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Detection of Some Virulence Genes of *Pseudomonas aeruginosa* Isolated from Different Clinical Specimens by Multiplex PCR, Khartoum, Sudan

الكشف عن بعض جينات الضراوة للزائفة الزنجارية التي تم عزلها من عينات سريرية مختلفة بواسطة تفاعل سلسلة البوليميرايز المتعدد في الخرطوم, السودان

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By:
Mazar Mustafa DafaAllah Abdeen
B.Sc in Medical Laboratory Science (Microbiology)
University of Khartoum (2014)

Supervised by:
Dr. Hisham Nouraldayem Altayeb Mohammed
B.Sc, M.Sc and Ph.D
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بسم الله الرحمن الرحيم

الاية

(ذَٰلِكَ الْفَضْلُ مِنَ اللَّه وَكَفَىٰ بِاللَّهِ عَلِيمًا)

صدق الله العظيم

سورة النساء الاية (70)

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 Salma Abdallah, Islam Jumaa, Duha Abdallah and Alaa Ibrahim

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

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ABSTRACT

Pseudomonas aeruginosa possesses a variety of virulence factors that may contribute to its pathogenicity, it also frequently resistant to many commonly used antibiotics. The aim of this study was to detect *P. aeruginosa oprI, oprL, toxA, exo S, nan1* and *LasB* virulence genes by Multiplex Polymerase Chain Reaction (PCR).

A total of 81isolates of *P. aeruginosa* recovered from urine, wound , blood, otitis and respiratory tract during the period from 15th March to 18th of June were collected and reidentified by conventional and molecular methods. positive isolates for *oprI* and *oprL* genes were further tested for the presence of other virulence genes and antibiotic susceptibility testing.

The antibiotic susceptibility test found that all 70 (100%) isolates were resistant to amoxicillin and amoxyclav, and mostly sensitive to imipenem 64(91.4%), gentamicin 56 (80%), ciprofloxacin 50 (71.4%), and ceftazidime 33(47.1%).

Multiplex PCR was used for detection of virulence genes of *P. aeruginosa.*

Only 70 (86%) isolates were positive for *oprI* and *oprL* genes, from these 70 isolates *toxA* gene detected in 60 (85.7%), *lasB* gene in 64 (91.4%), *exoS* gene in 37(52.9%) and *nan1* gene in 23(32.9%).

There was significant correlation between presence of virulence genes and susceptibility to antibiotics.

ملخص الدراسة

جرثومة الزائفة الزنجارية تمتلك مجموعة متنوعة من عوامل الضراوة التي قد تسهم في إمراضيتها، كما أنها كثيرا ما تكون مقاومة للعديد من المضادات الحيوية الشائعة الاستخدام. كان الهدف من هذه الدراسة الكشف عن جينات الضراوة أوبري,أوبرل, توكسا, اكسوس ونان1 باستخدام تفاعل سلسلة البوليميراز المتعدد.

تم جمع ما مجموعه 81 عزلة من الزائفة الزنجارية من البول، الجرح، الدم، الاذن والجهاز التنفسي خلال الفترة من 15 مارس إلى 18 يونيو واعادة اختبارها بواسطة الطرق التقليدية والجزيئية. عزلت العزلات التي كانت إيجابية للجينات أوبري و أوبرل مزيد من التجارب لوجود جينات الضراوة الأخرى واختبار الحساسية للمضادات الحيوية.

وجد اختبار الحساسية للمضادات الحيوية أن جميع العزلات (70)كانت مقاومة لأموكسيسيلين وأموكسيكلاف، ومعظمها حساسة ل إيميبينيم 64 (91.4٪)، جنتاميسين 56 (80٪)، سيبروفلوكساسين 50 (71.4٪)، و سيفتازيديمي 33 (47.1 ٪)

تم استخدام تفاعل البوليميراز التسلسلي المتعدد للكشف عن جينات الضراوة للزائفة الزنجارية.

كانت 70 عزلة (86٪) فقط إيجابية لجينات أوبري و أوبرل، من 70 عزلة جين توكسا تم اكتشافها في 60 (85.7٪)، جين لاسب في 64 (91.4٪)، جين إكسوس في 37 (52.9٪) و نان 1 جين 23 (32.9٪).. كان هناك ارتباط كبير بين وجود جينات الضراوة والحساسية للمضادات الحيوية.

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1.1 Introduction

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 (Barbara, 1996; 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 (Govan, 1996).

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;Barbara, 1996). It is found in the skin of some healthy persons and has been isolated from the throat (5%) and stool (3%) of nonhospitalized patients (Barbara, 1996).

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; Zeng, 2004). 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 (Barbara, 1996).

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 (Ferguson, 2008; Mesquita *et al.,* 2013), making susceptibility testing essential, combination of gentamicin and carbenicillin is frequently used to treat severe *Pseudomonas* infections (Barbara, 1996).

1.2 Rationale

P. aeruginosa one of the leading causes of nosocomial infections, is 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), particularly in people with compromised immune systems including burn sufferers , cancer and AIDS patients (Barbara, 1996), moreover increasing emergence of multidrug-resistant *P. aeruginosa* (MDRPA) isolates during therapy is critical problem which is reported in 27%– 72% of patients with initially susceptible *P. aeruginosa*, leading to increased health care costs and prolonged hospitalization (Sonbol *et al.*, 2015;Holban *et al.*, 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 . A zinc metalloprotease called Las B has an elastolytic activity on lung tissue . The gene called *nan1* encodes a sialidase that is responsible for adherence to the respiratory tract (Nikbin *et al.*, 2012). This study attempt to determine the frequency of *P.aeruginosa* virulence factors since no such study was published in Sudan.

1.3 Objectives:-

1.3.1 General objectives

To detect virulence genes of *P. aeruginosa* isolated from different sites in Khartoum State using multiplex PCR.

1.3.2 Specific objectives

1-To isolate and identify of *P. aeruginosa* by phenotypic and genotypic methods.

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

3- To detect *P.aeruginosa* virulence factors (*oprI, oprL, toxA, lasB, exoS* and *nan1*) by multiplex PCR.

4-To test the correlation between virulence factors and susceptibility to antibiotics and bacterial isolation site.

2. Literature review

2. *Pseudomonas aeruginosa:-*

2.1 Definition

P. aeruginosa was first obtained in pure culture by Gessard in 1882 from wounds that had produced blue-green discoloration (Forkner, 1960).

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) (Bergey’s manual of Systematic Bacteriology, 2001). 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 (Bergey’s Manual of Systematic Bacteriology, 2001).

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 (Cheesbrough, 2002), 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.(Brook *et al.,* 2004).

2.2 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;Cheesbrough, 2002), 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 (Govan, 1996). 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(Govan, 1996).

2.3 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 exist in multiple immuno-types. Lipopolysaccharide and pyocin (bacteriocin) susceptibility can be used to typed *P.aeruginosa* (Barbara, 1996; 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 (Brook *et al.*, 2004). 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 (Brook *et al*., 2004).

2.4 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 (Ferguson, 2008; Holban *et al.,* 2013). These large array 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).This study focus on detection of *oprI , oprL, toxA, lasB, exoS, nan1* genes that encode for one of the most important virulence factors of *P.aeruginosa* by molecular technique.

2.5 Virulence factors of *P.aeruginosa*:

2.5.1 *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 pseudomonads, and therefore it was used as phylogenetic marker for the classification of rRNA group I pseudomonad (Pirnay *et al.*, 2002; De vos *et al.*,1993), 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.aeruginosa 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 (Lim *et al.*, 1997).

2.5.2 *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).

2.5.3 *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 (Morihara and Tsuzuki, 1977) 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-terminal 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 (Kessler *et al.*, 1998).

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.

2.5.4 *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; Iglewski *et al.*, 1978). 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 (Iglewski *et al.*, 1978).

