**Sudan University of Science and Technology**

College of Graduate Studies

# Molecular detection of Virulence Genes oprI,ToxA and nan-1 of *Pseudomonas aeruginosa* Isolated from Different Clinical Specimens by Multiplex PCR, Khartoum, Sudan

الكشف الجزيئي عن جينات العوامل الامراضية او بي ار و تو اكزا اي و نان في الزائفة الزنجارية المعزولة من العينات السريرة في ولايه الخرطوم في السودن

A Thesis submitted in partial fulfillment of the
Requirements for the Award the Degree of
**Master of Medical Laboratory Science (Microbiology)**


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بسم الله الرحمن الرحيم

**الاية**

فَتَعَالَى اللَّهُ الْمَلِكُ الْحَقُّ ۗ وَلَا تَعْجَلْ بِالْقُرْآنِ مِنْ قَبْلِ أَنْ
يُقْضَىٰ إِلَيْكَ وَحْيُهُ ۖ وَقُلْ رَبِّ زِدْنِي عِلْمًا ﴿١١٤﴾

صدق الله العظيم

سورة طه الايه (114)

## Dedication

I dedicate this thesis to whom I love and admire

My mother and my Father.

To my sisters and my brothers.

To my friends

And all those who have helped with their advice and efforts.

**Acknowledgment**

All thank to ALMIGHTY ALLAH for giving me strength and courage to complete this work and made all the things possible.

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**Abstract**

*Pseudomonas aeruginosa is* one of the leading causes of nosocomial infections, was estimated to be involved in 10% to 22.5% of the hospital-acquired infections (HAI) as well in adults as in children. Furthermore is frequently life-threatening and often challenging to treat because it expresses a combination of bacteria-associated factors (intrinsic and acquired antimicrobial resistance, expression of different virulence factors. This study attempt to determine the virulence genes of *P.aeruginosa* which had correlation with their antibiotic resistance using multiplex Polymerase Chain Reaction. Total of *50 Pseudomonas aeruginosa* clinical isolates were collected from different hospitals, the samples were isolated on CLED and identified by gram stain, biochemical test and sensitivity test was carried, and were confirmed by Gene detection. The purity of the extracted DNA was determined by running the DNA sample on 1.5% agarose gel, All outcome data were analyzed by using Statistical Package for Social Sciences. The oprI genes were detected in 48 isolates (96%) and negative in 2 isolate (4%). They were recovered from urine 17 (94%), wound 10 (90%), ear swab 3 (100%), sputum 8(100%), blood 6(100%) high vaginal swabs 2 (100%) and body fluid 2(100%).The ToxA gene was detected in 47(94%) and negative in 3 isolate. nan1 gene was detected in 6(12%) and were negative in 44(88). There was significant difference in the prevalence of virulence genes among the different sites. The gene **ToxA gene** was harbored in all .samples, especially in urine samples which was significantly higher than wounds specimen. Comparing to other genes, **Nan1 gene** was higher percent in sputum 2 (25 %) and wound samples 2 (18 %). All 50 isolates were cultured in Mueller-hinton medium using 0.5 Mcfarland turbidity standard for 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. The overall results revealed that there was significant strong association between the presence of (oprI, toxA and nan1) genes and sensitive to Ciprofloxacin (p-value 0.4-0.3 and 0.1 respectively). This study showed that the oprI and Toxa genes are commonly disseminated among the *P. aeruginosa*. The differences in the distributions of virulence genes in the isolated strains need further studies to find out the actual role of these genes of *P. aeruginosa*

in their resistance to antibiotics. PCR showed that all *P. aeruginosa* strains do not necessarily have similar virulence genes. It seems that simultaneous use of oprI genes provides more confident detection of *P. aeruginosa* by PCR .

**مستخلص البحث**

الزائفة الزنجارية هي أحد الأسباب الرئيسية للعدوى النوزومية، ويقدر أنها تشارك في10 ٪ إلى22.5 ٪ من العدوى المكتسبة من المستشفى وكذلك في البالغين كما هو الحال عند الأطفال .علاوة على ذلك، غالبًا ما يكون العلاج مهددًا للحياة وغالبًا ما يكون صعبًا لأنه يعبر عن مجموعة من العوامل المرتبطة بالبكتيريا) المقاومة الجوهرية والمكتسبة لمضادات الميكروبات، والتعبير عن عوامل الفوعة المختلفة .تحاول هذه الدراسة تحديد جينات الفوعة للزائقه الزنجارية .التي لها علاقة بمقاومتها للمضادات الحيوية باستخدام تفاعل البوليميراز المتسلسل متعدد الإرسال .تم جمع ما مجموعه 50 عزلة إكلينيكية الزائفه الزنجارية من مستشفيات مختلفة، وتم عزل العينات على CLED وتم تحديدها بواسطة صبغة جرام، وتم إجراء اختبار كيميائي حيوي واختبار حساسية، وتم تأكيدها بواسطة اكتشاف الجين .تم تحديد نقاء الحمض النووي المستخرج من خلال تشغيل عينة الحمض النووي على هلام أغاروز بنسبة1.5 ٪، وتم تحليل جميع بيانات النتائج باستخدام الحزمة الإحصائية للعلوم الاجتماعية .تم اكتشاف جينات oprI في 48 عزلة96) ٪ (وسلبية في 2 عزلة4) ٪ .(تم استردادها من البول 17 94)٪(، الجرح90) 10 ٪(، مسحة الأذن100) 3 ٪(، البلغم100) 8 ٪(، الدم100) 6 ٪ (مسحات مهبلية عالية100) 2 ٪ ( وسائل الجسم100) 2 ٪ (تم اكتشاف جين ToxA في94) 47 ٪ (وسلبي في 3 عزلة .تم اكتشاف جين نان 1في12) 6 ٪ ( وكان سلبيًا في .(88) 44 كان هناك اختلاف كبير في انتشار جينات الفوعة بين المواقع المختلفة .تم إيواء جين ToxA في جميع. العينات، خاصة في عينات البول التي كانت أعلى بكثير من عينة الجروح .بالمقارنة مع الجينات الأخرى، كان الجين Nan1أعلى بالمائة في البلغم25) 2 ٪ (وعينات الجروح18) 2 ٪ .(تم زراعة جميع العزلات الخمسين في وسطا التزريع المناسب لاختبار الحساسيه باستخدام 0.5 معيار التعكر لاختبار حساسية المضادات الحيوية ضد 30) Amikacin ميكروغرام(، 10) colistin ميكروغرام(، 30) aztreonam ميكروغرام(، 5) ciprofloxacin ميكروغرام(، 10) gentamicinميكروغرام (كشفت النتائج الإجمالية أن هناك ارتباطًا قويًا كبيرًا بين وجود الجينات oprI) و toxA و nan1)والحساسة لـ) Ciprofloxacin قيمة 0.3-0.4 p و 0.1 على التوالي. أظهرت هذه الدراسة أن جينات oprI و Toxa تنتشر بشكل شائع بين الزائفه الزنجاريه .تحتاج الاختلافات في توزيعات جينات الفوعة في السلالات المعزولة إلى مزيد من الدراسات لمعرفة الدور الفعلي لهذه الجينات من الزائفة الزنجارية في مقاومتها للمضادات الحيوية .أظهر اختبار البرمله أن جميع سلالات الزائفة الزنجارية لا تحتوي بالضرورة على جينات فوعة مماثلة .يبدو أن الاستخدام المتزامن لجينات oprI يوفر اكتشافًا أكثر ثقة لـ الزائفة الزنجارية بواسطة اختبار البرمله.

