Sudan University of Science and Technology

College of Graduate Studies

Antimicrobial Susceptibility and Molecular Detection of Carbapenem Resistant Genes NDM-1 and IMP-1 of *Pseudomonas aeruginosa* Isolated from Clinical Specimens in Khartoum State-Sudan

اختبار حساسية المضادات الحيوية والكشف الجزيئي لجينات مقاومة الكاربابينيم ان دي ام و اي ام بي في الزائفه الزنجاريه المعزولة من عينات سريرية في ولاية الخرطوم- السودان

A Thesis Submitted in Partial Fulfillment of The
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الآيـــة

{ٱللَّهُ لَآ إِلَٰهَ إِلَّا هُوَ ٱلۡحَيُّ ٱلۡقَيُّومُۚ لَا تَأۡخُذُهُۥ سِنَةٞ وَلَا نَوۡمٞۚ لَّهُۥ مَا فِي ٱلسَّمَٰوَٰتِ وَمَا فِي ٱلۡأَرۡضِۗ مَن ذَا ٱلَّذِي يَشۡفَعُ عِندَهُۥٓ إِلَّا بِإِذۡنِهِۦۚ يَعۡلَمُ مَا بَيۡنَ أَيۡدِيهِمۡ وَمَا خَلۡفَهُمۡۖ وَلَا يُحِيطُونَ بِشَيۡءٖ مِّنۡ عِلۡمِهِۦٓ إِلَّا بِمَا شَآءَۚ وَسِعَ كُرۡسِيُّهُ ٱلسَّمَٰوَٰتِ وَٱلۡأَرۡضَۖ وَلَا يَـُٔودُهُۥ حِفۡظُهُمَاۚ وَهُوَ ٱلۡعَلِيُّ ٱلۡعَظِيمُ}

{سُورَةُ البَقَرَةِ :٢٥٥}

صدق الله العظيم

سورة البقره (255)

DEDICATION

May god have mercy on you , my soul`s beloved , and compensate me with you in his heaven . I had a wonderful ***father*** *.. The soul of my dear father*

To My heaven and the source of my happiness and pride . If you are not my mother ,I was going to wish you were my ***mother*** *.. For you , My lovely* ***"Nawal"***

I offer the fruit of my efforts to my dear ***Brothers*** *and beloved grandfather* ***Mohamed Abdallah alkhider*** *also grandmother* ***Mariam Abbas Alhassan***

For each an honest living and for supporting and encouraging me to believe in myselve.

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ABSTRACT

Carbapenems are the most powerful β-lactam antibiotics against bacteria especially multidrug resistant isolates like *Pseudomonas aeruginosa (P.aeruginosa).* This study was conducted to detect carbapenem resistance genes of *P. aeruginosa* from clinical specimens in Khartoum State in period from January _ June).

A total of Fivety (n=50) isolates of *p. aeruginosa* were included in this descriptive cross sectional laboratory based study. *P aeruginosa* were isolated from urine, blood, wound swab , sputum and Body fluids samples, which were obtained from different hospitals in Khartoum State. The samples were collected from both males and females with different age using non-self-constructing information list. The isolated organism was stored in 20% Glycerol peptone media and was inoculated on CLED agar. Antibiotic susceptibility tests were carried out using Kirby Bauer technique. DNA was extracted using boiling method. The isolates were tested for the presence of carbapenem resistance genes (NDM, IMP) using Multiplex PCR.

Out of 50 *P. aeruginosa* isolates, 19 (38%) were positive for one or both carbapenem resistance genes; 6 (12%) were positive for NDM gene and 13(26%) were positive for IMP gene. Tow isolates possess both genes.

The study concluded that, there was a high percentage and multiplicity of carbapenem resistance genes among *P. aeruginosa* isolates in Khartoum State.

المستخلص

الكاربابينيمات(بيتا لاكتام) من أقوى المضادات الحيوية ضد البكتيريا وخاصة المقاومة للأدوية في السودان لذا أجريت هذه الدراسة للكشف عن جينات مقاومة الكاربابينيم لبكتريا الزائفه الزنجاريه المعزولة من عينات سريرية في ولاية الخرطوم في الفترة من يناير الي يونيو2022 .

شملت هذه الدراسة الوصفية خمسين (ن = 50) من الزائفه الزنجاريه المعزولة من عينات البول، الدم، الجروح والبلغم وسوائل الجسم التي جمعت من مختلف المستشفيات في ولاية الخرطوم. تم عزل الزائفه الزنجاريه من الذكور والإناث من مختلف الأعمار. ومن ثم تم حفظها في وسط الجليسرول بيبتون بتركيز 20 %لحين زرعها في الاجار المناسب لها. أجريت اختبارات الحساسية للمضادات الحيوية باستخدام تقنية كيربي باور. استخلص الحمض الوراثي النووي باستخدام طريقةالتسخين والتبريد ومن ثم تم اختبارها لوجود جينات مقاومة الكاربابينيمجينات المقاومه

من خمسين عينه من الزائفه الزنجاريه تم ايجاد جينين من جينات مقاومه الكاربابينيم (38%) 19 عينه اعطت نتيجه ايجابيه , 13 (26%) اعطت نتيجه ايجابيه لجين (اي ام بي) بينما 6 (12%) (ان ار ام)

مما تبين أعلاه خلصت الدراسة إلى أن هناك نسبة عالية وتعددية في جينات مقاومة الكاربابينيم في الزائفه الزنجاريه المعزولة في ولاية الخرطوم .

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LIST OF ABBRIVATIONS

MDR/PA	Multidrug resistant *P. aeruginosa*
MBL	Metallo beta lactamases
IMP	imipenemase
VIM	Verona integrin-encoded metallo-β-lactamase
NDM	New Delhi metallo-β-lactamase
OXA	oxacillinases
CLSI	Clinical and Laboratory Standards Institute
ESBL	extended spectrum beta lactamases
CLED	Cystine Lactose Electrolyte Deficient
MDR	Multi Drug Resistant
MDR/P	Multidrug resistant *P. aeruginosa*
XDR	Extensively Drug Resistant
SUST	Sudan University of Science and Technology
CRE	Carbapenem resistant Enterobacteriales

CHAPTER I

INTRODUCTION

1.1. Introduction

Pseudomonas aeruginosa, a member of the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella.pneumoniae, Acinetobacterbaumannii, P. aeruginosa, and Enterobacter* species) group of pathogens, is responsible for lifethreatening nosocomial infections, especially in critically ill and immunocompromised patients and is known by potential drug resistance mechanisms . Multidrug resistant *P. aeruginosa* (MDR/PA) is a matter of great concern as it not only causes severe and fatal infections but also increases the length of hospital stay, resulting in increased treatment costs . Carbapenems are effective antibiotics against MDR-PA infections . However, their use in the management of infections is threatened by the development of carbapenem-resistant *P. aeruginosa* (CRPA) strains Resistance to the carbapenems in *P. aeruginosa* is often caused by impermeability through alteration or loss of the porin OprD , increased expression of an efflux pump , or the production of class B metallo-β-lactamases (MBLs) (Dogonchi, *et al* . 2018) *P. aeruginosa*, one of the most common opportunistic pathogen associated with nosocomial infections, including pneumonia, urinary tract infections, and wound infections .Although carbapenems are often used as a therapeutic agent for treating infections caused by *P. aeruginosa*, the high emergence of carbapenem resistance significantly decreases their usefulness (Shahin 2021) .

Resistance of *Pseudomonas aeruginosa* to different classes of antibiotics such as penicillin, cephalosporin, quinolone, aminoglycoside and carbapenem .Metallo beta lactamases (MBL) are enzymes that catalyze the hydrolysis of broad-spectrum beta lactam antibiotics including carbapenems ,carbapenems are the most effective against Gram negative and Gram-positive bacteria demonstrating broad range antibacterial activity. Resistant to carbapenems especially in Gram negative pathogens is global public health issue because of the spread of transferable carbapenemase encoding genes . The antibiotic resistant elements could be transferred to

other Gram-negative bacterial strains and contribute towards spread of antimicrobial resistance rate making the treatment of infected patients complex. It is therefore, important to know about the epidemiology and resistance mechanisms of *Pseudomonas aeruginosa* in order to control and prevent multi drug resistant pathogenic strains to overcome possible health risks (Amjed Ali *.etal ,(*2021) . Carbapenems are the antibiotics which are utilized for treating multidrug-resistant *P.aeruginosa* (MDRP) isolates. Carbapenem antibiotics used to be effective agents against MDRP when first presented. However, the growing prevalence of carbapenemresistant *P. aeruginosa* (CRPA) has turned into a severe health problem recently . These strains lead to high mortality rates in patients infected by *P. aeruginosa* and there are also few effective drugs against them. Colistin is a key antimicrobial agent used to treat *P. aeruginosa* infections . Resistance to carbapenems can be related to the production of carbapenemase enzymes such as serine carbapenemases (containing KPC and GES enzymes), metallo-β-lactamases (MBLs) such as IMP, VIM, and NDM enzymes, and oxacillinases (such as OXA enzymes) . blaIMP and blaVIM are the most frequently acquired MBLs. However, the recently emerged NDM type (New Delhi metallo-β lactamases) is becoming the most important carbapenemase (Farajzadeh *et al* 2019) .