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 (Yahr *et al.*, 1995).

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.

2.5.5 *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 (Lanotte *et al.*, 2004). 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 (Cacalano *et al.*, 1992)

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.

2.6 Clinical finding of *P.aeruginosa* infection

P.aeruginosa cause a variety of infections in clinical practice primarily in immunocompromised patients (Rossolini and Mantengoli, 2005). 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 (Brook *et al.*, 2004)

2.7 *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 (Ferguson, 2008). 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 (Zeng, 2004; Ferguson, 2008).

2.7.1 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 (Zeng, 2004). 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* (Zeng,

2004). Even the low permeability membrane of *P.aeruginosa* can only prolong the half-equilibration time of most antibiotics for a few minutes (Nikaido, 1989). Therefore, a second mechanism of antibiotic resistance is usually required in addition to a low permeability barrier.

2.7.2 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 (Ferguson, 2008;Zeng 2004). 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 (Ferguson, 2008).

2.7.3 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 (Ferguson, 2008), 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 (Zeng, 2004).

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 (Zeng, 2004). 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. (Zeng, 2004).

2.8 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). Amoxicilin, Amoxiclav, Ceftazidime and Imipenem are the four beta-lactam antibiotics that used in this study. *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 (Rossolini and Mantengoli, 2005). 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 ((https:/amrls.cvm.msu.edu /pharmacolog/antimicrobials/antibiotics-of veterinary-importance). 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 Mantengoli, 2005).

3. Materials and Methods

3.1 Study design

Descriptive cross-sectional study.

3.2 Study area

The study was carried out in Khartoum State (Microbiology Laboratory College, Sudan University of Science and Technology).

3.3 Study Period

The study was conducted during the period from15th March to 18thof June, 2017

3.4 Samples

A total of 81 *P.aeruginosa* isolates obtained from different hospitals (Military hospital, Suba hospital, ENT hospital) representing Khartoum state`s hospitals.

3.5 Ethical consideration

Ethical approval to conduct this study was obtained from the college of Graduate Studies, Sudan University of Science and Technology. Permission was obtained from the hospitals in each of the centers that were included in the study.

3.6 Growth examination

Standard amount of preserved *P.aeruginosa* isolates were inoculated separately into this suitable media;

3.6.1. MacConkey agar:

For isolation of Gram negative bacteria and to differentiate lactose fermenting organisms from non-lactose fermenting organisms (Cheesbrough, 2006).

3.6.2 Muller Hinton agar

This media was used for antimicrobial susceptibility testing
(Cheesbrough, 2006).

3.6.3 Nutrient agar

This media was used for purification and short preservation of organisms.
(Collee *et al.*, 1996).

3.7 Colony morphology

MacConkey agar: Produce smooth pink colony (Collee *et al.*, 1996).

3.8 Gram Stain

The procedure was carried out according to Cheesbrough, (2006) as follows; smear was prepared from overnight culture on a clean and dry slide. The smear was left to air dry. Fixation was done by rapid pass of the slide three times through the flame of a Bunzen burner then allowed to cool before staining. Crystal violet stain was added to smear for 30–60 seconds, and then washed by tap water. Lugol‟s iodine (mordant) was added for 30-60 minutes then washed by tap water and decolorized rapidly (few seconds) with acetone alcohol and washed immediately by tap water. Finally, the smear was covered with Saffranin stain for 2 minutes and washed by tap water. The back of slide was wiped clean and placed in a draining rack for smear to air dry. Drop of oil was added to the dried smear and examined under the light microscope by oil lens 100X.

3.9 Biochemical testing

The following tests have been done:

3.9.1 Oxidase test

according toCheesbrough, (2006) a piece of filter paper was placed in a clean petri-dish and 3 drops of freshly prepared oxidase reagent was added. A colony of the tested organism was removed using wooden stick and smeared on the filter paper. *P.aeruginosa* give purple colour.

3.9.2 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 shaked gently and examined for red color ring within 10 mints. *P.aeruginosa* is negative indole (no red ring appear) according to Cheesbrough, (2006).

3.9.3 Citrate utilization test

The test organism was inoculated into 2 ml of Simmon citrate medium with bromo-thymol blue as indicator using sterile straight wire. Then the medium was incubated at 37°Cfor 24 hrs, change in colour of the indicator from light green to blue colour mean a positive result. *P.aeruginosa* is citrate positive according to Cheesbrough, (2006).

3.9.4 Urease production test

The test organism was inoculated into slope surface of Christensen's urea medium with phenol red as indicator using sterile straight wire, the medium was incubated at 37 °Cfor 24 hrs, change in colour of the indicator to red-pink mean a positive result. *P.aeruginosa* is differential according to Cheesbrough, (2006).

3.9.5 Kligler Iron Agar (KIA)

The tested organism was inoculated in KIA medium, using a straight wire loop, agar butt was stabed, the opening was closed and then the top slope was streaked (as zigzag). The medium was incubated at 37°Cfor 24 hrs, glucose fermentation, lactose fermentation, H_2Sproduction, gas production was looked for. *P.aeruginosa* give red butt and slope , produce no gas or H_2S according to Cheesbrough, (2006).

3.10 Antimicrobial susceptibility testing

Susceptibility pattern was done by disk diffusion method. All the isolated organisms were put into appropriate media for antibiotic susceptibility test. Disc diffusion tests were performed and interpreted according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2007). All tests were performed on Muller-Hinton agar plates (pH 7.2-7.4). The surface was lightly and uniformly inoculated by sterile cotton swab stick. Prior to inoculation, the swab stick was dipped into bacterial suspension having visually equivalent turbidity to 0.5 McFarland standards. The swab stick was then took out and squeezed on the wall of the test tube to discard extra suspension, after inoculation the following discs were placed; amoxicillin (25mcg), amoxyclav (30mcg), ciprofloxacin (5mcg), gentamicin (10mcg), ceftazidime (30mcg), imipenem (10mcg). Inoculated plates were incubated at 37 degree for 24 hours. On the next day, Inhibition zones were measured in millimeter (mm) by using a ruler over the surface of the plate with the lid open. They were held a few inches above a black, nonreflecting background and illuminated with reflected light. Results were recorded and graded as resistant (R) and sensitive (S), according to the reference zone of inhibition of particular antibiotic (NCCLS, 2001).

3.11 Molecular characterizations

3.11.1. DNA Extraction for Polymerase Chain Reaction

Genomic DNA (templates) for PCR amplification were extracted from overnight growth of bacterial isolates on nutrient agar (several colony) suspended in 1000 µL of sterile deionized water, and boiled for 15 minutes. After centrifugation of the boiled samples at 14000 g for 10 minutes, supernatant was stored at -20°C as a template DNA stock (Yamamoto *et al*., 1995).

3.11.2. Gel electrophoresis of extracted DNA

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

3.11.3. Preparation of 10 X Tris Borate EDETA (TBE) buffer

Amount of 108 gm. Tris base were weighed and added to 55gm of boric acid and 40 ml of 0.5M EDTA then dissolved into 1 liter deionized water pH 8.0.

3.11.4. Preparation of 1X TBE buffer

Ten ml of 10 X TBE buffer was added to 90 ml deionized water and heated until completely dissolved.

3.11.5. Preparation of ethidium bromide solution

Ten milligrams of ethidium bromide powder were dissolved into 500 µl deionized water, and kept into brown bottle.

3.11.6 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).

3.11.7. Polymerase Chain Reaction Amplification

The primers were synthesized by (Macrogen, Korea).Specific primers were used to amplify the *oprI, oprL, toxA, lasB, exoS* and *nan1* genes as indicated in table (3).