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## List of Abbreviation

PCR:Polymerase chain reaction
CSF :Cerebrospinal fluid
CF: Cystic fibrosis
ETT: endotracheal tube aspirates
TTSS: Type IIIsecretion system
MBL: metallo-B-lactamases
ESBL:extended spectrum beta lactamases
AK:Amikacin
ATM:Aztreonam
CAZ: Ceftazidime
CIP: Ciprofloxacin
CT:Colistin
CN: Gentamicin
IMP:Imipenem
NOR:Norofloxacin
TPZ: Piperacillin
SSI : surgical site infection

# CHAPTER ONE

# INTRODUCTION

# CHAPTER ONE

# 1.INTRODUTION

## 1.1. Background

*Pseudomonas aeruginosa* is a Gram-negative rod measuring 0.5 to 0.8 μm by 1.5 to 3.0 μm. It belongs to the group of γ-Proteobacteria and it possesses a remarkable metabolic versatility, almost all strains are motile by means of a single polar flagellum (Mazar .2017; Mesquita *et al.*, 2013). It is strict aerobic, although it can achieve anaerobic growth by using nitrogen as a terminal electron acceptor in the absence of oxygen (Mazar .2017).

*P.aeruginosa* existing both as a ubiquitous environmental organism inhabits soil, water, and vegetation and as an opportunistic pathogen, within the hospital, *P. aeruginosa* finds numerous reservoirs: disinfectants, respiratory equipment, food, sinks, taps, and mops (Mesquita *et al.*, 2013; Mazar .2017). It is found in the skin of some healthy persons and has been isolated from the throat (5%) and stool (3%) of nonhospitalized patients (Mazar .2017).

This opportunistic pathogen may infect virtually any tissue, Urinary tract infections, cystic fibrosis, pneumonia, meningitis, otitis externa, infection is facilitated by the presence of underlying disease or by a breakdown in nonspecific host defenses (Rossolini and Mantengoli, 2005). *P. aeruginosa* is amongst the leading causes of nosocomial pneumonia, especially in mechanically ventilated patients, called Ventilator Associated Pneumonia (VAP) which is the most difficult to be treated thus mortality due to this bacteria has been presented as high as70% and directly mortality rates are almost 40%.(Joodzadeh *et al.*, 2016).

*P.aeruginosa* produces many factors that contribute to its virulence. These can be classified into cell-associated and secreted virulence factors. Cell-associated factors include flagella, pilli and lipopolysaccharide (LPS) it involved in the attachment, motility and the delivery of effector proteins into the host cells, as well as being involved in the establishment of persistent infections (Mesquita *et al.*, 2013; Mazar .2017).

Secreted virulence factors are causing extensive tissue damage, invasion and dissemination, maintaining a persistent inflammatory state and leading to pathogenesis success, it include Type

III secretion system (TTSS), proteases, pyocyanin, *P. aeruginosa* might also secrete two siderophores to accumulate iron: pyochelin and pyoverdine. Other secreted virulence factors such as phospholipase C, histamine and leukocidin (Mesquita *et al.,* 2013). TTSS is a main virulence factor for pathogenesis of *P. aeruginosa* that uses the TTSS to carry effector toxins (*ExoS, ExoU, ExoY,* and *ExoT*) into host cells causing cell necrosis and cytoskeleton damaging that help pathogen to invade host cells (Joodzadeh *et al.,* 2016), proteases (LasB elastase, LasA elastase or staphylolysin, alkaline protease and protease IV) enhancing the ability of *P. aeruginosa* to invade tissues and interfering with host defence mechanisms (Mesquita *et al.*,2013). Pyocyanin, however, facilitate colonization of *P aeruginosa* by retards the growth of some other bacteria (Mazar .2017).

Due to lower outer membrane permeability and to active efflux of antibiotics in *P.aeruginosa* that lead to a significant number of strains show innate and acquired resistance against a wide range of antimicrobial agents (e.g. β-lactams, aminoglycosides, fluoroquinolones) resulting in difficulty in treating infections (Mazar M.2017; Mesquita *et al.,* 2013), making susceptibility testing essential, combination of gentamicin and carbenicillin is frequently used to treat severe *Pseudomonas* infections (Mazar .2017). ).This study focus on detection of *oprI* , , *toxA* and *nan1* genes that encode for one of the most important virulence factors of *P.aeruginosa* by molecular technique.

**1.2. Rationale:**

*P. aeruginosa* one of the leading causes of nosocomial infections, was estimated to be involved in 10% to 22.5% of the hospital-acquired infections (HAI) as well in adults as in children (Holban *et al.,* 2013), it is responsible for variety of systemic infections, include urinary tract infections, respiratory system infections, skin and soft tissue infections, bone and joint infections, bacteraemia and also considered the prime lung pathogen of cystic fibrosis (CF) patients (Mesquita, 2013), Furthermore Virulent *P.aeruginosa* is frequently life-threatening and often challenging to treat because it expresses a combination of bacteria-associated factors (intrinsic and acquired antimicrobial resistance, expression of different virulence factors) (Sonbol *et al.,* 2015; Holban *et al.,* 2013). Each one of these factors bring different mechanisms that contributing to *P.aeruginosa* pathogenesis for example Protein biosynthesis is inhibited by exotoxin A and virulence factor exoenzyme S is secreted by a type III section system . concerning will the virulence genes and factors of *p.aeruginosa* ,so This study attempt to determine of virulence genes of *p.aeruginosa* which had correlation with antibiotic resistance using PCR . ).

## 1.3 Objectives:-

### 1.3.1 General objectives

To detect virulence genes (OprI ,TOXA and Nan-1)of *P. aeruginosa using molecular technique.*

### 1.3.2 Specific objectives

1-To perform *P. aeruginosa* susceptibility testing to commonly used antibiotics by disc diffusion method.

2- To detect *P.aeruginosa* virulence genes (*oprI, toxA* and *nan1*) by multiplex PCR

3--To find out the correlation between virulence genes and results of antimicrobial sensitivity

4 to determine association between virulence genes of *pseudomonas* and sex, type of samples and antimicrobial sensitivity.

# CHAPTER TWO
# LITERATURE REVIEW

# CHAPTER TWO

# 2.LITERATURE REVIEW

## 2.1. *Pseudomonas aeruginosa:*

### 2.1,1 Definition of *Pseudomonas aeruginosa:*

*P. aeruginosa* was first obtained in pure culture by Gessard in 1882 from wounds that had produced blue-green discoloration (Mazar .2017).

The word‘aeruginosa’ comes from the Latin word for verdigris or copper rust. This describes the blue-green bacterial pigment seen in laboratory cultures *of P. aeruginosa.* It is a Gram-negative, mesophilic, aerobic rod (measuring 0.5to 0.8 μmby1.5to3.0μm) (Mazar .2017).They are oxidase positive or negative, catalase positive, non-acid fast rods,. It is motile, with polar flagella and do not ferment carbohydrates, do not fix nitrogen and are not photosynthetic. Most species fail to grow in acidic conditions (pH 4.5 or lower) and do not require organic growth factors. The optimum growth temperature for most strains is 28 ºC, but many are capable of growth between 4- 45 ºC (Mazar .2017).

*P.aeruginosa* usually produces large, flat, haemolytic colonies on blood agar, also grows well on nutrient agar, MacConkey agar and other media containing bile salts and also on cetrimide agar (Mazar .2017), it produces colonies with a fluorescent greenish color. It often produces the nonfluorescent bluish pigment pyocyanin, which diffuses into the agar. 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(Mazar .2017).

### .2.1.2 Normal habitat

It is a ubiquitous organism present in a variety of environmental niches, because it is able to tolerate a variety of physical conditions and survive on minimal nutritional requirements, it is frequently found in hospital environment especially in moist places such as sink, bowl drains, cleaning buckets and humidifiers. It is also found growing in eye drops ointment, weak antiseptic solution (Mesquita *et al.,* 2013; Mazar .2017), in community settings it is found in swimming

pools, whirlpools, hot tubs, contact lens solution, home humidifiers, soil and rhizosphere, and vegetables (Mesquita *et al.,* 2013).

Normally the human faecal carrier rate for *P. aeruginosa* is less than 10%. However, this carrier rates increase 30% after 3 weeks with the length of stay of patient in hospitals, and this represent a distinct risk of endogenous infection (Mazar .2017).

Isolation of *P.aeruginosa* from healthy carriers or environmental sites is significant only when there is a risk of transfer to compromised patients. e.g by nurse hand or through respirators(Mazar 2017).