1.2. Rationale

Carbapenems have become the drug of choice for the treatment of severe nosocomial infections caused by Gram-negative bacilli. Carbapenem resistant Enterobacteriales (CRE) is a considerable health problem globally and are associated with increased mortality also There is a significant challenge in controlling the spread of carbapenemases, Surveys of the molecular epidemiology of carbapenemases revealed that the dissemination of carbapenemases, including NDM, VIM, IMP, OXA-48 and KPC producers, are rapid and widespread among healthcare facilities (Elbadawi *et al* 2021) .In Africa, data on the prevalence and distribution of carbapenem resistance among the multidrug resistant Gram-negative bacteria is still limited (Mushi *et al.*, 2014). In addition, detection of carbapenem resistance genes producing bacteria may be difficult based on routine antibiotic susceptibility testing (Satir *et al.*, 2016). Considering the fact that there were no comprehensive studies about prevalence of carbapenem resistance genes in Sudan so detection of those genes in *P. aeruginosa* from clinical specimens will be of a great value. Such data serve an important role in understanding the spread of this pathogen and help in good treatment for it .

1.3. Objectives

1.3.1. General objective

To detect carbapenem resistance genes of *Pseudomonas aeruginosa* from clinical specimens in Khartoum State.

1.3.2. Specific objectives

1- To identify the organism under study using conventional and PCR based method .

2- To test the susceptibility of *Pseudomonas aeruginosa*to antimicrobial agents using disc diffusion method.

3- To detect the carbapenem resistance genes of *Pseudomonas aeruginosa*(,NDM -1, IMP-1) using multiplex PCR.

4- To find out the relation between resistance to antimicrobial agents and the presence of carbapenem resistance genes of *Pseudomonas aeruginosa*.

5- To correlate between the presences of carbapenem resistance genes with different clinical specimens and age groups

CHAPTER II

LITERATURE REVIEW

2.1.Pseudomonas aeruginosa

The *P. aeruginosa* is widely distributed in nature and is commonly present in moist environments in hospitals. It can colonize normal humans, in whom it is a saprophyte. It causes disease in humans with abnormal host defenses (Jawetz 2007). *P. aeruginosais* ubiquitous microorganism that's can be found in soil, water, humans, animals, plants, sewage, hospital and considered part of the normal flora (Siegel *et al*. 2007).It is very difficult to eliminate since it shows intrinsic resistance too many types of chemotherapeutic antibiotics and drugs (Gould, 2008*). P. aeruginosais* the main occupant and the most abundant organism on earth (Bonomo, 2006). *P. aeruginosa* it has an outer membrane which contains Protein F (OprF). OprF functions as a porin, allowing certain molecules and ions to come into the cells and maintaining the bacterial cell shape (Carmeli *et al*., 2010). Similarly to its flagellum, *P. aeruginosa* pili contribute greatly to its ability toadhere to mucosal surfaces and epithelial cells (Gould, 2008)

P. aeruginosa is motile and rod shaped, measuring about 0.6 × 2 μm. It is gram negative and occurs as single bacteria, in pairs, and occasionally in short chains . *P aeruginosa* is an obligate aerobe that grows readily on many types of culture media, sometimes producing a sweet or grapelike or corn taco–like odor. Some strains hemolyze blood. *P aeruginosa* forms smooth round colonies with a fluorescent greenish color. It oft en produces the non fluorescent bluish pigment pyocyanin, which diff uses into the agar. Other Pseudomonas species do not produce pyocyanin. Many strains of *P aeruginosa* also produce the fluorescent pigment pyoverdin, which gives a greenish color to the agar . Some strains produce the dark red pigment pyorubin or the black pigment pyomelanin. *P aeruginosa* in a culture can produce multiple colony types.

P.aeruginosa from colony types may also have different biochemical and enzymatic activities and different antimicrobial susceptibility patterns. Sometimes it is not clear if the colony types represent different strains of *P aeruginosa* or are variants of the same strain. Cultures from patients with cystic fi brosis (CF) often yield *P aeruginosa* organisms that

form mucoid colonies as a result of over production of alginate, an exopolysaccharide. In CF patients, the exopolysaccharide appears to provide the matrix for the organisms to live in a biofilm . *P aeruginosa* grows well at 37–42°C; its growth at 42°C helps differentiate it from other *Pseudomonas* species in the fluorescent group. It is oxidase positive. It does not ferment carbohydrates, but many strains oxidize glucose. Identification is usually based on colonial morphology, oxidase positivity, the presence of characteristic pigments, and growth at 42°C. Differentiation of *P aeruginosa* from other *pseudomonads* on the basis of biochemical activity requires testing with a large battery of substrates (Jawetz 2007).

2.2. Pathogenesis

P aeruginosa is pathogenic only when introduced into areas devoid of normal defenses, such as when mucous membranes and skin are disrupted by direct tissue damage as in the case of burn wounds; when intravenous or urinary catheters are used; or when neutropenia is present, as in cancer chemotherapy. The bacterium attaches to and colonizes the mucous membranes or skin, invades locally, and produces systemic disease. These processes are promoted by the pili, enzymes, and toxins described earlier. Lipopolysaccharide plays a direct role in causing fever, shock, oliguria, leukocytosis and leukopenia, disseminated intravascular coagulation, and adult respiratory distress syndrome. *P aeruginosa* and other *pseudomonads* are resistant to many antimicrobial agents and therefore become dominant and important when more susceptible bacteria of the normal microbiota are suppressed (Jawetz 2007).

2.3.Clinical Findings

P aeruginosa produces infection of wounds and burns, giving rise to blue-green pus; meningitis when introduced by lumbar puncture or during a neurosurgical procedure; and urinary tract infection when introduced by catheters and instruments or in irrigating solutions. Involvement of the respiratory tract, especially from contaminated respirators, results in necrotizing pneumonia. The bacterium is often found in mild otitis externa in swimmers. It may cause invasive (malignant) otitis externa in patients with diabetes. Infection of the eye, which may lead to rapid destruction of the eye, occurs most commonly after injury or surgical procedures. In infants or debilitated persons, *P aeruginosa* may invade the bloodstream and result in fatal sepsis; this occurs commonly in patients with leukemia or lymphoma who have received antineoplastic drugs or radiation therapy and in patients with severe burns. In most *P aeruginosa* infections, the symptoms and signs are nonspecific and are related to the organ involved. Occasionally, verdoglobin (a breakdown product of hemoglobin) or fluorescent pigment can be detected in wounds, burns, or urine by ultraviolet fluorescence. Hemorrhagic necrosis of skin occurs often in sepsis caused by *P aeruginosa*; the lesions, called ecthyma

gangrenosum, are surrounded by erythema and often do not contain pus. *P aeruginosa* can be seen on Gram-stained specimens from ecthyma lesions, and culture results are positive. Ecthyma gangrenosum is uncommon in bacteremia caused by organisms other than *P aeruginosa*. A form of folliculitis associated with poorly chlorinated hot tubs and swimming pools can be seen in otherwise healthy persons (Jawetz 2007).

The risk is higher when it comes to cystic fibrosis 90%. It is considered one of the top three most frequent worst visual diseases (Pagani *et al*. 2005).

P. aeruginosa is rarely infects healthy people that is why it is considered opportunistic. It can cause nosocomial pathogen of immunocompromised individuals such as AIDS, cancer, cystic fibrosis or traumatic patients (Pagani *et al.,* 2005). *P. aeruginosa* typically infects the airway, urinary tract, burns, woundsand also causes other blood infections(Siegel *etal*., 2007). It is also the cause of infections associated with hot tubs and contaminated contact lens solutions (Carmeli *et al.,* 2010).

P. aeruginosa can cause serious infections that are associated with high risk groups such neutropenic patients in an infection of septic shock(Hidron *et al*., 2008). Premature infants and neutropenic cancer patients can be at high risk if they get gastrointestinal infections (Jones and Masterton, 2001). Moreover, pneumonia can be critical with cystic fibrosis patients and so is a skin and soft tissue infection in case of burns victims and patients with wound infections (Siegel *et al.*(2007). It is the most common cause of infections of the outer ear (otitis externa), and is the most frequent colonizer of medical devices (e.g., catheters) (Gould, 2008). *Pseudomonas* can be spread by equipment that gets contaminated and is not properly cleaned or on the hands of healthcare workers (Hidron *et al*.2008). Pseudomonas can, in rare circumstances, cause community acquired pneumonias, as well as ventilator associated pneumonias, being one of the most common agents isolated in several studies(Carmeli *et al.,* 2010).