PCR was done by multiplex PCR, amplification was done using TECHNE® Ltd peltier thermal cycler (Germany), DNA amplifies was done using Maxime PCR Premix kit (iNtRON, Korea),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, 200 µM of each dNTP, and 1 U Taq DNA polymerase, 0.5 µL of each of the virulence gene-specific primers (5 µL), 2 µL of template DNA and 16 µL of WFI (water for injection). The amplification conditions included three steps: heating at 95°C for 5 min; 33 cycles of denaturation at 95°C for 30 sec , annealing at 58°C for 30 sec for (*oprI.oprL, toxA* and *nan1* genes) and for (*lasB* and *exoS* genes) annealing at 60°C for 30 sec, and extension at 72°C for 30sec; 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**	**Amplified region (bp)**
oprI	PS1, 5'-ATG AAC AAC GTT CTG AAA TTC	**250**

	TCT GCT-3’ PS2, 5’-CTT GCG GCT GGC TTT TTC CAG-3’	
oprL	PAL1, 5’-ATG GAA ATG CTG AAA TTC GGC-3’ PAL2, 5’-CTT CTT CAG CTC GAC GCG ACG-3’	**500**
lasB	lasB lasf 5’ GGA ATG AAC GAA GCG TTC TC 3’ las r 5’ GGT CCA GTA GTA GCG GTT GG 3’	**300**
toxA	toxA toxf 5’ GGT AAC CAG CTC AGC CAC AT 3’ tox r 5’ TGA TGT CCA GGT CAT GCT TC 3’	**352**
exoS	exoS exo f 5’ CTT GAA GGG ACT CGA CAA GG 3’ exo r 5’ TTC AGG TCC GCG TAG TGA AT 3’	**504**
Nan1	nan1 nan f 5’ AGG ATG AAT ACT TAT TTT GAT 3’ nan r 5’TCA CTA AAT CCA TCT CTG ACC CGA TA	**1316**

3.11.8. Visualization of the DNA products

The gel casting tray was put into the electrophoresis, tank flooded with 1x TBE buffer just to cover the gel surface, 10 µl of PCR products from each

40samples 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 (100 V, 500 mA, UK). The electrophoresis was carried out at 75Volts for 30 minutes 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).

Reading of DNA product:

The expected band size: 250bp, 300bp, 352bp, 500bp, 504bp, 1316bp.

The negative result: there is no bands appear against the ladder.

3.12 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 0.5 was consider significant for the association between variables (IBM, 2012).

3.13 Safe disposal

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

4.Results

A total of 81 *P.aeruginosa* isolates were collected during the period 15th May to 18th of June 2017. The *oprI* and *oprL* genes were detected in only 70 isolates (86.4%). They were recovered from urine 27 (38%), wound 21 (30%), ear swab 9 (13%), sputum 7(10%) and blood 6(9%).

The overall results revealed that out of 70 *P.aeruginosa* which were positive for *oprI* and *oprL* genes confirmed by molecular detection, the *toxA* gene was detected in 60(85.7%), *lasB* gene in 64(91.4%), *exoS* gene in 37(52.9%) and *nan1* gene in 23(32.9%) figure (4.1), (4.2), (4.3), (4.4) respectively. 3 isolates from urine lack the four genes.

4.1 Association between the presence of virulence genes and samples

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

4.1.1 *oprI* and *oprL* genes

The two genes was detected in 70 isolates out of the 81 isolates.

4.1.2 *ToxA* gene

The gene was harbored in all blood samples, in wound samples was significantly higher than urine, ear swab and pulmonary samples as indicated in Table (4.1).

4.1.3 *LasB* gene

All the samples from ear and pulmonary were harbored the gene 100%, in blood it was found in 66% (4) of samples as indicated in Table (4.1).

4.1.4 *ExoS* gene

The gene was significantly higher in ear swab samples (88%) than other sites, in urine and pulmonary samples the percent nearly the same, in wound was found in 61% (13), and 3% (2) in the blood as indicated in Table (4.1)

4.1.5 *Nan1* gene

Comparing to previous genes this gene was not found in high percentage, the higher percent was found in blood 66% (4) following by wound samples 47% (10) as indicated in Table (4.1)

Table (4.1) the presence of *P.aeruginosa* virulence genes in different samples

	Sample sites					P value (chi-square)
Virulence genes	Urine n=27(%)	Wound n=21(%)	Ear swab n=9(%)	Pulmonary tractn=7(%)	Blood n=6(%)	
toxA^{+}	21(77%)	20(95%)	7 (77%)	6 (85%)	6(100%)	.000
lasB^{+}	24(88%)	20(95%)	9(100%)	7(100%)	4(66%)	.000
exoS^{+}	11(40%)	13(61%)	8(88%)	3(42%)	2(33%)	.000
Nan1^{+}	5(18%)	10(47%)	2(22%)	2(28%)	4(66%)	.000

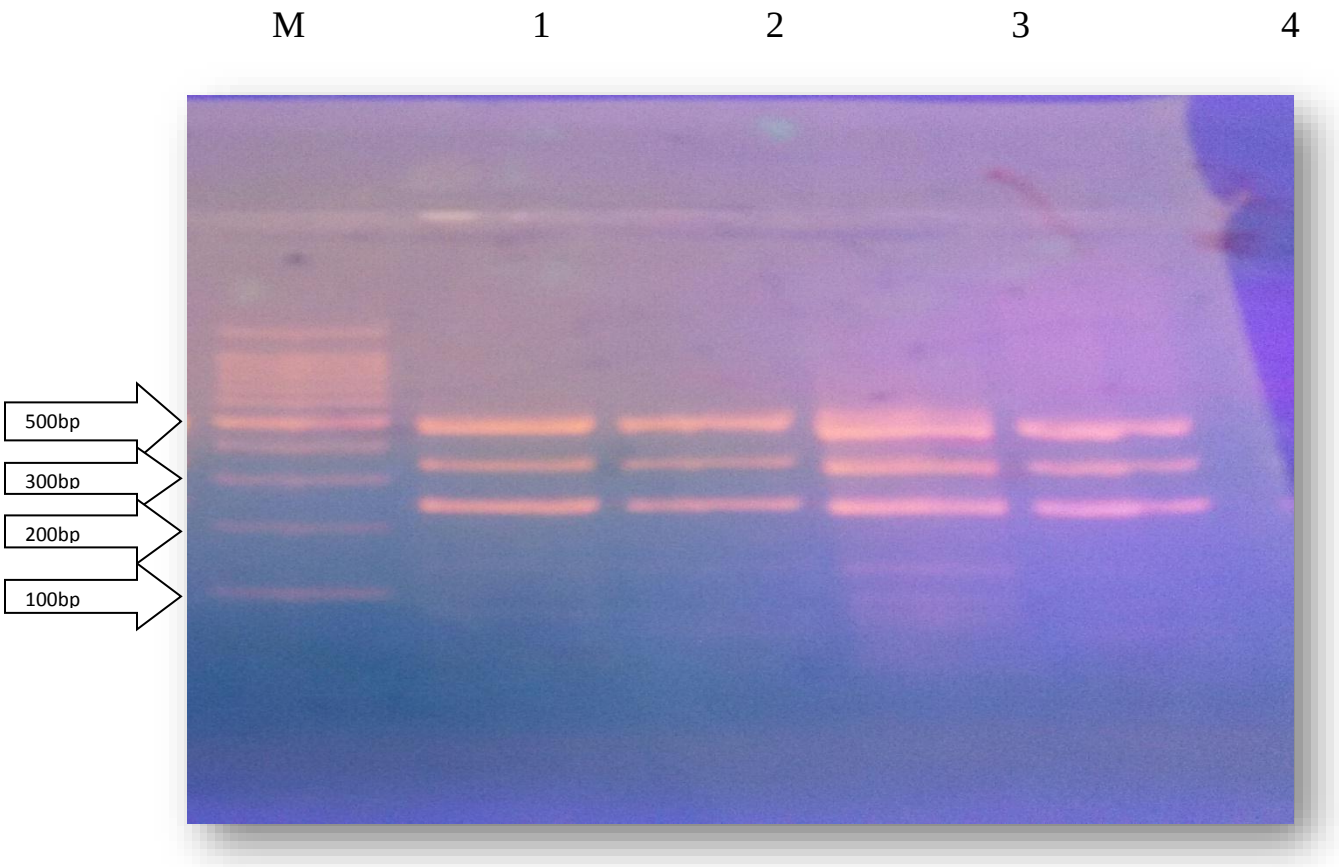

Figure (4.1) Amplification of *oprI* (250bp), *oprL* (500bp) and *toxA* (352bP) from clinical isolates of *P.aeruginosa :* M 100bp ladder, lane 1,2,3,4, samples positive for all the three genes.