**2.1.3 Antigenic structures of *P.aeruginosa***

The cell envelope of *P.aeruginosa*, consists of three layers: the inner or cytoplasmic membrane, the peptidoglycan layer, and the outer membrane. The outer membrane is composed of phospholipid, protein, and lipopolysaccharide (LPS) (Barbara, 1996). The lipopolysaccharide is responsible for many of the endotoxic properties of the organism and it is less toxic than that of other Gram-negative, it exists in multiple immuno-types. Lipopolysaccharide and pyocin (bacteriocin) susceptibility can be used to typed *P.aeruginosa* (Mazar .2017; Brook *et al*., 2004). Pili, which may be antiphagocytic and aids in bacterial attachment, thereby promoting colonization. The Polysaccharide capsule is responsible for the mucoid colonies seen in culture from patients with cystic fibrosis(Mazar .2017).Most strains of *Pseudomonas* produce extra cellular enzymes including elastases, proteases and two hemolysins, a heat labile phospholipase c and heat stable glycolipid. Many strains of *P.aeruginosa* produce exotoxin A which causes tissue necrosis (Mazar .2017).

**2.1.4 Pathogenesis of *P.aeruginosa*:-**

*P.aeruginosa* is an opportunistic pathogen causing a wide range of human infections. It is pathogenic only when introduced into areas devoid of normal defenses (Streeter and Katouli, 2016). The pathogenicity of *P.aeruginosa* is multifactorial, in which a variety of virulence factors are involved to help the bacteria to adhere and invade the host by damaging the host's immune responses and forming a barrier to antibiotics (Mazar .2017; Holban *et al.,* 2013). These large arrays of virulence factors can be classified into cell-associated and secreted virulence factors (Holban *et al.,* 2013; Mesquita *et al.,* 2013). These include protease enzymes, mucoid exopolysaccharide, pili, exotoxin A, lipopolysaccharide, pigments, lipase, haemolysin, histamine, exoenzyme S, leukocidin and rhamnolipids.

These virulence factors encode by different genes, since the genome of *P.aeruginosa* is one of the highest among all bacterial genomes (Mesquita, 2013).

### 2.2. Virulence factors of *P.aeruginosa*:

#### 2.2.1 *P.aeruginosa oprI* and *oprL* genes:

The *oprI* gene is coding for the outer membrane lipoprotein I, and the *oprL* gene is coding for the outer membrane lipoprotein L both genes are conserved among the fluorescent *pseudomonad*s, and therefore it was used as phylogenetic marker for the classification of rRNA group I pseudomonad (Mazar .2017), these specific outer membrane proteins play important roles in the interaction of the bacterium with the environment as well as the inherent resistance to antibiotics (Khattab *et al.,* 2015).Since these genes are conserved and found only in this organism (Khattab *et al.,* 2015), different studies were done to identify *P.aeruginos*a using these two genes. De Vos *et al*. (1997) by designing a multiplex PCR assay based on *oprI* and *oprL* genes for molecular detection of *P. aeruginosa* showed that the specificity and sensitivity of the PCR assay were 74 and 100%, respectively. Lavenir *et al.*(2007) also noted that all of *P. aeruginosa* strains contained the *oprI* and *oprL* genes. Nikbin *et al*. (2012) and Khattab *et al*. (2015) also noted that all of *P.aeruginos* harbored *oprI* and *oprL* genes.The product of *oprI* is very immunogenic it developed both mucosal and humoral immunity and can be used as a carrier for fused peptides to elicit antibodies without adjuvant (Mazar .2017).

#### 2.2.2 *P.aeruginosa toxA* gene

Encode for exotoxinA, it is a major member of the type II secretion system (T2SS) of *P.aeruginosa* (Yousefi *et al.,* 2015), it has enzymatic activity and belongs to the mono-ADP ribosyl transferase family it is specified asNAD+-diphthamide-ADP-ribosyl transferase (Michalska and wolf, 2015) which inhibits protein synthesis by ADP-ribosylation of eukaryotic elongation factor 2 (Yousefi *et al.,* 2015) and enables *P.aeruginosa* to damage tissue for dissemination and nutrition supply and to increase its survival rate (Michalka and wolf, 2015).

The regulation of *P.aeruginosa* exotoxinA (PE) expression is complex, different studies established a relation between PE expression and iron metabolism. The PE structural gene, *toxA*, is regulated at the transcriptional level by the gene products of the regAB operon, and both of them are mediated by Fur-regulated alternative sigma factor, which had initially been identified as a positive activator for the production of the siderophore pyoverdin (PvdS). The transcription of regAB and toxA after induction of the P tac-pvdS gene was monitored in cells grown in high-iron

medium, the bacterium produces pyoverdine that specifically chelate iron ions with high affinity. Interestingly, in the presence of iron ions, pyoverdine was found to activate signaling pathway for the up-regulation of PE expression, the efficient up take of iron is one important factor for *P.aeruginosa* allowing the colonization of the host (Ochsner *et al.*, 1996; Michalka and wolf, 2015).

Distribution of exotoxinA may differe among different clinical isolates as suggested by different studies. Khattab *et al. (*2015) found that among 30 *P.aeruginosa* isolates collected from pulmonary tract, burn and blood presence of *tox A* gene in clinical samples was different. Isolates from burn and pulmonary tract was significantly higher than that from blood (P<0.001). Nikbin *et al*.(2012) found that among 268 *P. aeruginosa* isolates collected from pulmonary tract, burn and wound, the presence of *toxA* gene in isolates from burn was significantly higher than pulmonary tract ($P < 0.05$). A total 156 *P.aeruginosa* clinical isolates were obtained from urine, cerebrospinal fluid (CSF), sputum, abdominal discharge (AD), endotracheal tube aspirates (ETT), eye, blood, and wound, the Frequency of *toxA* among wound samples was significantly higher than urine isolates (P< 0.05) and distribution of *toxA* among other sources was not significantly different (Yousefi *et al.,* 2015).

**2.2.3 *P.aeruginosa lasB* gene**

This gene is encoded for elastase a member of T2SS of *P.aeruginosa* (Sabharwal *et al.,* 2014), it is zinc metalloendopeptidase one of the several extracellular enzymes that produced by *P.aeruginosa*, it called elastase because it is active on elastin (Mazar M.2017).but also degrade collagen and inactivate human immunoglobulin G , serum a1-proteinase inhibitor, and several complement components and this indicate the importance of this enzyme as virulence factor (Bever and Iglewski, 1988) that is closely related to thermolysin is synthesized as a preproenzyme (53.4 kDa) with a classical signal peptide and a covalently linked 18-kDa amino-te2minal propeptide, The 2.4-kDa signal sequence is removed upon passage through the inner membrane into the periplasm, where the propeptide is rapidly cleaved off by autoproteolysis. This step is apparently necessary for elastase secretion because an active site mutation blocks the secretion of elastase as well as enzymatic activity and processing. When *lasB* is expressed in *Escherichia coli*, autocatalytic cleavage of the propeptide occurs independently of any other *P.aeruginosa* gene product(s). The elastase propeptide acts as an inhibitor of the enzyme. After autoprocessing, it remains noncovalently associated with the mature domain to form an inactive propeptide-elastase

complex, and it is in this form that elastase is found within the periplasm of *P. aeruginosa* . The propeptide also functions as an intramolecular chaperone required for correct folding and secretion competence. Elastase translocation through the outer membrane is mediated by a complex extracellular protein export apparatus (*Xcp*) of at least 12genes that bears significant homology to the pullulanase (type II; general secretory pathway) export apparatus of *Klebsiella oxytoca.* Although the propeptide is required for elastase secretion, the involved and the fate of the propeptide remain unknown (Mazar .2017).

Implies the importance of *LasB* factor to survival of *P. aeruginosa* in various settings, Nikbin *et al*. (2012) and Khattab *et al*. (2015), *lasB* gene was harbored in all clinical isolates tested *P.aeruginosa* isolates.