However, research indicates salicylic acid can inhibit pyocyanin production.One in ten hospital-acquired infections is from Pseudomonas (Bonomo , 2006). Since these bacteria like moist environments, such as hot tubs and swimming pools, they can cause skin rash or swimmer's ear (Gould, 2008).The most common cause of burn infections is *P. aeruginosa*. The bacterium is frequently associated with osteomyelitis involving puncture wounds of the foot(Siegel *etal.*,2007).

This gives them the ability to resist many defenses, including anti-Pseudomonas antibiotics such as ticarcillin, ceftazidime, tobramycin, and ciprofloxacin, because once the bacteria sense that their outer layer of biofilm is being destroyed, the inner layers will grow stronger

to reestablish the community (Hidron *et al*.2008). *P.aeruginosa* is also resistant to many antibiotics and chemotherapeutic agents due to their intrinsic resistance (Carmeli *et al*., 2010).

This is caused by the low permeability to antibiotics of the outer membrane and by the production of β-lactamases against multidrug efflux pumps and βlactam antibiotics (Siegel *et a*l. 2007). *P. aeruginosa* can be transmitted to a host via fomites, vectors, and hospital workers who are potential carriers for multiply-antibiotic-resistant strains of the pathogen (Hidron *etal*.2008). Controlled infection of burn wounds on animal and plant models with *P. aeruginosa* strains devoid of pili and flagella demonstrate a trend of decreased virulence (Hidron *et al*., 2008). Without these morphological virulence factors,

the bacteria exhibit a substantially decreased survival rate at the wound site and a decreased ability to disseminate within the host organism (Carmeli *et al*., 2010). Thespread of *P. aeruginosa* within host organisms is also dependent on the microorganism's elastase production and other protease mechanisms (Hidron *et al.,* 2008). Bacterial elastase and other bacterial proteases degrade the host's proteins, including the structural proteins within membranes, disrupting the host's physical barriers against the spread of infection (Bonomo . 2006).

2.4.Treatment of *Pseudomonas aeruginosa*

Traditionally, significant infections with *P aeruginosa* have not been treated with single-drug therapy because the success rate is low with such therapy and the bacteria can rapidly develop resistance when single drugs are used. An extended spectrum penicillin such as piperacillin active against *P aeruginosa* is used in combination with an aminoglycoside, usually tobramycin. Other drugs active against *P aeruginosa* include aztreonam; carbapenems such as imipenem or meropenem; and the fluoroquinolones, including ciprofloxacin. Of the cephalosporins, ceftazidime, cefoperazone, and cefepime are active against *P aeruginosa*; ceftazidime is often used with an aminoglycoside in primary therapy of *P aeruginosa* infections, especially in patients with neutropenia. The susceptibility patterns of *P aeruginosa* vary geographically, and susceptibility tests should be done as an adjunct to selection of antimicrobial therapy. Multidrug resistance has become a major issue in the management of hospital-acquired infections (HAI) with *P aeruginosa* because of acquisition of chromosomal β-lactamases, extended-spectrum β-lactamases, porin channel mutations, and efflux pumps (Jawetz 2007).

These antibiotics must all be given by injection, with the exceptions of fluoroquinolones, aerosolized tobramycin and aerosolized aztreonam (Bonomo and Szabo, 2006). In the rare

occasions where infection is superficial and limited (for example, ear infections or nail infections), topicalgentamicin or colistin may be used (Hidron *et al.,* 2008).

Use of blactamase inhibitors such as sulbactam is being advised in combination with antibiotics to enhance antimicrobial action even in the presence of certain level of resistance (Andrade *et al*., 2003). Combination therapy after rigorous antimicrobial susceptibility testing has been found to be the best course of action in the treatment of multidrug-resistant *P. aeruginosa* (Paterson, 2006). Some next generation antibiotics that are reported as being active against *P. aeruginosa* included oripenem, ceftobiprole and ceftaroline (Andrade *et al*., 2003). However, these require more clinical trials for standardization (Gould, 2008). Therefore, research for the discovery of new antibiotics and drugs against *P.aeruginosa* is very much needed (Hidron*et al.*,2008).

2.5. Antibiotic resistance mechanism

One of the most worrisome characteristics of *P. aeruginosa* is its low antibiotic susceptibility, which is attributable to a concerted action of multidrug efflux pumps with chromosomally encoded antibiotic resistance genes and the low permeability of the bacterial cellular envelopes (Bonomo , 2006).

In addition to this intrinsic resistance, *P. aeruginosa* easily develops acquired resistance either by mutation in chromosomally encoded genes or by the horizontal gene transfers of antibiotic resistance determinants (Andrade *et al*., 2003).

Hyper-mutation favors the selection of mutation-driven antibiotic resistance in *P.aeruginosa* strains producing chronic infections, whereas the clustering of several different antibiotic resistance genes in integrons favors the concerted acquisition of antibiotic resistance determinants (Falagas *et al*., 2006). Some recent studies have shown phenotypic resistance associated to biofilm formation or to the emergence of small-colony variants may be important in the response of *P. aeruginosa* populations to antibiotics treatment (Hidron *et al.*, 2008).

*P. aeruginosa*has immense potential to develop resistance against antibiotic as is evident from the fact that its genome contains the largest resistance island with more than 50 resistance genes (Goossens, 2003).

Presence of antibiotic-degrading enzymes such as extended-spectrum β-lactamases like PER-1, PER-2, VEB-1,AmpCcephalosporinases, carbapenemases like serine oxacillinases, metallo-b-lactamases, OXA-type carbapenemases, aminoglycoside-modifying enzymes, among others have been reported (Hidron *et al.,* 2008).

2.6. Multidrug resistance *P aeruginosa*

While the prevalence of *P. aeruginosa* in the last two decades has remained stable, the prevalence of resistant strains has increased dramatically (Magiorakos *et al.,* 2011).

Multidrug resistance **(**MDR) is defined as non-susceptibility to at least one agent in three or more antimicrobial categories . Extending drug resistant (XDR) is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories). Pandrug resistant (PDR) is defined as non-susceptibility to all agents in all antimicrobial categories (i.e. no agents tested as susceptible for that organism). Thus, a bacterial isolate that is characterized as XDR will also be characterized as MDR. Similarly, a bacterial isolate would have to be XDR in order for it to be further defined as PDR (Magiorakos *etal*., 2011). Moreover, the use of standard terminology will optimize epidemiological surveillance systems, facilitating the exchange of information between the medical community, public health authorities and policy makers in order to promote the prudent use of antimicrobials and other public health measures (Paterson 2006).

Resistant *P. aeruginosa* infections are associated with high mortality, morbidity, and increased resource utilization and costs (Falagas *et al.,* 2006). Further, the acquisition of resistance during anti-pseudomonal therapy among initially susceptible isolates and the emergence of MDR isolates make treatment even more challenging (Paterson 2006).

2.7.Carbapenems

Carbapenems (imipenem, meropenem) are classified as b-lactam antibiotics. The introduction of carbapenems into clinical practice provided agreat advance in treatment of serious bacterial infections caused by beta lactamase producing bacteria. Due to broad spectrum of activities and stability to hydrolysis by most beta- lactamases (ESBLs and AmpC b-lactamases) carbapenems have become the drug of choice for treatment of infections caused by penicillin or cephalosporin resistant Gram-negative bacilli, especially ESBL producing gram-negative infections3. Carbapenem resistance has been frequently observed in non fermenting bacilli, such as *P. aeruginosa* and *Acinetobacter spp*. Resistance to carbapenem is due to decreased outer membrane permeability, increased efflux system, alteration of penicillin binding proteins and carbapenem hydrolyzing enzymes carbapenemase. These carbapenemases are class B metallo-b-lactamases (IMP,VIM,SPM, NDM) or class D oxacillinases (OXA 23 to OXA 27) or class A clavulanic acid inhibitory enzymes (SME, NMC, IMI, KPC). Metallo-b-lactamases are able to hydrolyze all b-lactams except monobactam5. Class B metallo-b-lactamas requires a divalent cations of zinc as

cofactors for enzyme activity. The IMP and VIM genes responsible for MBL production are horizontally transferable via plasmids and can rapidly spread to other bacteria. VIM and IMPs are the most frequent MBLs acquired by gram negative bacilli7. MBL producing *P.aeruginosa* was first reported in Japan in 1991 since then its incidence has been reported from various parts of the world. VIM-1 was first reported in *P. aeruginosa* in Italy in 1997, followed by reports of VIM-2 in France and Greece. NDM-1 represents the recent type of mobile metallo blactamase to appear but is behaving differently in terms of rapidity of its spread and scope of organism in which it is found10. NDM was predominantly found in *Enterobacteriaceae* and mostly in *Klebsiella. pneuminiae* and *Escherecia.coli* isolates. Cases among gram negative non fermenters such as *Acinetobacter spp* that produce NDM have also been reported. More recent publications report cases also among *Pseudomanas spp*. that produce NDM. The ability to spread not only among Enterobacteriaceae but also among other bacteria l families like *Pseudomanaceae* implies the possibility for numerous new NDM-1 cases to be detected in the near future. Because of its ability to spread, carbapenem resistance has become a serious concern11. Immediate detection of MBL producing *P. aeruginosa* is important to prevent the spread of organism within and between hospitals and to accurately treat infections caused by these organisms. To reduce healthcare cost and prolonged hospital stay, a regular monitoring of incidence b-lactamase producing organisms has become a need of time (Aleya Farzana *et al* 2022) .