1 2 M 3 4 5

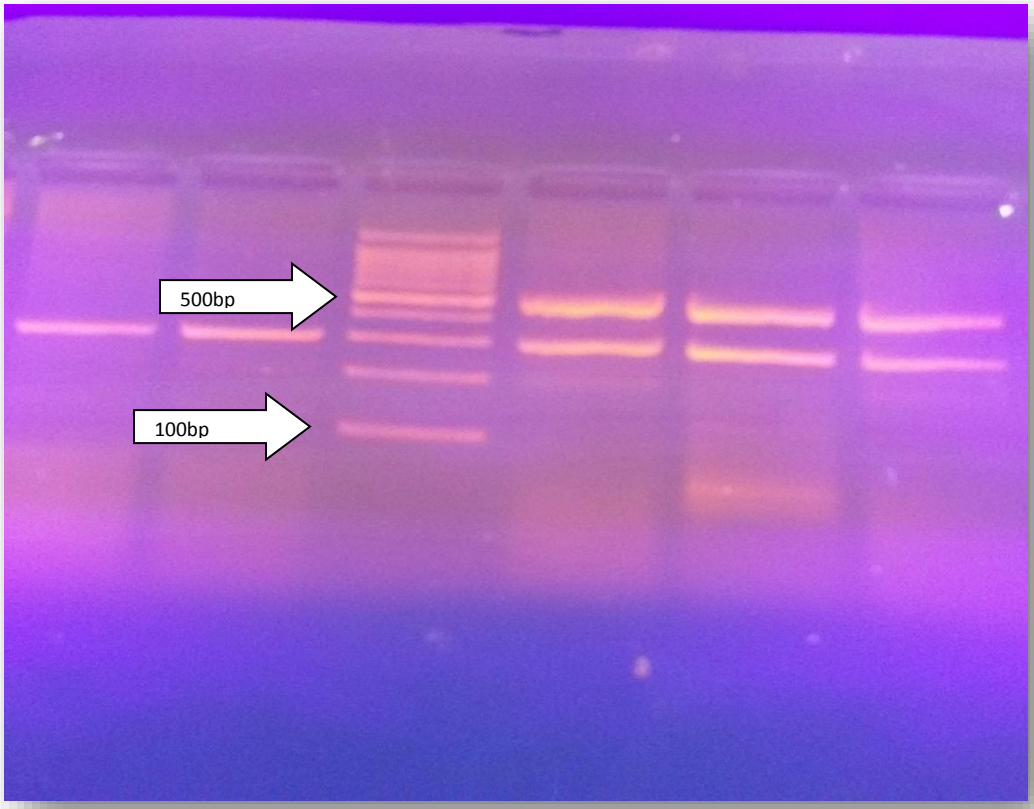

Figure (4.2) Amplification of *lasB* (300bp) and *exoS* (504bp) genes from clinical isolates of *P.aeruginosa*: M 100bp ladder, lane 1,2 samples positive for only *lasB* gene, lane 3,4,5, samples positive for both *lasB* and *exoS* genes.

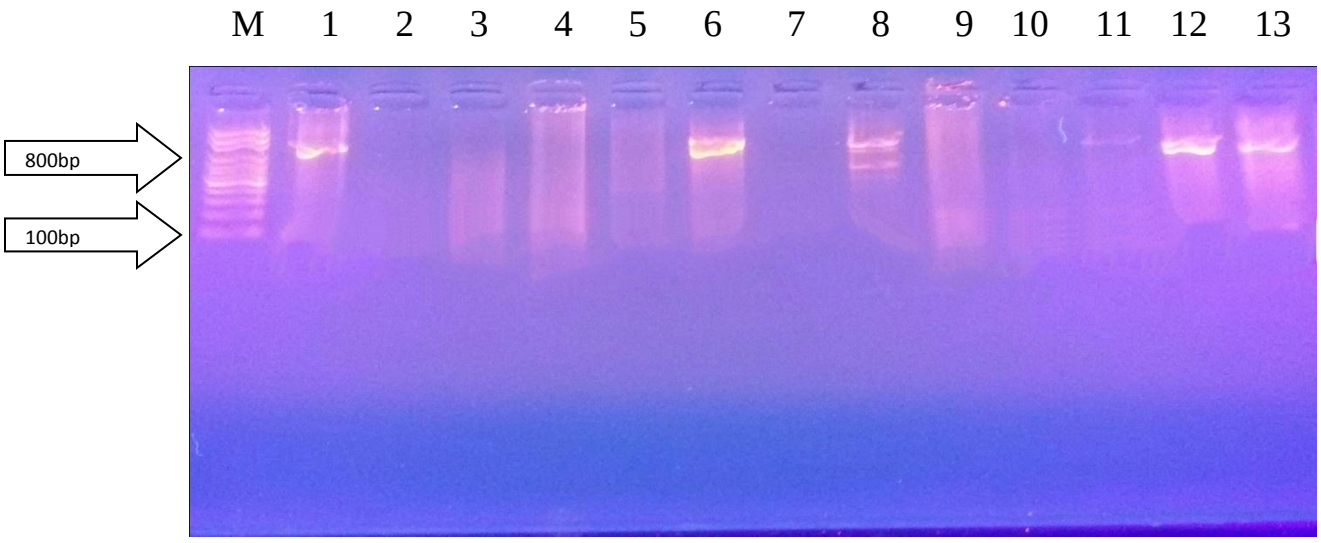

Figure (4.3) Amplification of *nan1* (1316 bp) gene from clinical isolates of *P.aeruginosa*: M 100bp ladder, lane 1, 6, 8, 11, 12 and 13 samples positive for *nan1*gene, lane 2, 3, 4, 5, 7, 9 and 10 samples negative for *nan1*gene.