**2.2.4 *P.aeruginosa exoS* gene**

This gene is encoded for exoenzyme S is a member of Type III secretion system (TTSS) of *P.aeruginosa*, is an adenosine diphosphate ribosyltransferase (Mesquita *et al.,* 2013:Mazar M.2017). It involved in stages of colonization, invasion and dissemination of infection (Yousefi *et al.,* 2015), inhibit Protein biosynthesis (Nikbin *et al.,* 2012) and disrupt the actin cytoskeleton of host cells, ultimately causing apoptosis (Mesquita *et al.,* 2013), distinct from *Pseudomonas* toxin A by several tests: it is not neutralized by toxin A antibody, it is destroyed rather than potentiated by pretreatment with urea, and it is more heat stable (Mazar .2017).

The synthesis of exoenzyme S by *P.aeruginosa* is highly regulated. exoenzymeS expression requires the growth of *P.aeruginosa* under environmental conditions that correspond to low concentrations of cations In addition; a regulatory operon termed the exoenzyme S *trans*-regulatory locus is required for exoenzyme S synthesis (Mazar .2017).

Khattab *et al*. (2015) detected exoS gene in 46.6% of *P.aeruginosa* isolates and the difference between *exoS* prevalence in isolates from pulmonary tract and burn isolates was statistically significant higher than that from blood. In study done by Nikbin *et al*. (2012) 268 *P aeruginosa* isolates including 100 strains recovered from burn, 50 from wound and 118 from pulmonary tract infections were obtained from patient, difference between *exoS* prevalence in isolates from pulmonary tract and burn isolates was statistically significant ($P < 0.05$), while in study done by Yousefi *et al*. (2015) the distribution of *exoS among* clinical samples showed no significant differences.

### 2.2.5 *P.aeruginosa nan1* gene

Encode for the enzyme sialidase that is responsible for adherence to the respiratory tract (Nikbin *et al.,* 2012) Several arguments suggest that nan1 encodes a sialidase, an enzyme theoretically able to release sialic acid from sialylated gangliosides, thus increasing the amount of asialoGM1, a major receptor for adherence to the respiratory tract (Mazar M.2017).it is production was found to be increased under hyperosmolar conditions. Neuraminidase was active against a range of substrates expected to be present in the respiratory tract, including a2, 3-linked sialic acids as found in sialyllactose as well as the sialic acid residues present on epithelial cell surfaces. In the respiratory tract there are several potential substrates for the action of neuraminidase. The glycoproteins which comprise respiratory mucins are highly sialylated and epithelial membranes have abundant sialylated ganglioside and other glycoconjugate components (Mazar .2017).

Nikbin *et al*. (2012) found that *nan1* gene, was found in 55 (46.6%) of 118 isolates from pulmonary tract, 15 (30%) of 50 from wound and 4 (4%) of 100 from burn specimens, and the prevalence of *nan1* gene was significantly higher in isolates of pulmonary tract than burn specimens ($P < 0.05$), whilein study by Khattab *et al*. (2015) the prevalence of *nan*1 gene was significantly higher in isolates of pulmonary tract and burn specimens than isolates from blood.

### 2.3 Clinical finding of *P.aeruginosa* infection:

*P.aeruginosa* cause a variety of infections in clinical practice primarily in immunocompromised patients (Mazar .2017).

It is the prime pathogen of cystic fibrosis patient in which mucus hypoxia in airways promotes *P.aeruginosa* colonization and establish permanent residency in the airways, resulting in the recurrence of chronic lung infections (Streeter and Katouli, 2016), non-CF patients are also susceptible to respiratory tract colonization of *P.aeruginosa*, especially patients with chronic obstructive pulmonary disease (COPD), Colonization of the respiratory tract is initiated by the contamination of medical equipment and/or cross-colonization from other (Streeter and Katouli, 2016). It cause urinary tract infection when introduced by catheters and instrument or surgery (Barbara, 1996), catheterization of the urinary tract is the major cause of nosocomial acquired-UTI by *P.aeruginosa* (Streeter and Katouli, 2016). It colonize severe burns and wound infections giving rise to green blue pus wound infections (Streeter and Katouli, 2016), it may cause invasive (malignant) otitis externa in diabetic patient. Infections of the eye which may lead to rapid destruction of the eye occur most commonly after injury or surgical procedures. In infants or a

debilitated person, *P. aeruginosa* may invade the blood stream and result in fatal sepsis. This occurs commonly in patients with leukemia or lymphoma who received antineoplastic drugs or radiation therapy; it causes meningitis when introduced by lumber puncture (Mazar .2017).

**2.4 *P.aeruginosa* antibiotics resistant mechanisms:-**

*P. aeruginosa* has been living in the soil for millions of years in the presence of antibiotic producing bacilli, actinomycetes and moulds, therefore it has developed resistance to a variety of their naturally occurring antibiotics. Moreover, *P. aeruginosa* maintains antibiotic resistant plasmids, and is able to transfer these genes by means of the bacterial processes of transduction and conjugation. Only a few antibiotics are effective against *Pseudomonas*, including someβ - lactams, aminoglycosides and fluoroquinolones, and even these antibiotics are not effective against all strains, this limits future therapeutic uses of antibiotics against this bacterium and increases rates of mortality (Mazar .2017).

the resistance could arise through several mechanisms, including antibiotic-modifying enzymes and intrinsic resistance mechanisms; such as decreased outer membrane permeability and upregulation of multidrug efflux pumps (Mazar .2017).

**2.4.1 Membrane Permeability**

The major permeability barrier in bacteria membrane is the lipid bilayer structure but some bacteria further protect themselves by making an additional structure. Gram negative bacteria are surrounded by a second membrane called an outer membrane, which functions as a very effective barrier, it is composed of an unusual lipid known as lipopolysaccharide (LPS) act as an efficient barrier against rapid penetration of many antibiotics, and use special class of proteins known as porins, to bring essential nutrients into the cell , These porins also make the influx of antibiotics almost impossible because of their narrow openings, *P.aeruginosa* lacks the typical high–permeability porins but instead has low-efficiency porins (Ferguson, 2008), *P.aeruginosa* has been known to have 10- to 100-fold lower outer-membrane permeability, as compared to other gram-negative bacteria (Mazar .2017).

The determining factor for *P. aeruginosa* outer membrane permeability to antibiotics is the major porin *OprF*, and possibly other known porins, such as *OprB* and *OprD* (Mazar .2017).

Even the low permeability membrane of *P.aeruginosa* can only prolong the half-equilibration time of most antibiotics for a few minutes (Mazar .2017).Therefore, a second mechanism of antibiotic resistance is usually required in addition to a low permeability barrier.

### 2.4.2 Efflux Pumps

Both Gram-negative and Gram-positive bacteria commonly produce proteins, which localized at membrane and act as multidrug efflux pumps that pumping out undesirable chemicals, including anionic detergent and various antibiotics, so that concentrations of the antibiotic inside the bacterial cell are kept low and ineffectual, drug efflux pumps have broad substrate specificity and, therefore may deal with a wide range of drugs of completely unrelated pharmacological classes. So far, four such efflux pumps have been characterized in *P. aeruginosa mexAB-oprM* (β-lactams), *mexXY-oprM* (aminoglycosides), *mexCD-oprJ* and *mexEF-oprN* (carbapenems and quinolones) along with several important regulatory factors to control the expression level of these genes (Mazar .2017).The genes encoding these pumps can be found on plasmids, transposons or even as part of integrons, which facilitates widespread dissemination of the genes. However, several of these pumps are already encoded in microbial genomes (Mazar .2017).

### 2.4.3 Enzymatic Modification

Antibiotic-modifying enzymes play an important role in *P.aeruginosa* antibiotic resistance (Zeng, 2004), they are wide ranges of enzymes and use many strategies to confer antibiotic resistance. Some of these enzymes inactivate the antiabiotic by hydrolysing susceptible bonds which are central to the antibiotics' biological activities (β-lactamases), whereas others modify the antibiotic resulting in structural alterations that impair target binding as aminoglycoside modifying enzymes (Mazar .2017), these enzymatic genes are commonly carried by mobile genetic elements such as plasmids and transposons in *P.aeruginosa*, and in other cases are chromosomally encoded. These enzymes are either originated from antibiotic-producing microorganisms or derived from normal metabolic enzymes (Mazar .2017).

