≤15	16-21	≥22
Ceftazidime	CAZ-30	30µg	≤14	15-17	≥18
Ciprofloxacine	CIP-5	5 µg	≤15	16-20	≥21
Colistin	CT-10	10 µg	≤10	---	≥11
Gentamicin	CN-10	10 µg	≤12	13-14	≥15
Imipenem	IMP-10	10 µg	≤13	14-15	≥16
Norfloxacin	NOR-10	10 µg	≤12	13-16	≥17
Piperacillin/tazobactam	TPZ-100/10	100/10 µg	≤17	---	≥18

[1]performance standards for antimicrobial disk susceptibility, M100-S[32]

APPENDIX IV

Preparation of reagents and culture media

Cystine Lactose Electrolyte Deficient Agar:

pH (at 25°C) =7.3+/-0,2

Ingredients: gms/litter lactose 10.0, pancreatic digest of gelatin 4.0, pancreatic digest of casein 4.0, beef extract 3.0, L-cystine 0.128, bromothymol blue 0.02, agar 15.0.

Directions: Suspend 36 grams in 1000ml distilled water. Heat to boiling to dissolve the medium completely sterilize by autoclaving at 15Ibs pressure (121°C) for 15 minutes. Cool to 45°C- 50°C, mix well pour into sterile petri plates.

Prepare Crystal Violet Stain:

Dissolve 2 g crystal violet in 20 ml of 95% ethyl alcohol.

Dissolve 0.8 g ammonium oxalate monohydrate in 80 ml deionized water.

Mix the crystal violet and ammonium oxalate monohydrate solutions to make the crystal violet stain. Filter the stain if necessary.

Prepare Safranin solution:

Add 20mg safranin powder to a 100ml beaker.

Pour 20ml distilled water in the beaker and make 0.1% safranin staining solution by constant stirring.Filter the staining solutions to avoid particles.

Acetone-alcohol decolorizer:

To make 1 litre, 500 ml of Acetone and 475 ml of Ethanol absolute add to 25 distilled water.

Kliger Iron Agar (KIA):

pH (at 25°C) =7.4+/-0.2

Ingredients: Gms/litter Peptic digest of animal tissue 15.0 Beef extract 3.00 Yeast extract 3.00 Proteose peptone 5.00 Lactose 10.00 Dextrose 1.00.

Directions: Suspend 57.5 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by Autoclave at 15 ibs pressure (121°C) for 15 min. Mix and pour. Set as slope with butt.

Simmon's Citrate Agar:

pH (at 25°C) =6.8+/-0.2

Ingredients: Gms/litter Magnesium sulphate 0.02, Ammonium dihydrogen phophate 1.00 Dipotassium phosphate 1.00 Sodium citrate 2.00 Sodium chloride 5.00 Bromothymol blue 0.08 Agar 15.00.

Directions: Suspend 24.28 grams in 1000ml distilled water. Heat to boiling to dissolve the medium completely sterilize by autoclaving at 15Ibs pressure (121°C) for 15 minutes. Cool to 45°C- 50°C, mix well pour into sterile tubes

Christensen's Urea agar:

pH (at 25°C) =7.3+/-0.1

Ingredients: Gms/litter Peptic digest of animal tissue 1.50 Dextrose 1.00 Sodium chloride 5.00 Monopotassium phosphate 2.00 Phenol red 0.012 Agar 15.00

Directions: Suspend 24.51 grams in 1000ml distilled water. Heat to boiling to dissolve the medium completely sterilize by autoclaving at 15Ibs pressure (121°C) for 15 minutes. Cool to 50°C and aseptically add 50ml sterile 40% urea solution, mix well pour into sterile tubes.

Indole broth:

pH (at 25°C) 7.5±0.2

Ingredients: Gms / Litre Casein enzymic hydrolysate 10.000 Sodium chloride 5.000

Directions: Suspend 15 grams in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Dispense into tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Semisolid media for motility:

pH (at 25°C) 7.2±0.2

Ingredients: Gms / Litre Tryptose 10.000 Sodium chloride 5.000 Agar 5.000 Final

Directions: Suspend 20 grams in 1000 ml purified / distilled water. Heat to boiling to dissolve the medium completely. Dispense in tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Allow tubed medium to cool to 45-50°C in an upright position.

Mueller Hinton Agar:

pH (at 25°C) =7.3+/-0.1

Ingredients Gms/litter Beef, infusion from 300.00 casein acid hydrolysate 17.50 starch 1.50 Agar 17.00.

Directions: Suspend 38.0 grams in 1000ml distilled water. Heat to boiling to dissolve the medium completely sterilize by autoclaving at 15Ibs pressure (121°C) for 15 minutes. Cool to 45°C- 50°C, mix well pour into sterile petri plates.

McFarland Standard Turbidity tube 0.5:

Ingredients Sulphuric acid 1 %, dehydrated barium chloride 1.16 g and distilled water 100 ml

Prepare 1% w/v solution of barium chloride by dissolve 1.16 g of dehydrated barium chloride in 100 ml of distilled water. Mix 19.9 ml of sulphuric acid with 0.1ml of barium chloride.

Preparation of 1X TAE buffer:

50X TAE (Thermo Scientific, Lithuania) was diluted to 1 L10X as a stock solution by adding 200 ml of 50X to 800 ml of distilled water, then 1L working solution of 1X was prepared using 100 ml of 10X diluted by 900 ml of distilled water.