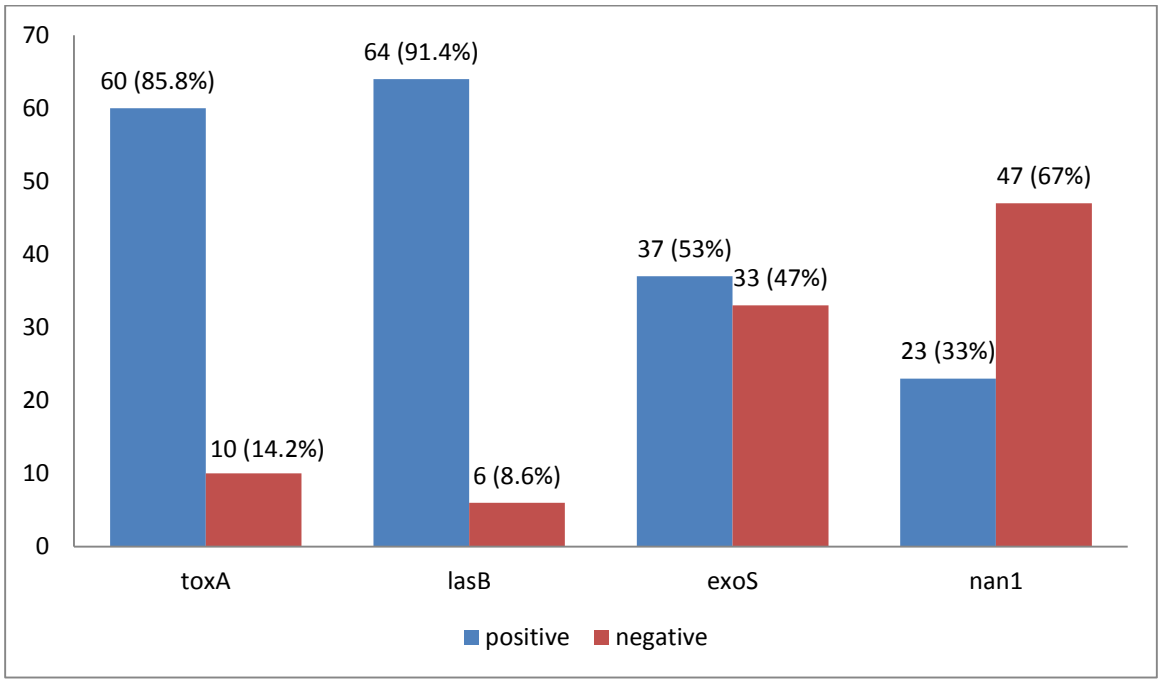

Figure (4.4) The occurrence of *P. aeruginosa* virulence genes.

4.2. The association between the presence of virulence genes and antibiotics susceptibility.

All 70 isolates were cultured in Mueller-hinton medium using 0.5 Mcfarland turbidity standard for antibiotic susceptility testing against amoxycilin (25mcg), amoxyclav (30mcg), ciprofloxacin (5mcg), gentamicin (10mcg), imipenem (10mcg) and ceftazidime (30mcg)) discs.

4.2.1 Amoxycillin

The overall results revealed that total of 70 samples were resistant for Amoxycillin and there was significant strong association between the presence of (*oprI*, *oprL*, *toxA*, *lasB*, *exoS*, *nan1*) genes and resistance to Amoxycillin (p-value 0.000) table (4.2) figure (4.5).

Table (4.2) the association between presence of virulence genes and Amoxycillin

genes	*oprI*		*oprL*		*toxA*		*lasB*		*exoS*		*Nan1*	
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Sensitive (n=0)	0	0	0	0	0	0	0	0	0	0	0	0
Resistant (n=70)	70	0	70	0	60	10	64	6	37	33	23	47
Total	70	0	70	0	60	10	64	6	37	33	23	47
p-value	0.000		0.000		0.000		0.000		0.000		0.000	

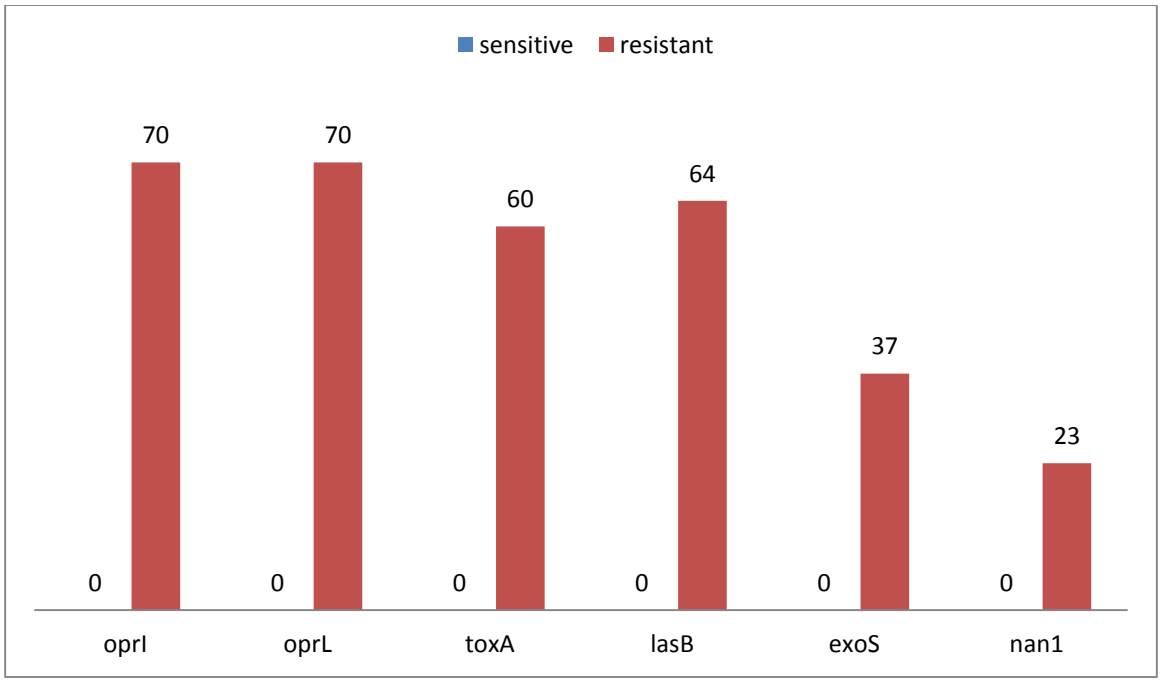

Figure (4.5) Result of the occurrence of virulence genes and Amoxycillin

4.2.2 Amoxyclav

The overall results revealed that total of 70 samples were resistant for Amoxyclav and there was significant strong association between the presence of (*oprI*, *oprL*, *toxA*, *lasB*, *exoS*, *nan1*) genes and resistance to Amoxyclav (p-value 0.000) table (4.3) figure (4.6).

Table (4.3) the association between presence of virulence genes and Amoxyclav

genes	*oprI*		*oprL*		*toxA*		*lasB*		*exoS*		*Nan1*	
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Sensitive (n=0)	0	0	0	0	0	0	0	0	0	0	0	0
Resistant (n=70)	70	0	70	0	60	10	64	6	37	33	23	47
Total	70	0	70	0	60	10	64	6	37	33	23	47
p-value	0.000		0.000		0.000		0.000		0.000		0.000	