### 2.7.4 Mutational Resistance

*P. aeruginosa* is capable of acquiring resistance through mutation in specific chromosomal genes these mutations can fall into a large number of regulatory genes controlling the function of membrane permeability, efflux pumps, or other antibiotic-resistance genes (Mazar M.2017). Oliver *et al*. (2000) examined CF isolates and found that hyper mutable *P. aeruginosa* mutants ( with an extra ordinarily high mutation rate (about 100-fold higher than that of spontaneous mutation) in one third of the CF isolates examined, easily generating clones with increased levels of resistance to various antibiotics(Mazar .2017).

### 2.5 Antibiotics and *P.aeruginosa*:

*P.aeruginosa* is frequently resistant to many commonly used antibiotics (Mesquita *et al.*, 2013).in this study six antibiotics have been used to test susceptibility of isolated *P.aeruginosa* , four of them are β-lactam antibiotics this group of antibiotics consist of four member that contain in the core of its structure nitrogen-containing beta-lactam ring which is key to the mode of action of this group, Beta lactam antibiotics target the penicillin-binding proteins or PBPs - a group of enzymes found anchored in the cell membrane, which are involved in the cross-linking of the bacterial cell wall. The beta-lactam ring portion of this group of antibiotics binds to these different PBPs, rendering them unable to perform their role in cell wall synthesis. This then leads to death of the bacterial cell due to osmotic instability or autolysis. (https://amrls.cvm.msu.edu /pharmacolog /antimicrobials/antibiotics-of veterinary-importance). *P.aeruginosa* resist the beta-lactam antibiotics by producing enzymes (β -lactamases) that target and hydrolyze the peptide bond of the β-lactam ring to inactivate the antibiotics, *P. aeruginosa* is able to produce various β-lactamases, including extended-spectrum β-lactamases (ESBL), metallo-β-lactamases (MBL) and chromosomalcephalosporinase (AmpC). From aminoglycoside antibiotics gentamycin is used, it bind to the 30s ribosomal sub-unit inside the bacterial cell and cause a misreading of the genetic code. This subsequently leads to the interruption of normal bacterial protein synthesis (https://amrls.cvm.msu.edu/pharmacolog /antimicrobials /antibiotics-of veterinary-importance). Acquired resistance to aminoglycosides can be due to the production of aminoglycoside-modifying enzymes, the most prevalent aminoglycoside-modifying enzymes found in *P.aeruginosa* are the acetyl-transferases AAC(6')- II (resistance to gentamicin, tobramycin and netilmicin), AAC(3)-I (resistance to gentamicin), AAC(3)-II (resistance to gentamicin, tobramycin and netilmicin) and AAC(6')-I (resistance to tobramycin, netilmicin and amikacin), and the adenylyl-transferase ANT(2 ')-I (resistance to gentamicin and tobramycin). Or mutation in the efflux systems that reduced aminoglycoside uptake this mode of resistant has broad spectrum than modified enzymes (Mazar .2017).

Ciprofloxacin is another antibiotic used in this study representing the fluoroquinolones antibiotics, that have been shown to bind to the DNA gyrase-DNA complex and interrupt a process that leads to the negative supercoiling of bacterial DNA. This disruption leads to defects in the necessary supercoiling, and render the bacteria unable to multiply and survive Acquired resistance to fluoroquinolones can be due either to mutations that cause the up-regulation of

efflux systems, or to mutations of the target of fluoroquinolones DNA gyrase, followed by topoisomerase IV as the secondary target (Streeter and Katouli, 2016;Rossolini and Mazar .2017)

# CHAPTER THREE

# MATERIALS AND METHODS

# CHAPTER THREE

# 3. MATERIALS AND METHODS

## 3.1Study design

This study design was a descriptive cross-sectional laboratory base study.

## 3.2Study 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 lab.

## 3.3Study duration

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

## 3.4Sample size

A total of fifty non-repetitive *P.aeruginosa* (n=50), from different clinical specimens (18 Urine, 11 Wound swabs, 6 Blood, 2 High vaginal swabs, 8 Sputum, 3 Ear swabs, and 2 Body fluid).

## 3.5. Sampling technique

Non-probability convenience sampling technique.

## 3.6. Data collection procedure

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

## 3.7. Ethical clearance:

Ethical approval to conduct this study was obtained from the research committee of medical Laboratory College of, Sudan University of Science and Technology.

## 3.8. Laboratory methods

### 3.8.1Sample purification and identification

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

### 3.8.2Colonial 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.

### 3.9 Gram stain

The procedure was carried out according to ( Tille,2015) 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.

### 3.10. Biochemical test

### 3.10.1Oxidase 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 phenylenediaminedihydrochloride). The purple color that appears within 60 seconds indicates positive results according to ( Mackie and Mccartney,1953).

### 3.10.2. 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) – dimethylaminobenzaldehyde) 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 and Mccartney,1953).

### 3.10.3. 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 (Tille,2015).

### 3.10.4. 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 (Mackie and Mccartney,1953).

### 3.10.5. Citrate 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 Mccartney,1953).

### 3.11. 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.

### 3.12. Antimicrobial Susceptibility test

All confirmed isolates were subculture from 15% glycerol in neutral agar getting overnight and pure growth. Disc diffusion tests 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. Following antimicrobial disc (Bioanalyse company, Turkey) were applied on the surface of the agar.

### 3.13 Molecular characterizations

### 3.13.1. 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.W 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 use.

**3.13.2. Gel electrophoresis of extracted DNA**

The purity of the extracted DNA was determined by running the DNA sample on 1.5% agarose gel..

**3.13.3 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) . The PCR assay was carried out in a total volume of 25 µL of mixture containing 2 µL Maxime 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 WFI (water for injection). 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 (oprI, toxA and nan1 genes) and extension at 72°C for 1min; and the final extension at 72°C for 5 min (Jalali et al., 2015).

**T able (3): Primers used for amplification of virulence genes of *P.aeruginosa* isolates (Khattab, 2015)**

| Amplified gene | Specific primer | Size |
|---|---|---|
| **oprI** | PS1, 5'-ATG AAC AAC GTT CTG AAA TTC TCT GCT-3'<br>PS2, 5'-CTT GCG GCT GGC TTT TTC CAG3' | 250 |
| **Toxa** | f 5' GGT AAC CAG CTC AGC CAC AT 3'<br>r 5' TGA TGT CCA GGT CAT GCT TC 3' | 352 |
| **Nan-1** | F 5' AGG ATG AAT ACT TAT TTT GAT 3'<br>R 5'TCA CTA AAT CCA TCT CTG ACC CGA TA | 1316 |

### 3.13.4 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 (120 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: 250bp, 300bp, 352bp, 1316bp.

Posotive result: there was bands appear against ladder

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

### 3.14 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, pvalue less than 0.5 was consider significant for the association between variables.

### 3.15. Safe disposal

After the end of the work all the materials was packaged into plastic bags for disposal after been burn using autoclave.

# CHAPTER FOUR

# RESULTS

# CHAPTER FOUR

# 4. RESULTS

## 4.1 Demographic data:

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 (36%) were isolated from urine samples, 11 (22%) from wound swabs, 6 (12%) from blood, 2 (4%) from high vaginal swabs, 8 (16%) from sputum, 3 (6%) from 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%) Demographic data show out of 50 the male 23 were (46%) where female were 27 (54%) with ratio 0.85 table (4.2), ages ranged from 4 to 76 years with an average of 49, majority of the samples n= 20 (40%) were found in the age group (40 – 60) year

**Distribution of gender among different age groups:**

|less than 20 age( 1 male (2%) and 0 female) ..20-40 age( 3 male(6%) and 10 female (20%)..40-60 age (10 male (20%) and 10 female (20%).. more than 60 age (9 male (18%) and 7 female (14%).