In the late 1960s, as bacterial β-lactamases emerged and threatened the use of penicillin, the search for β-lactamase inhibitors began in earnest (PappWallace *et al.,* 2011). After a series of experiments, thienamycin was firstly serve as the parent or model compound for all carbapenems which is a natural product from Streptomyces cattleya bacterium (Rao, 2012). To date, more than 80 compounds with mostly improved antimicrobial properties, compared to those of thienamycin (Papp-Wallace *et al.*2011). The term "carbapenem" is defined as the 4:5 fused ring lactam of penicillins with a double bond between C-2 and C-3 but with the substitution of carbon for sulfur at C-1(Rao, 2012). Since thienamycin displayed inhibitory microbiological activity against Gram-negative bacteria, unfortunately, it was found to be unstable in aqueous solution, sensitive to mild base hydrolysis, and highly reactive to nucleophiles, such as hydroxylamine, cysteine. The chemical instability of thienamycin stimulated the search for analogous derivatives with increased stability (Papp-Wallace *et al*., 2011). The first developed was the N-formimidoyl derivative, imipenem. Imipenem and a closely related carbapenem, panipenem, identified later, were more-stable derivatives of thienamycin and less sensitive to base hydrolysis in solution (*Papp-Wallace et al., 2011*). In

1985, imipenem (originally called MK0787) became the first carbapenem available for the treatment of complex microbial infections (Rao, 2012). Like its parent, thienamycin, demonstrated high affinity for PBPs and stability against βlactamases (*Papp-Wallace et al.,* 2011). Along the journey to the discovery of more-stable carbapenems with a broader spectrum, the other currently available compounds, meropenem, biapenem, ertapenem, and doripenem, were developed, and several novel carbapenems were identified. These novel carbapenems included antipseudomonalcarbapenems, anti-methicillin-resistant *S.aureus* (MRSA) carbapenems (i.e., cationic and dithiocarbamate carbapenems), orally available carbapenems, trinemcarbapenems, a dual quinolonylcarbapenem, and others (Papp-Wallace *et al.,* 2011). Several chemical approaches were developed for the synthesis of carbapenems since fermentation was not an efficient method for production. Natural products were often used as starting material for production of carbapenems, and the synthetic approach was largely influenced by the desired stereochemistry of the final compound (PappWallace *et al.,* 2011). As a class of β-lactams, carbapenems are not easily diffusible through the bacterial cell wall. They enter Gram-negative bacteria through outer membrane proteins (OMPs), also known as porins (Rao, 2012). After transversing the periplasmic space, carbapenems "permanently" acylate penicillin binding proteins (PBPs) which are enzymes (i.e., transglycolases, transpeptidases, and carboxypeptidases) that catalyze the formation of peptidoglycan in the cell wall of bacteria. Carbapenems act as mechanism-based inhibitors of the peptidase domain of PBPs and can inhibit peptide crosslinking as well as other peptidase reactions. A key factor of the efficacy of carbapenems is their ability to bind to multiple different PBPs (Papp-Wallace *et al.,* 2011). In general, imipenem, panipenem, and doripenem are potent antibiotics against Gram-positive bacteria (Rao, 2012). Meropenem, biapenem, ertapenem, and doripenem are slightly more effective against Gramnegative organisms (Rao, 2012). Carbapenems can also be combined with other antimicrobials to treat serious infections (Papp-Wallace *et al*., 2011). Combination therapy is a subject of intense interest, since the emergence of MDR pathogens often requires us to treat patients with more than one antibiotic *(*Papp-Wallace *et al.,* 2011). Some combinations demonstrate positive effects, such as extending the spectrum or working additively or synergistically (Papp-Wallace *et al*., 2011). Adverse effects include increased resistance to one of the drugs used in the combination, as well as a lack of synergy and strain dependence *(*Papp-Wallace *et al.,* 2011). Nephrotoxicity, neurotoxicity, and immunomodulation have been reported with the use of carbapenems, and thus predisposing factors should be considered when administering any carbapenem (Papp-Wallace *et al*., 2011). In addition, the use of carbapenems can alter

the intestinal microflora and select for carbapenem-resistant isolates (Rao, 2012). 2.2.1. Mechanisms of resistance against carbapenems Mechanisms of resistance to carbapenems include production of βlactamases, efflux pumps, and mutations that alter the expression and/or function of porins and PBPs. Combinations of these mechanisms can cause high levels of resistance to carbapenems in bacteria such as *K. pneumoniae, P. aeruginosa*, and *A. baumannii* (Rao, 2012). Carbapenem resistance in Gram-positive cocci is typically due to the result of substitutions in amino acid sequences of PBPs or acquisition/ production of a new carbapenem resistant PBP (Papp-Wallace *et al*., 2011). Carbapenem resistance in Gram-negative bacteria is often due to production of β-lactamases; expression of efflux pumps, as well as porin loss and alterations in PBP (Papp-Wallace *et al.,* 2011). Production of β-lactamases appears to be the most widespread cause of carbapenem resistance (Rao, 2012). In addition to carbapenemases, resistances to carbapenems can also occur due to over production of certain AmpC beta-lactamases (Rao, 2012). Carbapenem hydrolyzing enzymes (also known as carbapenemases) are broadly divided into two types based on the reactive site of the enzymes; serine carbapenemases and metallo-β-lactamases (Rao, 2012). Carbapenemases represent the most versatile family of β-lactamases, with a breadth of spectrum unrivaled by other β-lactam-hydrolyzing enzymes, many of these enzymes recognize almost all hydrolyzable β-lactams, and most are resilient against inhibition by all commercially viable β-lactamase inhibitors (Queenan . 2007) .The advent of carbapenems in the 1980s heralded a new treatment option for serious bacterial infections.They have a broad spectrum of activity and are stable to hydrolysis by most of the beta lactamases, including the extended spectrum beta lactamases (ESBL) and the Amp C beta lactamases. In recent years there has been anincrease in carbapenem resistance among Gram negative bacteria in the Indian subcontinent. Resistance mechanisms include lack of drug penetration (*i.e.*, porin mutations and efflux pumps) and/or carbapenem hydrolyzing betalactamase enzymes (Amudhan *etal* 2012).

2.8.Molecular detection of carbapenemases genes

Among the carbapenemase types in *Pseudomonas* spp, according to the molecular classification of Ambler, there are serine *β*-lactamases such as KPC (*Klebsiella pneumoniae* carbapenemases) and GES (Guiana extended-spectrum) in Class A and OXA-198 (Oxacillinases-198) in Class D . New metallo-beta-lactamase (MBL) enzymes, which may be responsible for the growing carbapenem resistance of non-fermentative Gram-negative (NFGN) bacilli, have been identified in recent years, which are spread worldwide . Many carbapenemases have been identified in *Pseudomonas* species and encompass metallo-*β*-

lactamases (MBL) in Class B, including imipenemase (IMP), Verona integron-mediated metallo-*β*-lactamase (VIM), Sao Paulo MBL (SPM), Seul imipenemase (SIM), Australian imipenemase (AIM), German imipenemase (GIM), Dutch imipenemase (DIM), and new Delhi metallo *β*-lactamase (NDM) (Tanriverdi *et al.*2022).

Molecular techniques remain the gold standard for the precise identification of carbapenemase genes (Nordmann *et al.*2012). Most of these techniques are based on PCR and may be followed by a sequencing step if a precise identification of the carbapenemase gene is needed (e.g. VIM type, KPC type, NDM type or OXA-48 type) (Nordmann .2012). They are either single or multiplex PCR techniques (Nordmann *et al.* 2012).

A PCR technique performed directly on colonies can give results within 4–6 h (or less when using real-time PCR technology) with excellent sensitivity and specificity. The main disadvantages of the molecular based technologies are their cost, the requirement for trained microbiologists and the inability to detect novel unidentified genes (Nordmann *et al*. 2012)

2.9 previous studies:

2.9.1 Previous studies of (NDM -1 / IMP -1)genes in Sudan:

In a study done in Khartoum, Sudan late 2012 by Abdelrazig and his colleague among 74 clinical isolates of *Pseudomonas aeruginosa* , 57 isolates were recognized to have IMP family genes (IMP-7 and IMP-10) using PCR assay (Adam 2018) . also Elbadawi revealed that phenotypic test versus Carbapenemase or ESBL gene detection were strongly correlated (P = 0.0000001; P = 0.01, respectively).The correlation between phenotypic and carbapenemase genes detection was highly significant for *K. pneumonia*, *E. coli*, *P. aerugnosa and A. baumannii* (P = 0.0000031; P = 0.00079; P = 0.015; P = 0.02, respectively) while for ESBL genes correlation was only significant for *P. aerugnosa* (P = 0.038) (Elbadawi *et al* 2021) . The frequency of ESBL producing isolates varied between hospitals (18.2–45.1%), although Khartoum Teaching Hospital had a large incidence of 45.1% (Ibrahim 2013) .Another study releaved that most common isolated species was *P. aeruginosa*, among which the predominant genes were blaOXA-51 and blaVIM. However, the second most common resistant gene was NDM-1 in *K. pneumoniae* (Mohamed *et al*. 2019) .