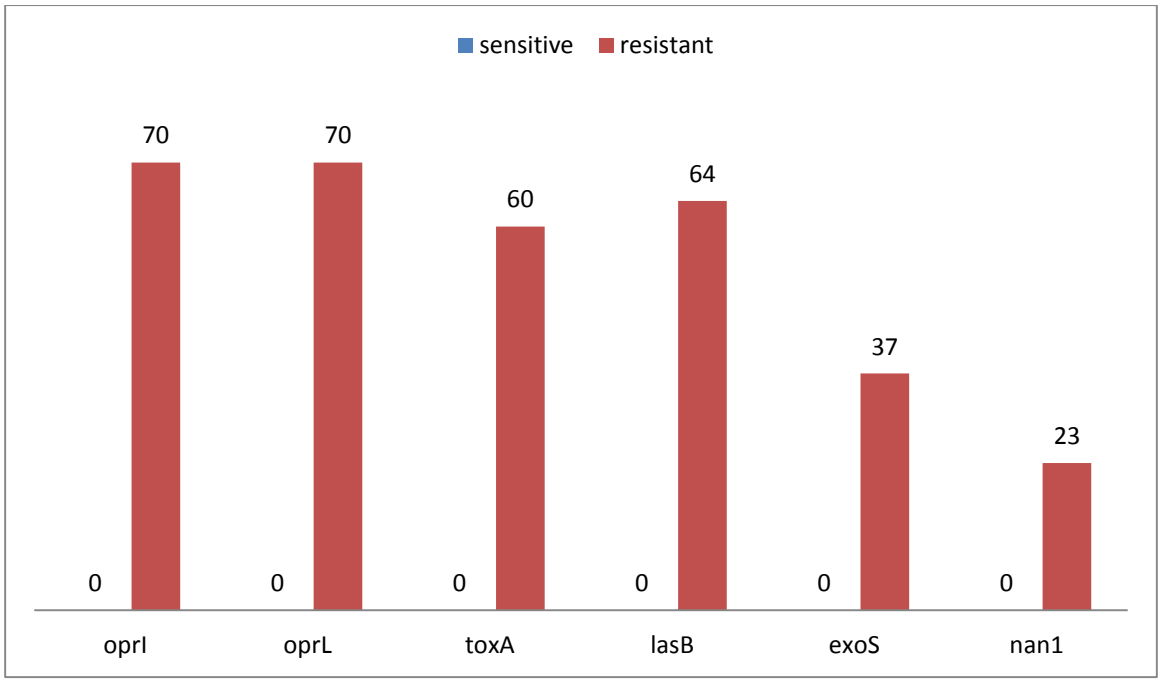

Figure (4.6) Result of the occurrence of virulence genes and Amoxyclav

4.2.3Ciprofloxacin

The overall results revealed that there was significant strong association between the presence of (*oprI, oprL, toxA, lasB, nan1*) genes and sensitive to Ciprofloxacin (p-value 0.000) total of 50 samples were sensitive for Ciprofloxacin, as indicated in Table (4.4), Figure (4.7).

Table (4.4) the association between presence of virulence genes and Ciprofloxacin

genes	*oprI*		*oprL*		*toxA*		*lasB*		*exoS*		*Nan1*	
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
sensitive	50	0	50	0	43	8	47	4	31	19	21	29
resistant	20	0	20	0	17	2	17	2	6	14	2	18
Total	70	0	70	0	60	10	64	6	37	33	23	47
p-value	0.000		0.000		0.000		0.000		0.000		0.000	

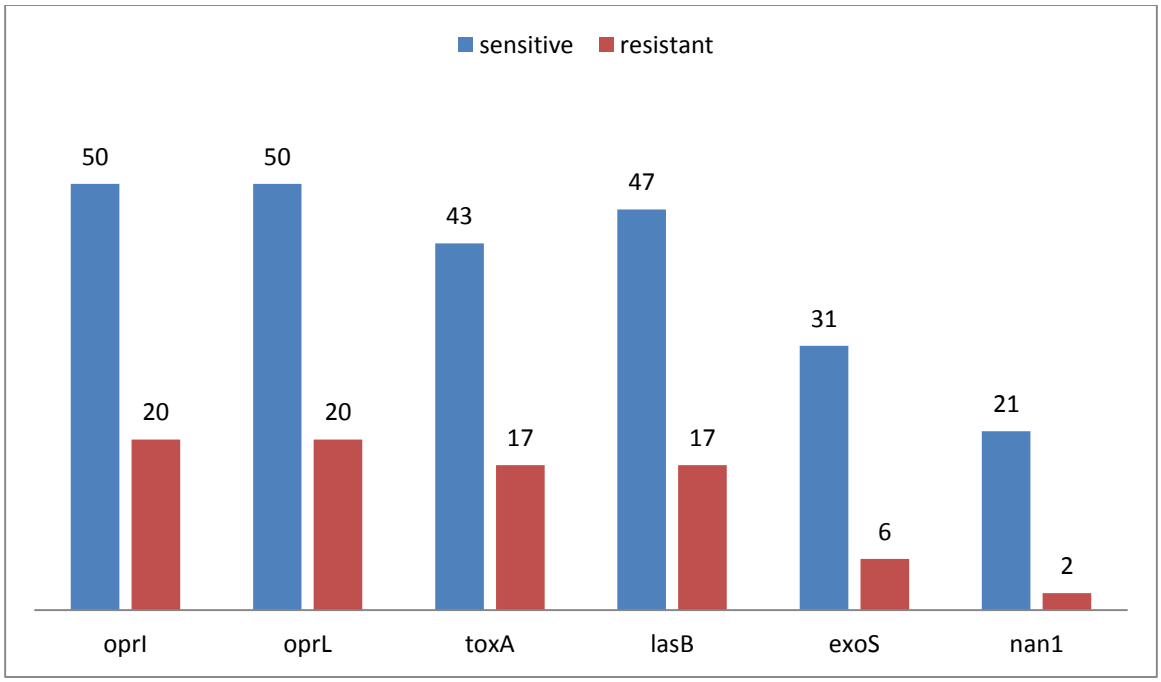

Figure (4.7) Result of the occurrence of virulence genes and Ciprofloxacin

4.2.4Gentamicin

There was significant strong association between the presence of (*oprI*, *oprL*, *toxA*, *lasB*,*exoS*, *nan1*) genes and sensitive to gentamicin (p-value 0.000), as indicated in Table (4.5), Figure (4.8).

Table (4.5) the association between presence virulence genes and Gentamicin

genes	*oprI*		*oprL*		*toxA*		*lasB*		*exoS*		*Nan1*	
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
sensitive	56	0	56	0	48	8	51	5	32	24	23	33
resistant	14	0	14	0	12	2	13	1	5	9	0	14
Total	70	0	70	0	60	10	64	6	37	33	23	47
p-value	0.000		0.000		0.000		0.000		0.000		0.000	

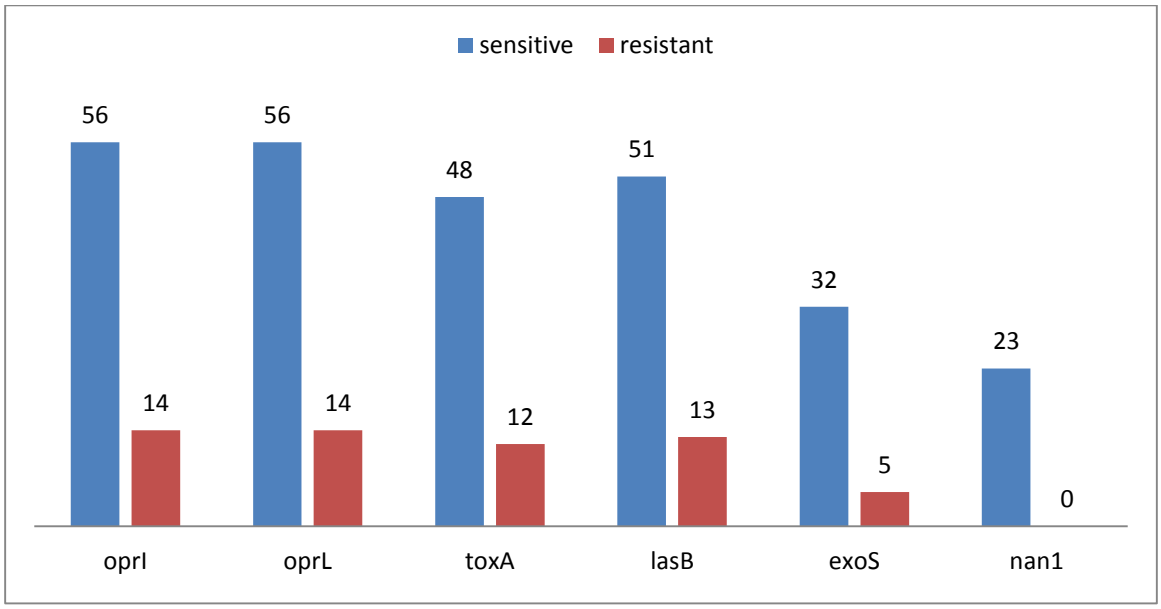

Figure (4.8) Result of the occurrence of virulence genes and Gentamicin

4.2.5 Imipenem

There was significant strong association between the presence of (*oprI, oprL, toxA, lasB,exoS, nan1*) genes and sensitive to imipenem (p-value 0.000), as indicated in Table (4.6), Figure (4.9).