**4.2 antimicrobial susceptibility tests:**

**Table (4.2). Antimicrobial resistance pattern of *P . aeruginosa* isolates**

| sample | CIP | | CN | | TPZ | | CAZ | | ATM | | AK | | IMP | | CT | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R | S | R | S | R | S | R | S | R | S | R | S | R | S | R | S |
| urine | 3 | 15 | 4 | 14 | 5 | 13 | 6 | 12 | 12 | 6 | 2 | 16 | 2 | 16 | 5 | 13 |
| wound | 3 | 8 | 5 | 6 | 3 | 8 | 4 | 7 | 5 | 6 | 5 | 6 | 0 | 11 | 1 | 10 |
| blood | 2 | 4 | 2 | 4 | 0 | 6 | 3 | 3 | 3 | 3 | 1 | 5 | 0 | 6 | 0 | 6 |
| HVS | 0 | 2 | 0 | 2 | 0 | 2 | 0 | 2 | 1 | 1 | 0 | 2 | 0 | 2 | 1 | 1 |
| Sputum | 1 | 7 | 2 | 6 | 1 | 7 | 2 | 6 | 7 | 1 | 3 | 5 | 0 | 8 | 2 | 6 |
| Ear swab | 2 | 1 | 1 | 2 | 2 | 1 | 1 | 2 | 1 | 2 | 1 | 2 | 0 | 3 | 1 | 2 |
| Body fluid | 0 | 2 | 1 | 1 | 0 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 2 | 0 | 2 |
| Total | 11 (22 %) | 39 (78 %) | 15 (30 %) | 35 (70 %) | 11 (22 %) | 39 (78 %) | 17 (34 %) | 33(66%) | 30 (60 %) | 20 (40 %) | 13 (26 %) | 37 (74 %) | 2 (4 %) | 48 (96 %) | 10 (20 %) | 40 (80 %) |

Key☹ R:Resistant ,S:Sensitive,CIP:Ciprofloxacin,IMP:Imipenem,CT:Colistin,CAZ:Ceftazidime ,TPZ:Piperacillin ,CN:Gentamicin ,ATM:Aztreonam ,AK:Amikacin

**Table (4.3): Association between sex and Antibiotics:**

**Male more resistant to antibiotics (30%) than female(25%) while female is most sensitive to antibiotics**

| **Antibiotic** | **Sensitive** | | **Resistant** | | **p-value** |
|---|---|---|---|---|---|
| **Sex** | **Male** | **Female** | **male** | **Female** | |
| **Ceftazidime** | 15 | 18 | 8 | 9 | 0,6 |
| **Aztreonam** | 11 | 10 | 12 | 17 | 0,6 |
| **Piperacillin** | 17 | 22 | 6 | 5 | 0,5 |
| **Colistin** | 21 | 19 | 2 | 8 | 0,1 |
| **Gentamicin** | 13 | 22 | 10 | 5 | 0,5 |
| **Amikacin** | **15** | **22** | **8** | **5** | **0,5** |
| Imipenem | 22 | 26 | 1 | 1 | 0,1 |
| Ciprofloxacin | **15** | **24** | **8** | **3** | **0,1** |
| Total | **129 (70%)** | **163 (75%)** | **55 (30%)** | **53 (25%)** | |

**4.2 Association between sex and virulence gene:**

A total of 50 *P.aeruginosa* isolates were collected from different hospital and different clinical specimen The oprI genes were detected in 48 isolates (96%) and negative in 2 isolate (4%) . They were recovered from urine 17 (94%), wound 10 (90%), ear swab 3 (100%), sputum 8(100%) , blood 6(100%) high vaginal swabs 2 (100%) and body fluid 2(100%) .the ToxA gene was detected in 47(94%),and negative in 3 isolate. nan1 gene in 6(12%) figure (4.1), (4.2) , respectively.

**Table (4.4): Association between sex and virulence gene:**

| | **OprI** | | **Toxa** | | **Nan1** | |
|---|---|---|---|---|---|---|
| **Sex** | (+ve) | (-ve) | (+ve) | (-ve) | (+ve) | (-ve) |
| **Male** | 21 | 2 | 21 | 2 | 4 | 19 |
| **Female** | 27 | 0 | 26 | 1 | 2 | 25 |
| **p-value** | 0,1 | | 0,4 | | 0,2 | |

**4.3. The percentages of virulence genes in different samples type**

There was significant difference in the percentages of virulence genes among the different sites.

**4.4. OprI gene:**

The gene was detected in 48 isolates out of the 50 isolates (96%).as indicated in table (4.5)

**4.5. ToxA gene**:

The gene was harbored in all samples, in urine samples was significantly higher (94%)than wound (90%). As indicated in table(4.5).

**4.6. Nan1 gene:**

Comparing to previous genes this gene was not found in high percentage, the higher percent was found in sputum 2 (25 %) and wound samples 2( 18 %) as indicated in Table (4.5)