2.9.2 Previous studies of (NDM -1 / IMP -1) genes in *pseudomonas* around the world

The first report of blaNDM-1 positive in *P. aeruginosa* came from Serbia (Jovcic . *et al* 2011) . blaNDM-1 producing *P. aeruginosa* is extremely rare (Shokri *et al* (2017) . To date there are no reports of co-harboring occurrence blaNDM-1 in *P.aeruginosa* isolates in Iran. Nevertheless, *P.aeruginosa* isolates producing three carbapenemase genes is rare and has

been reported in Brazil (blaSPM-1, blaKPC-2 and blaVIM-2) (Rizek . *et al* 2014) , Denmark (blaNDM-1, blaVIM-2, blaIMP-1) (Wang *et a,l* (2015), Bangladesh (blaNDM-1, blaVIM-2, blaIMP-1) (Farzana .*etal*,2013) and Turkey (blaVIM-1, blaVIM-2, and bla GES-5) (Malkocoglu, *et al* 2017). Although these cases are scarce and sporadic, information of its occurrence is vital because NDM-positive *P. aeruginosa* is an organism with potent colonization ability in the hospital for long periods (Johnson .2013) . To best of our knowledge, we report the first report of *P. aeruginosa* isolates producing four carbapenemases co-existence blaNDM-1, blaVIM-2, blaIMP-1 and blaOXA-10 from Iran. The acquisition of MB L carbapenemase blaNDM-1, blaVIM, blaIMP and blaSPM led to emergence of MDR or XDR *P. aeruginosa* (Flateau , *et al* , 2012) .

CHAPTER III

3. MATERIALS AND METHODS

3.1.Study design

This design was a descriptive cross-sectional laboratory base 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 Bahri Teaching Hospital). Laboratory tests were performed at the Sudan University of Science and Technology (SUST) and Exon Molecular Biology Laboratory.

3.3.Study duration

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

3.4.study sample

A total of fifty non-repetitive *P.aeruginosa* (n=50), isolated from different clinical specimens

3.5.Ethical clearance:

Ethical approval to conduct this study was obtained from scientific Research committee of college of Medical Laboratory Science and Hospital administration , Sudan University of Science and Technology.

3.6.Sampling technique

Non-probability convenience sampling technique.

3.7.Data collection procedure

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

3.8.Laboratory methods

3.8.1.Sample purification and identification

One colony from each isolate was sub-cultured on Cysteine Lactose Electrolyte Deficient media (CLED) and Nutrient agar (TM Media, India) and incubated aerobically at 37°C overnight, for further Re-identification and preservation.

3.8.2.Colonial morphology

P.aeruginosa showed smooth blue non-lactose fermenting mostly with eye shape characteristic colonies on CLED, some with pyocyanin (blue) or pyoverdine (blue to green) diffusible pigments.

3.8.3.Gram stain

The procedure was carried out according to Cheesbrough(2006) as follows; 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 minutes, then washed in tap water and decolorized rapidly (few seconds) with acetone alcohol and washed immediately 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. The drop of oil was added to the dried smear and examined under the light microscope by oil lens 100X.

3.8.4.Biochemical test

3.8.4.1Oxidase test

The single and pure colony was taken by wooden stick and put in a commercially available paper disk, with the substrate (1% tetramethyl-p-phenylenediamine-dihydrochloride). The purple color that appears within 60 seconds indicates positive results (Mackie and McCartney 1996) .

3.8.4.2.Indole test

A sterile loop was used to inoculate the tested organism into 2 ml peptone water, the tube was incubated at 37°Cfor 24 hrs. in the next day 0.5 ml of Kovac's reagent (4 (p) – di-methylamino-benzaldehyde) was added, it was shaken gently and examined for the red color in alcohol layer within 10 mints. P.*aeruginosa* is negative indole (no red ring appears) according to (Mackie & McCartney 1996) .

3.8.4.3.Urease test

The test organism was inoculated into the slope surface of Christensen's urea medium with phenol red as an indicator using sterile straight wire, the medium was incubated at 37 °C for

24 hrs, change in color of the indicator to purple-pink means a positive result. *P.aeruginosa* is differential according to(Cheesbrough 2006).

3.8.4.4.Kligler Iron agar

The tested organism was inoculated in KIA medium, using a straight wire loop, agar butt was stabbed, the opening was closed, then the top slope was streaked (as a zigzag). The medium was incubated at 37°Cfor 24 hrs, and glucose fermentation, lactose fermentation, H2Sproduction, and gas production were looked for *P.aeruginosa* give red butt and slope, produce no gas or H2S according to (Mackie and McCartney 1996) .

3.8.4.5.Citrate Utilization test

The tested organism was inoculated into 2 ml of Simmon citrate medium with bromothymol blue as an indicator using sterile straight wire. Then the medium was incubated at 37°Cfor 24 hrs, change in color of the indicator from light green to blue color or streaking of growth mean a positive result. P.*aeruginosa* is citrate positive according to (Mackie and McCartney1996) .

3.8.5.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.

3.8.6.Antimicrobial Susceptibility test

All confirmed isolates were subculture from 15% glycerol on nutrient agar getting overnight and pure growth. Modified Kirby Bauer were performed and interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2022). 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. Before inoculation, the cotton swab was dipped into bacterial suspension with visually equivalent turbidity to 0.5 McFarland standards. The swab was squeezed into the test tube wall to discard extra fluid. 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. antibiotic susceptility testing against Amikacin (30mcg), colistin (10 mcg), aztreonam (30mcg), ciprofloxacin (5mcg), gentamicin (10mcg), imipenem (10mcg) and ceftazidime (30mcg), piperacillin/tazobactam (100/10 mcg) discs.

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

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

3.8.7. Molecular characterizations

3.8.7.1 DNA Extraction

The boiling method was used for isolation of DNA template from bacteria,bacterial genomic DNA was extracted manually from fresh overnight incubated nutrient slope that suspended in 200ul of d H2O in 1.5 eppendrof tube ,bolied for 10 min at 100°C in thermal block incubator (chem-Tech),vortex, then cooling at -20°C following by centrifuged for 10 min at 12000xg. Supernatant were carefully collected and store at -20°C until analysed .

3.8.7.2. Gel electrophoresis of extracted DNA

The purity of the extracted DNA was determined by running the DNA sample on 1.5% agarose gel (Sambrook *et al*., 1989).

3.8.7.7.Polymerase Chain Reaction Amplification

PCR was done by multiplex PCR, amplification which done using Maxime PCR Premix kit (iTaq, Korea) and specific primer which sensitized by(Macrogen,korea) Specific primers were used to amplification indicated in table (3.2) .

The PCR assay was carried out in a total volume of 25 µL of mixture containing 2 µLMaxime PCR Premix containing 1X PCR buffer, 1.5 mM MgCl2, 200M µ of each dNTP, and 1 U Taq DNA polymerase, 0.5 µL of each of the virulence gene-specific primers (2 µL), 5 µL of template DNA and 13 µL of water for injection (WFI). The amplification conditions included three steps: heating at 95°C for 5 min; 34 cycles of denaturation at 95°C for 1 min , annealing at 58°C for 1min for (NDM -1 and IMP -1 genes) and extension at 72°C for 1min; and the final extension at 72°C for 5 min (Jalali *et al*., 2015).

Table (3.2): Primers used for amplification of carbapenem resistance genes of *P.aeruginosa* isolates

Target gene	Sequence(5'- 3')	Amplicon size (bp)	Reference
IMP-F	GGAATAGAGTGGCTTAAYTC	**Bla IMP -1 232**	L. Poirel *etal* ,(2010)
IMP-R	GGTTTAAYAAAACAACCACC		
NDM-F	GGTTTGGCGATCTGGTTTTC	**Bla NDM -1621**	L. Poirel *etal,* (2010)
NDM-R	CGGAATGGCTCATCACGATC		

3.8.7.8. Gel electrophoresis

The gel casting tray was put into the electrophoresis, tank flooded with 1x TBE buffer just to cover the gel surface, 2µl of PCR products from each 50samples was added to wells of electrophoreses, 5 µl of100-bp DNA ladder (iNtRON,Korea),was added to the well in each run. The gel electrophoresis apparatus was connected to power supply (150 V, 500 mA, UK). The electrophoresis was carried out at 120Volts for 2 Hours and the gel tray was removed from the electrophoresis apparatus and the buffer was discarded. Then the gel was visualized for DNA bands by U.V transilluminater and photographed (Uvitec – UK), (Jalali *et al*., 2015**).**

The expected band size: IMP -1 (232bp), NDM -1 (621bp) .