Table (4.6) the association between presence virulence genes and Imipenem

genes	*oprI*		*oprL*		*toxA*		*lasB*		*exoS*		*Nan1*	
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
sensitive	64	0	64	0	56	9	60	5	36	28	23	41
resistant	6	0	6	0	4	1	4	1	1	5	0	6
Total	70	0	70	0	60	10	64	6	37	33	23	47
p-value	0.000		0.000		0.000		0.000		0.000		0.000	

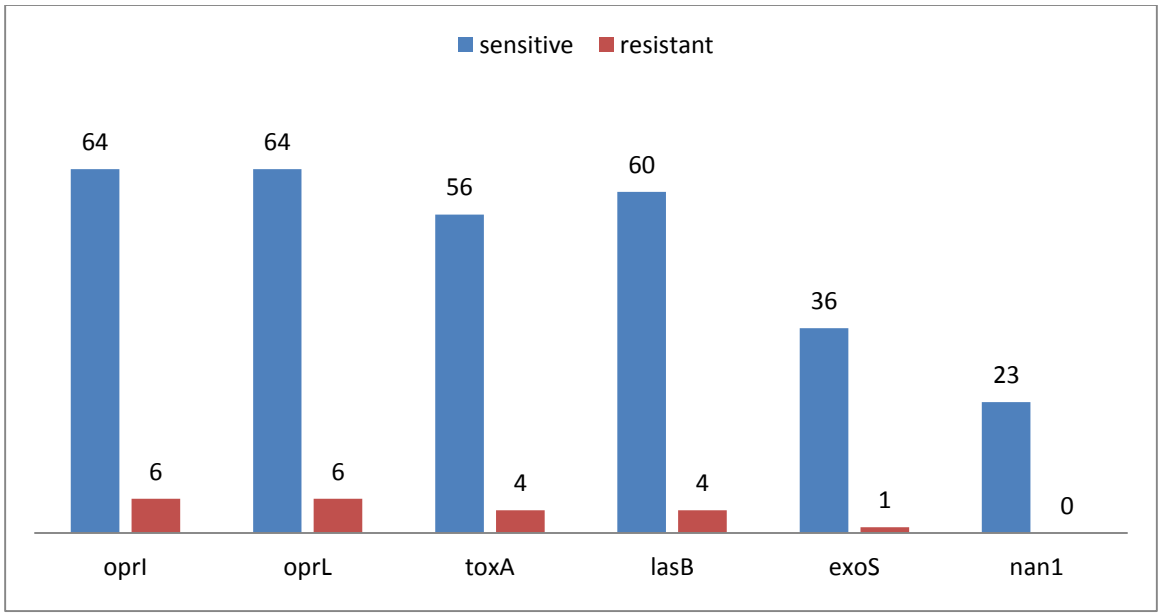

Figure (4.9) Result of the occurrence of virulence genes and Imipenem

4.2.6 Ceftazidime

The overall results revealed that total of 30 sample sensitive for Ceftazidime there was significant strong association between the presence of (*toxA*, *lasB*) genes to Ceftazidime antibiotic (p-value 0.000), also there was significant strong association between the presence of (*oprI*, *oprL*, *exoS*, *nan1*) gene and resistance to Ceftazidime (p-value 0.000), as indicated in Table (4.7), Figure (4.10).

Table (4.7) the association between presence virulence genes and Ceftazidime

Genes	*oprI*		*oprL*		*toxA*		*lasB*		*exoS*		*Nan1*	
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
sensitive	30	0	30	0	32	6	33	3	18	11	11	20
resistant	40	0	40	0	28	4	31	3	19	22	12	27
Total	70	0	70	0	60	10	64	6	37	33	23	47
p-value	0.000		0.000		0.000		0.000		0.000		0.000	

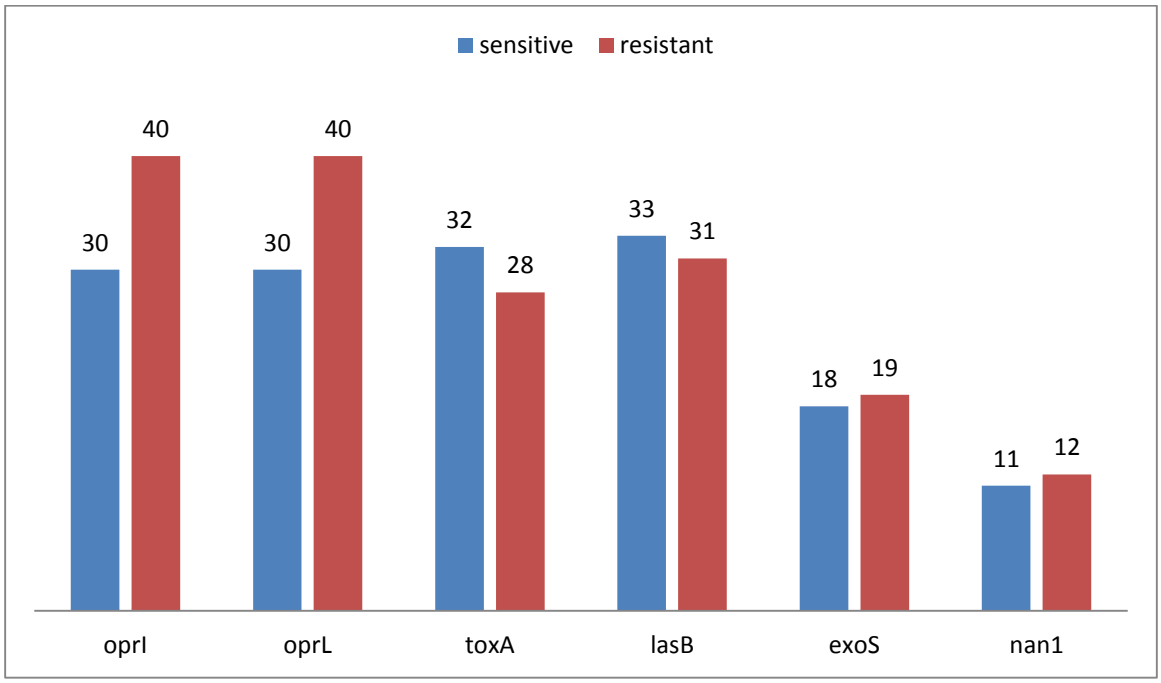

Figure (4.10) Result of the occurrence of virulence genes and Ceftazidime

P.aeruginosa isolates were mostly sensitive to imipenem 64(91.4%), gentamycin 56(80%), ciprofloxacin 50(71.4%), and ceftazidime 33(47.1%) figure (4.11).