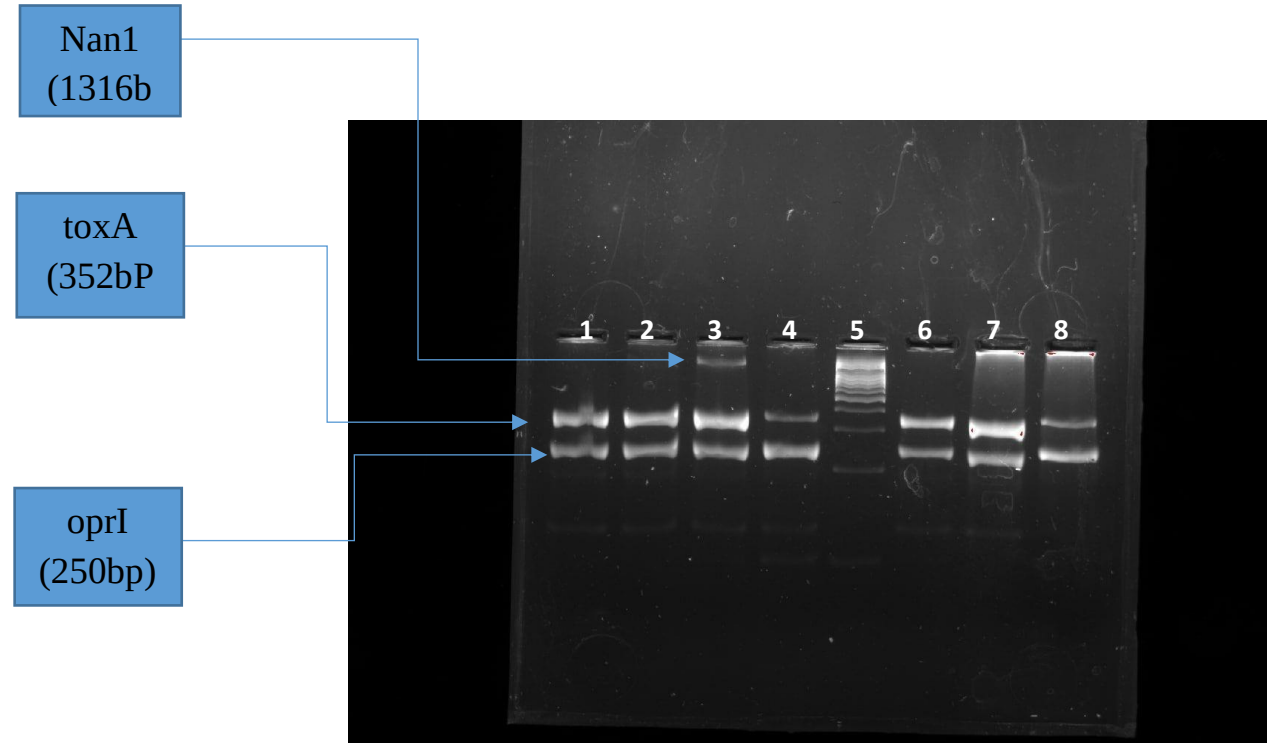



**Figure (4.1) Amplification of oprI (250bp) , Nan1 (1316bp) and toxA (352bP) from clinical isolates of *P.aeruginosa***

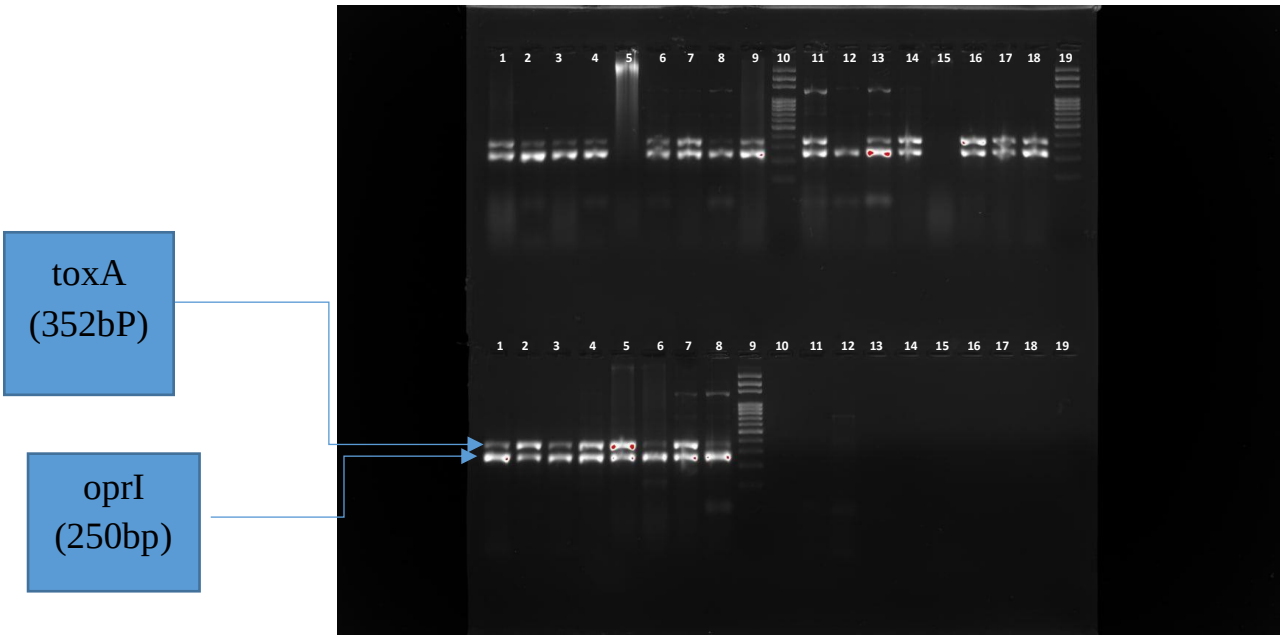



**Figure (4.2) Amplification of oprI (250bp), Nan1 (1316bp) and toxA (352bP) from clinical isolates of *P.aeruginosa***

**Table (4.5): The percentages of virulence genes in different samples type**

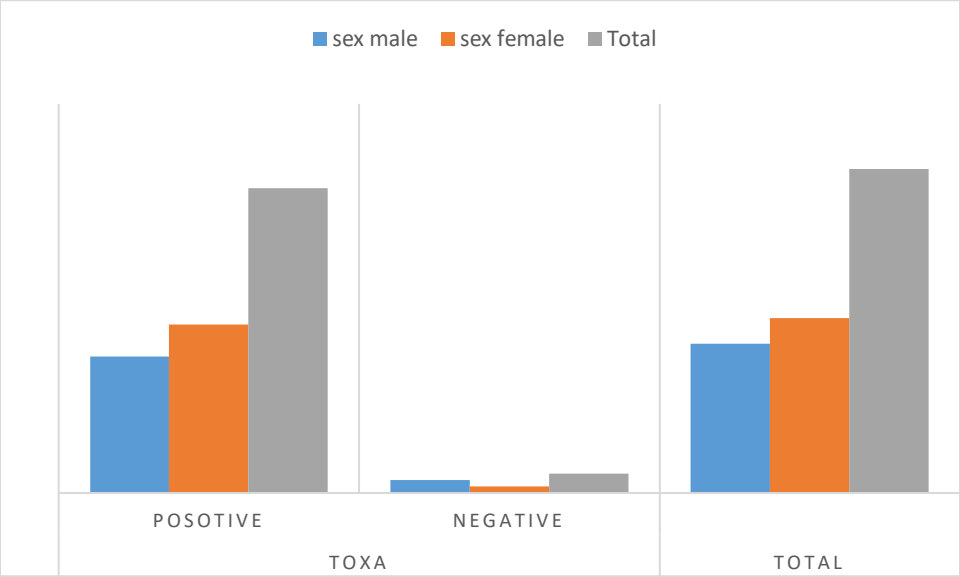
| Virulence genes | Sample sites | | | | | | P value (chi square |
|---|---|---|---|---|---|---|---|
| | Urine N=18 | Wound N=11 | Ear swab N=3 | Blood N=6 | Body fluid N=2 | Sputum N=8 | |
| **ToxA** | 17 | 10 | 3 | 5 | 2 | 8 | 0,7 |
| **Nan1** | 1 | 2 | 0 | 1 | 0 | 2 | 0,8 |
| **Opr** | 17 | 10 | 3 | 6 | 2 | 8 | 0,3 |

**4.5. The association between the presence of virulence genes and antibiotics susceptibility:**

All 50 isolates were cultured in Mueller-hinton medium using 0.5 Mcfarland turbidity standard for 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 (4.6): The association between the presence of virulence genes and antibiotics susceptibility**

| Antibiotic | Genes | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | OprI | | | ToxA | | | Nan-1 | | |
| | sensitive | Resistant | p-value | Sensitive | resistant | p-value | Sensitive | resistant | p-value |
| **Ciprofloxacin** | 37 | 11 | 0,4 | 36 | 11 | 0,3 | 6 | 0 | 0,1 |
| **Colistin** | 38 | 10 | 0,4 | 37 | 10 | 0,3 | 5 | 1 | 0,7 |
| **Gentamicin** | 33 | 15 | 0,3 | 32 | 15 | 0,2 | 6 | 0 | 0,09 |
| **Amikacin** | 35 | 13 | 0,4 | 34 | 13 | 0,2 | 6 | 0 | 0,1 |
| **Pipracillin** | 37 | 11 | 0,3 | 36 | 11 | 0,6 | 5 | 1 | 0,7 |
| **Impenem** | 46 | 2 | 0,7 | 45 | 2 | 0,7 | 6 | 0 | 0,6 |
| **Aztreonam** | 19 | 29 | 0,7 | 18 | 29 | 0,3 | 4 | 2 | 0,1 |
| **Ceftazidime** | 31 | 17 | 0,6 | 30 | 17 | 0,2 | 2 | 4 | 0,09 |