Posotive result: there was bands appear against ladder

The negative result: there was no bands appear against ladder.

3.9. Statistical analysis:-

All outcome data were analyzed by using Statistical Package for Social Sciences (SPSS; Version16). The outcome data arranged in tables and then entered into SPSS according to program guidelines analyzed by chi-squre, P.value less than 0.05 was consider significant for the association between variables.

CHAPTER FOUR

4.RESULTS AND DISCCUSSION

4.1 Results:

The present cross sectional study was conducted at Sudan University of Science and Technology (SUST) and "Exon" Molecular biology laboratory; during the period from January 2022 to June 2022 .

Clinical isolates of *P. aeruginosa* were collected over the study period from different hospitals (Royal care, Fedial, Alzaytouna, Omer Sawi, Alraqi, Khartoum ENT, and Bahri teaching hospital).

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 were show pigmentation 32 (74%), the most pigmented strain was found in urine (26%) and wound samples (20%) table (4.1).

Demographic data show out of 50 the male 23 were (46%),where female were 27 (54%) with ratio1 : 0.85 table (4.2). Ages ranged from 4 to 76 years, with an average of 49. Majority of the samples (37%) were found in the age group (40 – 60) year.

Approximately one-third 18 (36%) of isolates were resistant to Aztreonam, 10 (20%) were resistant to Ciprofloxacine and Colistin, 11 (22%) resistant to Gentamicin, Amikacin, and Pipracillin/tazobactam combinations,only one isolate was resistant to Imipenem, from 18 urine samples 3 (19%) isolates were resistant to Norfloxacin table (4.3).

Out of 50 *P. aeruginosa* isolates; only 14 (28%) were MDR according to the previously described definition , most isolates were sensitive to all antibiotics 10 (36%), 12 (24%) isolates resist to one antibiotic, 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.

The NDM-1genewas detected in 6 isolates (12%) and negative in 44 isolate (88%) . They were recovered from urine (8%), wound (4%), ear swab (0%), sputum (0%) , blood (0%) high vaginal swabs (0%) and body fluid (0%) . theIMP-1 gene was detected in 13(26%),and negative in 37(74%) isolate.

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

		Pigment appearance	
		Green Pigment	Non pigmented
Sample	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		
		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%)

Table (4.3) : Antimicrobial susceptibility pattern of *P . aeruginosa* isolates

Sample / antibiotic		Urine (N=18)	Wound swab (n=11)	Blood (N=6)	HVS (N=2)	Sputum (N=8)	Ear swab (N=3)	Body fluid (N=2)	Total (N=50)
CIP	Resistant	**3**	**3**	**2**	**0**	**1**	**2**	**0**	**11(22%)**
	Sensitive	**15**	**8**	**4**	**2**	**7**	**1**	**2**	**39(78%)**
CN	Resistant	**4**	**5**	**2**	**0**	**2**	**1**	**1**	**15(30%)**
	Sensitive	**14**	**6**	**4**	**2**	**6**	**2**	**1**	**35(70%)**
TPZ	Resistant	**5**	**3**	**0**	**0**	**1**	**2**	**0**	**11(22%)**
	Sensitive	**13**	**8**	**6**	**2**	**7**	**1**	**2**	**39(78%)**
CAZ	Resistant	**6**	**4**	**3**	**0**	**2**	**1**	**1**	**17(34%)**
	Sensitive	**12**	**7**	**3**	**2**	**6**	**2**	**1**	**33(66%)**
ATM	Resistant	**12**	**4**	**3**	**1**	**7**	**1**	**1**	**30(60%)**
	Sensitive	**6**	**7**	**3**	**1**	**1**	**2**	**1**	**20(40%)**
AK	Resistant	**2**	**5**	**1**	**0**	**3**	**1**	**1**	**13(26%)**
	Sensitive	**16**	**6**	**5**	**2**	**5**	**2**	**1**	**37(74%)**
IMP	Resistant	**2**	**0**	**0**	**0**	**0**	**0**	**0**	**2 (4%)**
	Sensitive	**16**	**11**	**6**	**2**	**8**	**3**	**2**	**48(96%)**
CT	Resistant	**5**	**1**	**0**	**1**	**2**	**1**	**0**	**10(20%)**
	Sensitive	**13**	**10**	**6**	**1**	**6**	**2**	**2**	**40(80%)**

Table (4.4) :Association between sex and Antimicrobial susceptibility results

Antibiotic	Sex group				
	male		Female		
	Sensitive	Resistant	Sensitive	Resistant	P.value
CAZ	**15**	**8**	**18**	**9**	**0.6**
AZT	**11**	**12**	**10**	**17**	**0.6**
TPZ	**17**	**6**	**22**	**5**	**0.5**
CT	**21**	**2**	**19**	**8**	**0.1**
CN	**13**	**10**	**22**	**5**	**0.5**

4.1 .NDM-1gene

The gene was detected in 6 isolates out of the 50 isolates.

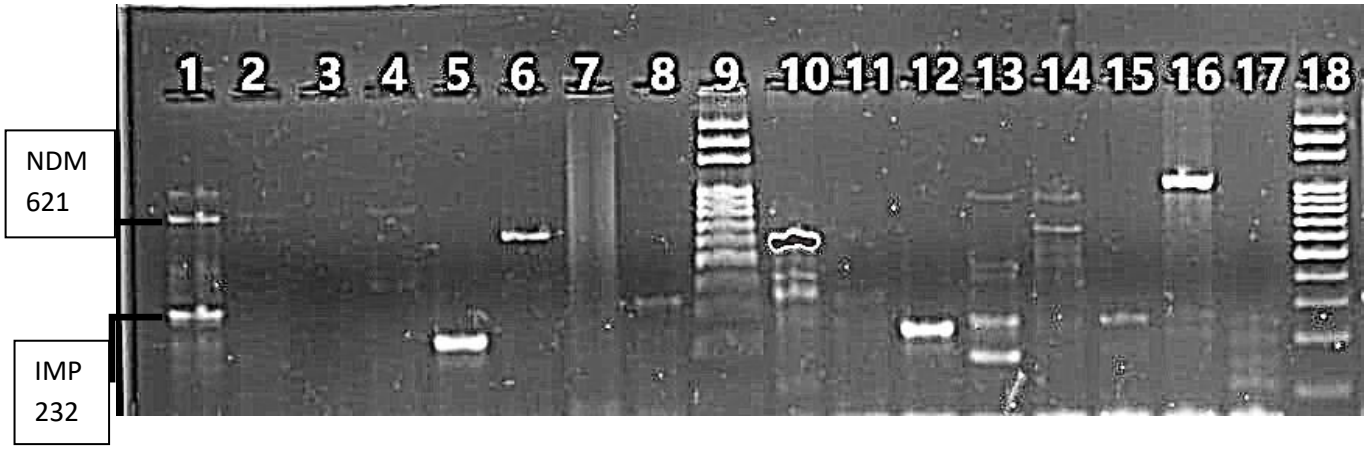

Figure (4.1) : Gel electrophoresis of NDM -1 gene

Lane: 9 and 18 ladder 100 bp.

Lane: 1/5/12/13/15/and 17 positive for IMP -1 product (232bp).

Lane:7/8 and 11 show negative results .

Lane: 1/2/4/13 /14 and16 show positive for NDM -1product (621bp)

4.2 .IMP-1 gene

The gene was harbored in13samples

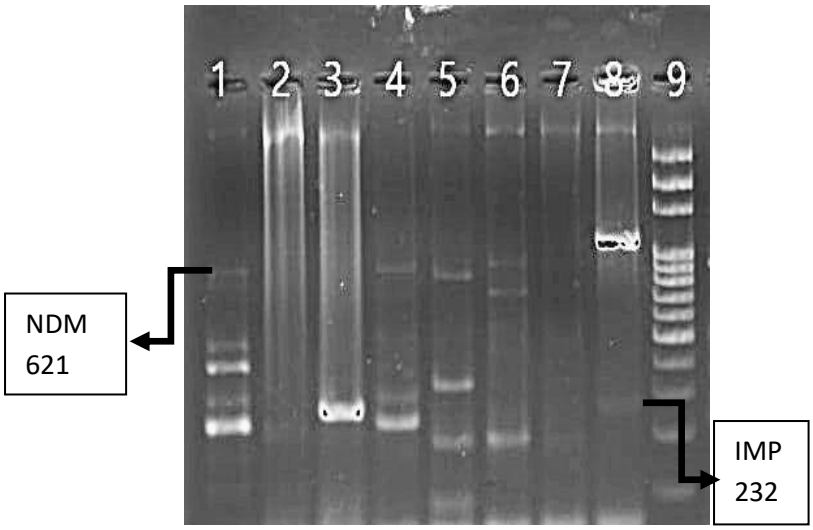

Figure (4.2) : Gel electrophoresis of IMP -1 gene

Lane: 9 ladder 100 bp.