**Figure(4.3) : The percentage of ToxA *P. aeruginosa* genes**

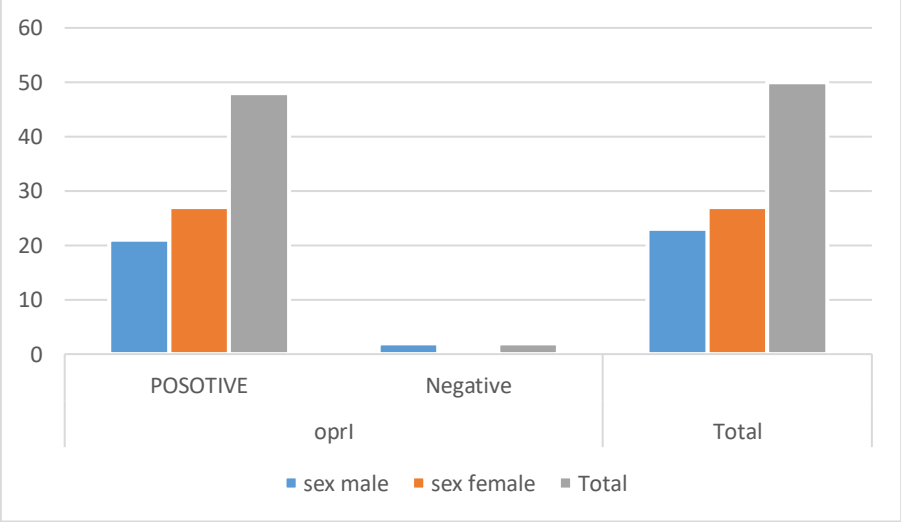



**Figure (4.4): The percentage of Opr1 *P. aeruginosa* gene**

# CHAPTER FIVE

# DISSCUTION, CONCLUTIONS AND RECOMMENDATIONS

# CHAPTER FIVE

## 5. DISCUSSION, RECOMMENATION AND CONCLUTIONS

### 5.1 Discussion:

*Pseudomonas aeruginosa* is an opportunistic pathogen capable of infecting virtually all tissues, and possesses a variety of virulence factors that may contribute to its pathogenicity ( Van Delden, & Iglewski, 1998) . And it is one of the most common causes of health care- associated infections (Fazeli, .,*etal*, 2017 ). *P. aeruginosa* contributes substantially to morbidity and mortality related to surgical site infection (SSI) worldwide, the third most commonly reported nosocomial infection.( Sapana .; *etal*,.2017]. , the result of this study showed higher occurrence of *P. aeruginosa* in the fe male (54 %), and found in the older patients (ages 40 60 years ) compared to the young, which agreed , with studies of Shewatatek et al. [ Ekrem Rokan DK.. .2014] in Ethiopia and Ekrem and R okan in AlSulaimania city, Iraq, where results of the studies showed higher occurrence of the bacterium in female and elderly patients( Shewatatek ,*etal*.2014) indicates that females in this age group are more active and involve in different clinical hygiene practices, even in hospital environment.. This result disagree with the study ( Al-Zaidi, J.R.2016) which higher occurrence of the bacterium in male and found in age group of 40-60years . A recent studies reveal *P. aeruginosa* is the most frequent pathogen that formed many of virulence factors example Tox , A oprI genes and nan1 gene ( Rhonda , 2012] . PCR results showed that, 48 out of 50 (96%) *P. aeruginosa* isolates was positive for the oprI gene with amplified size (250 bp) , Similarly to study, all isolate of *p.aeruginosa* (100%) were remarkably positive for oprI gene (Lavenir, 2007). The prevalence of *pseudomonas areugenosa* and percentage of virulence factors genes depend on several causes including nature of places, immune status of patients, degree of contamination and type and virulence of strain ( Khan, and Cerniglia, 1994).

Higher resistace of antbitics is aztreonam which combrised two third of pseudomonas aeruginosa.

Higher percentage of virulence genes (opr1,toxa) were detected among female than male this may be due to strain differences and large sample size of female than male (54% vs 46%).

The percentage of virulence genes in term of their site of infection was determined it was higher in urine followed by wound .our result different to( Elmaraghy 2019, higher percentage of virulence genes in wound followed by urine.

The study revealed that there was no correlation between virulence genes with antibiotics, there no studies to correlate with this result.

## 5.2 Conclusion:

In conclusion, this study showed that the oprI and Toxa genes are commonly disseminated among the *P. aeruginosa*. Also the differences in the distributions of virulence genes in the isolated strains need further studies for finding out the actual role of these genes of *P. aeruginosa* from different sources. PCR showed that all *P. aeruginosa* strains do not necessarily have similar virulence genes. It seems that simultaneous use of oprI genes provides more confident detection of *P. aeruginosa* by PCR .the most resistant of antibiotics in this study is Aztreonam while most sensitive is Imipenem

**5.3 Recommendations:**

1- use of oprI genes for detection of *P. aeruginosa* by PCR

2- Implementation of infection control measures will help in controlling the dissemination of virulence genes among *P. aeruginosa* isolates.

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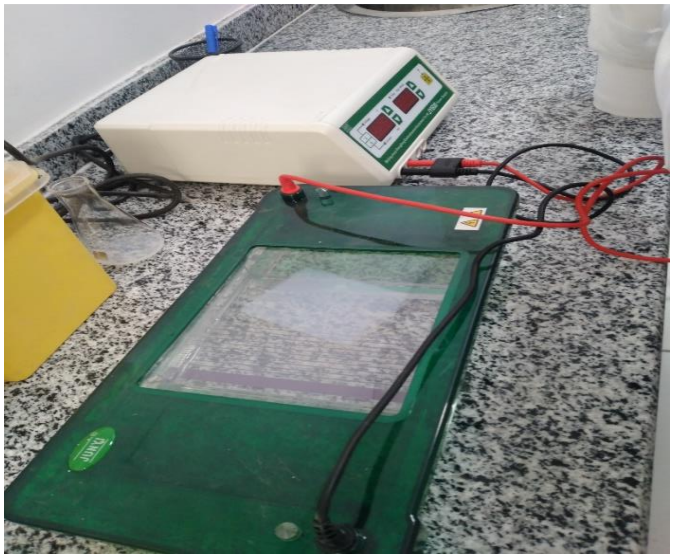
# APPENDICES

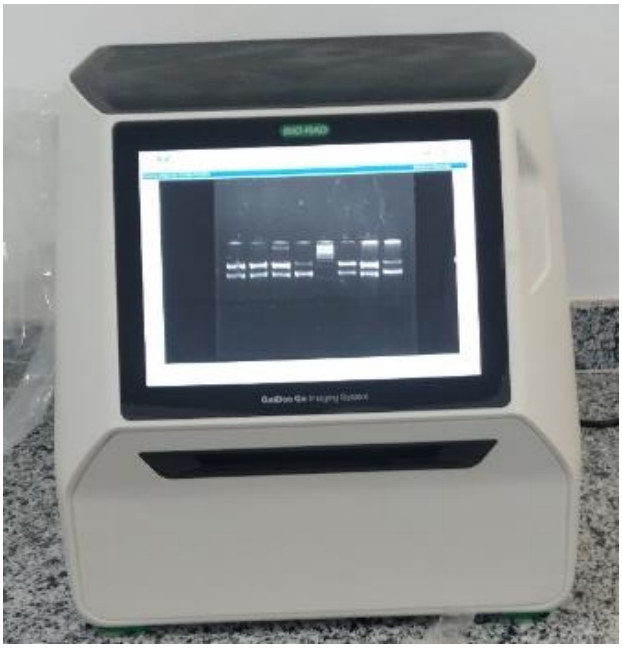
## Appendix I

## Color plates of instruments picture



**Color plate (1): Gel electrophoresis apparatus was connected to the power supply (JY600;Beijing –Dongfang, China)**


**Color plate (2): PCR machine**



**Color plate (3): Ultraviolet**

## Appendix II

### Antimicrobial drugs, abbreviations, concentration, and zone size interpretation[1]

| Name interpretation[1] | 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 |

## Appendix III

**1) Equipments and instruments**: -

light microscope.

-incubator 37°C

. -hot air oven.

-sensitive balance.

-refrigerator.

-Bunsen burner

-bacteriological loops

-straight wire.

forceps

-wooden stick.

-cotton

–Autoclave

-filter paper.

-physiological saline

. -distilled water.

-centrifugation.

-thermocycler.

-UV light machine.

-micropipettes.

-eppendorff tube

- tips

**2) Glass ware**:

-Petri dishes.

-Flasks 100,500,250ml

. -measuring cylinders 500and 1000ml.

-test tubes.

**3) Media and reagent:**

-CLED agar.

-Muller hinton agar.

- Kligler Iron Agar.

- Christensen's urea medium.

- Simmon citrate medium

. - Peptone water. - Kovac’s reagent.

-oxidase reagent.

-Gram stain set.

-Agarose powder

. -TBE buffer.

## Appendix VI

**Preparation of media**

**CLED Agar:**

Ph **(**at 25°C)=7.3+/-0,2

Formula:

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.

**Muller hinton agar M173-500G:**

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.

**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.

**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.

**Simmons citrate agarM099-500G:**

pH (at 25°C) =6.8+/-0.2

Formula:

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.

**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. Filter both the staining solutions to avoid particles.

**Acetone alcohol:**

**To make 1 litre:**

Acetone……..500 ml

Ethanol or methanol, absolute* ……..475 ml

Distilled water……25 m

**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. Mix 19.9 ml of sulphuric acid with 0.1ml of barium chloride.

**Preparation of 10 X TE buffer**

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

**Preparation of 1X TE 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

**Preparation of ethidium bromide solution**

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

**Preparation of agarose gel**

Amount of 2 gm of agarose powder dissolved by boiling in 100 ml 1X TBE buffer, then was cooled to 55°C in water bath, then, 5 µl of (10mg\ml) Ethidium bromides 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