Lane: 1/4/5and 6 positive for NDM -1 product (621bp).

Lane: 2/7 show negative results .

Lane: 1/3and 8 positive for IMP -1 product (232bp)

Table (4.5): Association between *P . aeruginosa* Resistant genes and infection sites

There was insignificant difference in the percentage of Resistant genes among the different infection sites.

Resistant genes	Sample sites						P value
	Urine N=18	Wound N=11	Ear swab N=3	Blood N=6	Body fluid N=2	Sputum N=8	
NDM-1	4	2	0	0	0	0	0.9
IMP-1	6	4	0	1	1	1	0.1

Table (4.6) :Association between the presence of NDM -1 gene and antibiotics susceptibility:

Antibiotic		**NDM -1**		**P.value**
		(+ve)	**(-ve)**	
ciprofloxacin	**R**	**2**	**15**	**9.7**
	S	**4**	**29**	
Colistin	**R**	**3**	**10**	**1.6**
	S	**3**	**34**	
Gentamicin	**R**	**2**	**20**	**5.8**
	S	**4**	**24**	
Amikacin	**R**	**0**	**6**	**3.5**
	S	**6**	**38**	
Pipracillin	**R**	**1**	**25**	**6.7**
	S	**5**	**19**	
Impenem	**R**	**2**	**21**	**5.2**
	S	**4**	**23**	
Aztreonam	**R**	**3**	**29**	**4.6**
	S	**3**	**15**	
Ceftazidime	**R**	**3**	**33**	**0.2**
	S	**3**	**11**	

Table (4.7) :Association between the presence of IMP-1gene and antibiotics susceptibility:

Antibiotic		IMP -1		P.value
		(+ve)	(-ve)	
ciprofloxacin	R	5	12	7
	S	8	25	
Colistin	R	3	10	7.9
	S	10	27	
Gentamicin	R	4	18	2.7
	S	9	19	
Amikacin	R	1	5	5.9
	S	12	32	
Pipracillin	R	7	19	8.8
	S	6	18	
Impenem	R	5	18	5.4
	S	8	19	
Aztreonam	R	8	24	8.3
	S	5	13	
Ceftazidime	R	10	26	0.6
	S	3	11	

4.2 Discussion

In this study, 50 *P.aeruginosa* isolates were collected from different hospitals in Khartoum State and were cultured on CLED agar. All isolates were identified using Multiplex PCR was used to detect two carbapenem resistance genes (NDM -1, IMP-1). The percentage of the *P. aeruginosa* isolates exhibiting resistance to carbapenems observed in this study (19 %). In addition the vast majority of these isolates also showed resistance to other b-lactams, aminoglycosides and fluoroquinolones.

Infections with blaNDM-1 producing isolates in non-endemic regions such as Europe and North America are often linked to visit and be hospitalized in endemic regions such as Indian subcontinent(Van der, 2012).

In the current study, most of isolates harboring carbapenem resistance genes were phenotypically susceptible to imipenem and most of them were positive for IMP gene. This confirm what Walsh, (2010) said that this gene is not strong and relies upon other synergistic mechanisms to mediate resistance against carbapenems. In addition to imipenem other antibiotics were analyzed in this study. Majority of the isolates showed varying degrees of resistance to the other antibiotics (ciprofloxacin, gentamicin and ceftazidime). Resistance to these antibiotics may also be due to the presence of ESBLs and other mechanisms like efflux pumps and porin mutations (Singh-Moodley and Perovic, 2016), which were not covered in this study.

In the current study, the most prevalent gene among the 50*P. aeruginosa* isolates was IMP gene (26 %). NDM gene was also present but on a smaller scale (12%, n =6). This is consistent with previous findings by (Candan and Aksöz, 2015) in Turkey, which detected OXA-48 gene (58%) and NDM gene (2%), (Al Tamimi *et al*., 2016) detected OXA-48 gene (64.7%) and NDM gene (5.8%) and (Shibl *et al*., 2013) detected OXA-48 gene (78%) and NDM gene (20%) both from Saudi Arabia, IMP gene were not detected in these studies

These variations in results may be because, they only detect resistant genes in carbapenem-resistant isolates or due to differences in method of detection as the first one used real-time PCR or differences in the sample size of tested organism and that was seen with the other two studies .

Of 50 *P. aeruginosa* isolates detected of having carbapenem resistance genes, 2 had multiple genes. This finding agree with (Ali and Omer, 2017) and (Satir *et al.,* 2016) which showed multiplicity of genes in their isolates. The presence of multiple resistance genes in one isolate have not been commonly detected in a large number of studies probably due to the limited

number of genes studied since most of the studies research on one or two genes (Satir *et al.*, 2016).

Regarding NDM and IMP gene, 6 (12%) and 13(26%) were positive respectively. Ceftazidime resistant Isolates showed significant association with both NDM and IMP gene as detected 3 , 10 isolates respectively.

CHAPTER V

5. CONCLUTIONS AND RECOMENDATIOS

5.1 Conclusions

In conclusion,The findings of the present study disclosed a high percentage andmultiplicity of carbapenem resistance genes among *P. aeruginosa* isolates in Khartoum State. This study showed that the NDM-1 and IMP-1 genes were commonly disseminated among the *P. aeruginosa* .

5.2 Recommendations

It is of great importance to make a unique and precise guideline for routine microbiology laboratories in order to detect carbapenemase producing isolates.

Extensive infection control measures in community and hospitals to study the spread of carbapenemase-producing isolates among patients in Sudan.

Sequencing of detected carbapenem resistance genes as well as further studies with large number of isolates required to validate the results of this study.

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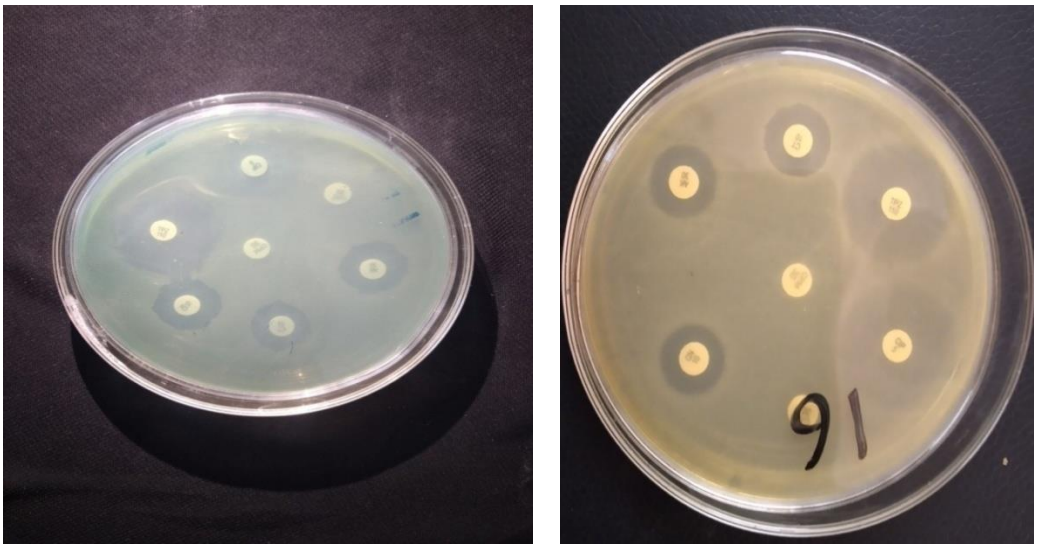
APPENDICES

Appendix (1)

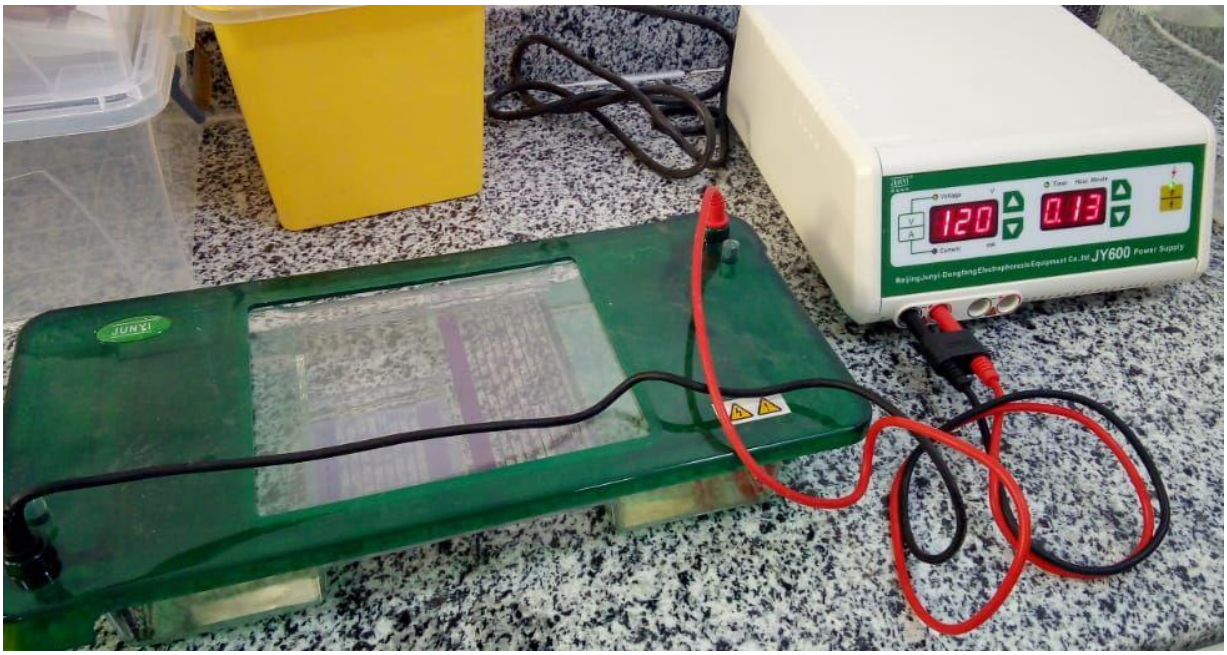
Color plates



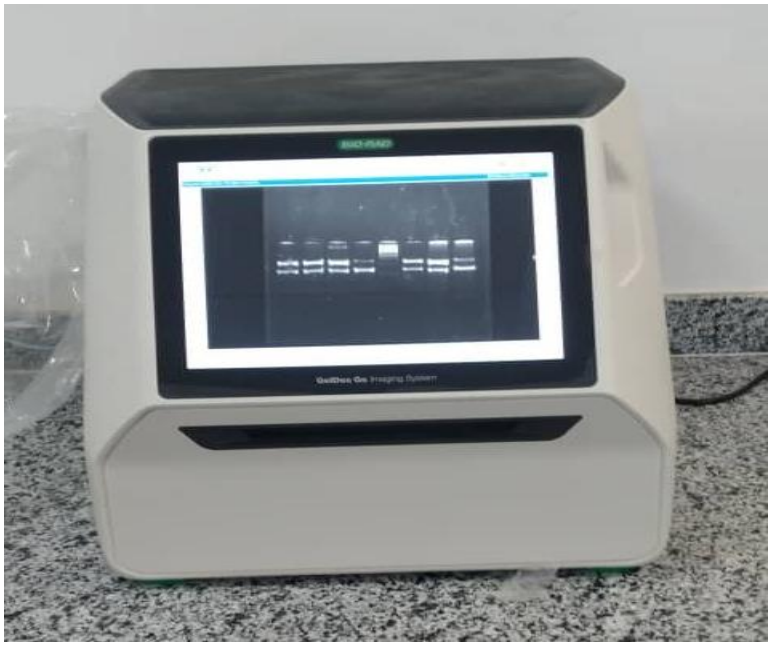
Color plate (1):Antimicrobials susceptibility test using Kirby Bauer disc diffusion method of *P. aeruginosa isolates*

Color plate (2): Thermal block incubator (Chem-Tech)

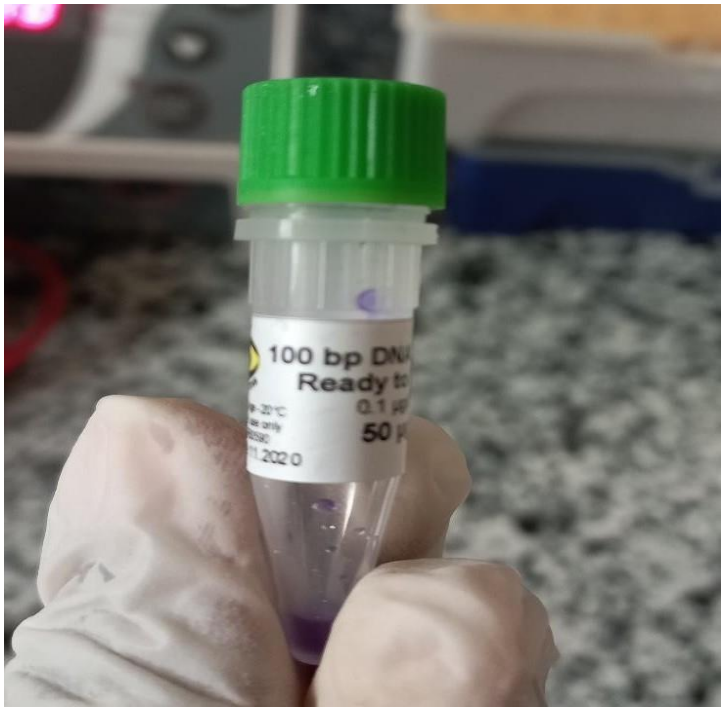
Color plate(3): Thermocycler PCR (Bio-Rad; C1000 touch, UK)



Color plate (4) :The gel electrophoresis apparatus was connected to the power supply (JY600; Beijing Junji-Dongfang, China)



Color plate (5): Ultraviolet Trans- illuminator (Bio-Rad, UK)



Color plate (6): 100 bp DNA leader

Appendix (II)

1. Equipments and instruments :

a. light microscope.
b. incubator 37°C
c. hot air oven.
d. sensitive balance.
e. refrigerator.
f. Bunsen burner
g. bacteriological loops
h. straight wire.
i. forceps
j. wooden stick.
k. cotton
l. Autoclave
m. filter paper.
n. physiological saline
o. distilled water.
p. centrifugation.
q. thermocycler.
r. UV light machine.
s. micropipettes.
t. eppendorff tube
u. tips

2. **Glass ware:**

a. Petri dishes.
b. Flasks 100,500,250ml
c. measuring cylinders 500and 1000ml.
d. test tubes.

3. **Media and reagent**:

a. CLED agar.
b. Muller hinton agar.
c. Kligler Iron Agar.

d. Christensen's urea medium.
e. Simmon citrate medium
f. Peptone water.
g. Kovac’s reagent.
h. oxidasedisc .
i. Gram stain set.
j. Agarose powder
k. TBE buffer.

Appendix (III)

Preparation of media

1.Cystine Lactose Electrolyte Deficient Agar:

Ph **(**at 25°C)=7.3+/-0,2Ingredients 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.

2.Kligler iron agarM078-500G:

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 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 tubes.

3.Urea agar baseM112s-500G:

pH (at 25°C) =7.3+/-0.1

Formula:

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.

4.Simmons citrate agarM099-500G:

pH (at 25°C) =6.8+/-0.2

Formula:

Ingredients Gms/litter Magnesium sulphate 0.02 Ammonium dihydrogenphophate 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.

5.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.

6. peptone water broth:

Composition** Ingredients Gms / Litre Casein enzymic hydrolysate 10.000 Sodium chloride 5.000 Final pH (at 25°C) 7.5±0.2 **Formula adjusted, standardized to suit performance parameters

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.

7.Semisolid media for motility:

Ingredients Gms / Litre Tryptose 10.000 Sodium chloride 5.000 Agar 5.000 Final pH (at 25°C) 7.2±0.2 **Formula adjusted, standardized to suit performance parameters 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.

8.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 the staining 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.

Transfer 20mg of fast green dye in another 100ml beaker. ...

Filter both the staining solutions to avoid particles.

Acetone - alcohol decolorizer

To make 1 litre :

- Acetone……..500 ml
- Ethanol or methanol, absolute* ……..475 ml
- Distilled water……25 ml

9.Mueller Hinton Agar:

pH (at 25°C) =7.3+/-0.1

Formula:

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.

10.McFarland Standard Turbidity tube 0.5:

Ingredients

Sulphuric acid ……..……..…….1 %

Dehydrated barium chloride ……..……..1.16 g

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. Mix19.9 ml of sulphuric acid with 0.1ml of barium chloride.

11.Preparation of 10 X TE buffer

10 X contains 500 Mm KCL,100MmTris HCL(PH 9.0 at 25 c) ,15 Mm Mgcl2 and 1% Triton X-100. This buffer is optimized for use with 200Um dNTP

12.Preparation of 1X TE buffer

Ten ml of 10 X TBE buffer was added to 90 ml deionized water

13.Preparation of ethidium bromide solution

Ten mg of ethidium bromide powder were dissolved into 500 µldw, and kept into brown bottle.

14. Preparation of agarose gel

Amount of 2 gm of agarose powder (iNtRON Biotechnology, Korea) dissolved by boiling in 100 ml 1X TBE buffer (Thermo Scientific, Lithuania), then was cooled to 55°C in water bath, then 2µl (0.2 for 10 µl) of Ethidium bromides (iNtRON Biotechnology, Korea) were added, mixed well and poured on to the casting tray that has been taped up appropriately and was equipped with suitable comb to form well in place. Any bubbles were removed and the gel was allowed to set at room temperature After solidification, the comb was gently removed and the spacer from the opened sides was removed (Jalali *et al.,* 2015)