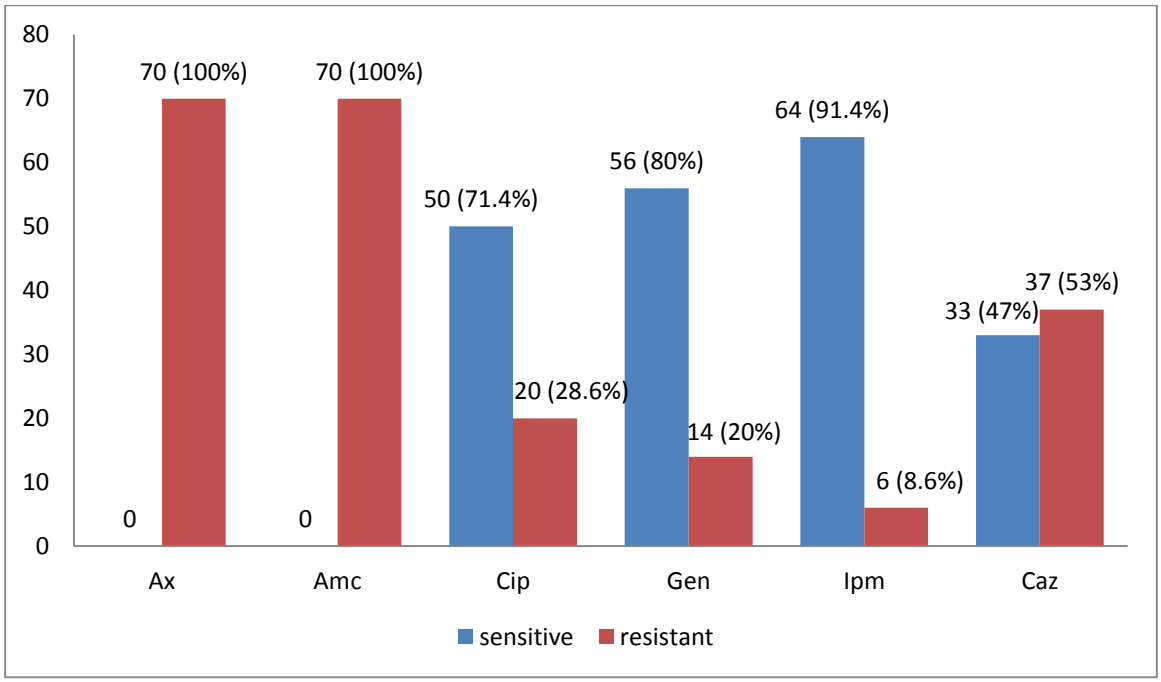

Figure (4.11) Pattern of antibiotics susceptibility against *P.aeruginosa*.

5. DISCUSSION

Identification of *P. aeruginosa* has traditionally relied on phenotypic methods. This is still the most accurate standard when dealing with typical isolates of *P. aeruginosa*, but in cystic fibrosis (CF) patients, *P. aeruginosa* isolates display unusual phenotypic reactions (Qin *et al.*, 2003). Moreover, biochemical testing takes long time to perform and requires extensive hands on work by the technologist. Molecular methods have been reported to be superior to the phenotypic methods for identification of *P. aeruginosa* (Qin *et al.*, 2003). De Vos *et al*. (1997) by designing a multiplex PCR assay based on *oprI* and *oprL* genes for molecular detection of *P.aeruginosa* the two genes were detected in all *P. aeruginosa* Lavenir *et al*. (2007) noted that all of tested *P. aeruginosa* strains contained the *oprI* and *oprL* genes, Nikbin *et al*. (2012) also noted that all of 268 *P. aeruginosa* isolates were remarkably positive for both *oprI* and *oprL* genes, the same result was reported by Khattab *et al*. (2015). According to these studies, detection of *P.aeruginosa* by PCR of *oprI* and *oprL* genes could be a reliable factor for rapid identification of *P. aeruginosa* in clinical samples, as these proteins are found only in this organism, (Nibkin *et al.*, 2012), differently in this study 70 out of 81 isolates were positive for both *oprI* and *oprL* genes, this result spotlighting the importance of molecular methods to identify organisms, to overcome the issue of wrong phenotypic identification.

ToxA gene was detected in 85.7% of *P. aeruginosa* isolates, the *ptxR* gene expression enhancer of *toxA* gene, was only detected in *P. aeruginosa* isolates, low sensitivity with *toxA* PCR screening is due to the fact that some isolates of *P. aeruginosa* do not carry this gene naturally (Khattab *et al.*, 2015). However, presence of *toxA* gene in clinical samples was different; according to table (4.1) the presence of *toxA* gene in isolates from blood and

wound was significantly higher than other sites (*P*-value 0.000), the same result obtained by Holban *et al.* (2013).

Pathogenicity of *P. aeruginosa* is multifactorial, *lasB* is one of the most important proteases of *P. aeruginosa* (Lomholt *et al.*, 2001). In this study from 70 isolates examined 64(91.4%) harbored *lasB* gene. This finding was nearly the same with previous reports Nikbin *et al*. (2012), and Khattab *et al*. (2015), in which all isolates were harbored lasB gene. , *lasB* gene also had been harbored in all of the environmental and clinical isolates (Cowell *et al.*, 2003), implies the importance of *LasB* gene for survival in various setting .

In this study difference between *exoS* gene prevalence in the isolates from wound and otitis infections was significantly higher than other sites (*P*-value 0.000) (Table 4.1). Although the prevalence of isolates from pulmonary tract infections that exhibited *exoS* gene (42%) in this study was lower than that previously reported studies Nikbin *et al*. (2012) and Khattab *et al*. (2015) but they similar with this study in that isolates from wound was significant. Since *P. aeruginosa* isolates ability to disseminate and breach epithelial barriers is linked to expression of the exoenzyme S (encoded by exoS gene) (Yahr *et al.*, 1995), this may explain why isolates from ear swab and wound had the higher prevalence.

About the *nan1* gene, the other virulence factor studied in this study it was found that the prevalence of *nan1* was significantly higher in isolates from blood and wound than isolates from other sites (*P*-value 0.000). The low prevalence of this factor among isolates from urine, ear infection, pulmonary infections may show that the role of this gene in these infections is less important than blood and wound infections. The differences in the distributions of virulence factor genes in the populations strengthen the probability that some *P. aeruginosa* strains are better adapted to the specific

conditions found in specific infectious sites (Lanotte *et al.,* 2004). The conflicting results of these studies may be due to the isolates from patients with different clinical and physiological conditions.

The second part of this research was to find out the role of the virulence genes in *P.aeruginosa* susceptibility to antibiotics. Tables4 (2, 3, 4, 5, 6,7) reveal the significant relationship between virulence genes of *P.aeruginosa* and susceptibility to antibiotics, all isolates was remarkably resistant to both amoxycillin and amoxyclav, also considerable percent of isolates were resistant to ceftazidime. About 14 isolates were resistant to three of the six antibiotic used, this finding agreement with Sonbol *et al.* (2015) but Joodzadeh *et al.* (2016) found that there was no relationship between presence of *exoS* gene and antibiotic resistant. The different geographic locations and the inappropriate use of antibiotics in our community may explain this drug resistant.

6.1 CONCLUSION

It seems that simultaneous use of *oprI* and *oprL* genes provides more confident detection of *P. aeruginosa* by PCR.

Determination of different virulence genes of *P. aeruginosa* isolates suggests that they are associated with different levels of intrinsic virulence and pathogenicity, this may have different consequence on the outcome of infections.

Significant correlations between studied virulence genes and source of infections obtained in this research indicate the potential risk of these isolates in nosocomial infections.

Significant correlations to antibiotics susceptibility bring more attention to the emergence of multi-drug resistant organism which is continuing to increase making the need for new agents more urgent.

6.2 RECOMMENDATION

1-Further studies are needed to identify other virulence factors of *P. aeruginosa* to determine the actual role of these genes in different clinical infectious caused by *P. aeruginosa*.

2- Large sample size is recommended to identify the prevalence of virulence genes.

3- Determination of antimicrobial resistant genes in association with virulence genes is a great importance.

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

5- Implementation of measures to control the use of antibiotics.

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Appendices

Appendix NO.1

Antibiotics susceptibility testing chart

Disk name	symbol	Concentration (mcg)	Resistant (mm or less)	Intermediate (mm)	Sensitive (mm or more)
Gentamicin	Gen	10	12	13-14	15
Ciprofloxacin	Cip	5	15	16-20	21
Amoxyclav	Amc	30	13	14-17	18
Ceftazidime	Caz	30	14	15-17	18
Amoxycillin	Ax	25	14	15-16	17
Imipenem	Ipm	10	15	16-18	19

Appendix No.2

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.

-goose.

-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:**

-MacConkey 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 No.3

Preparation of media

MacConkey agar M082-500G:

pH (at 25°C) =7.4+/-0.2

Formula:

Ingredients	**Gms/litter**
Peptone	20.00
Lactose	10.00
Sodium taurocholate	5.00
Neutral red	0.04
Agar	20.00

Directions:

Suspend 55.04 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:

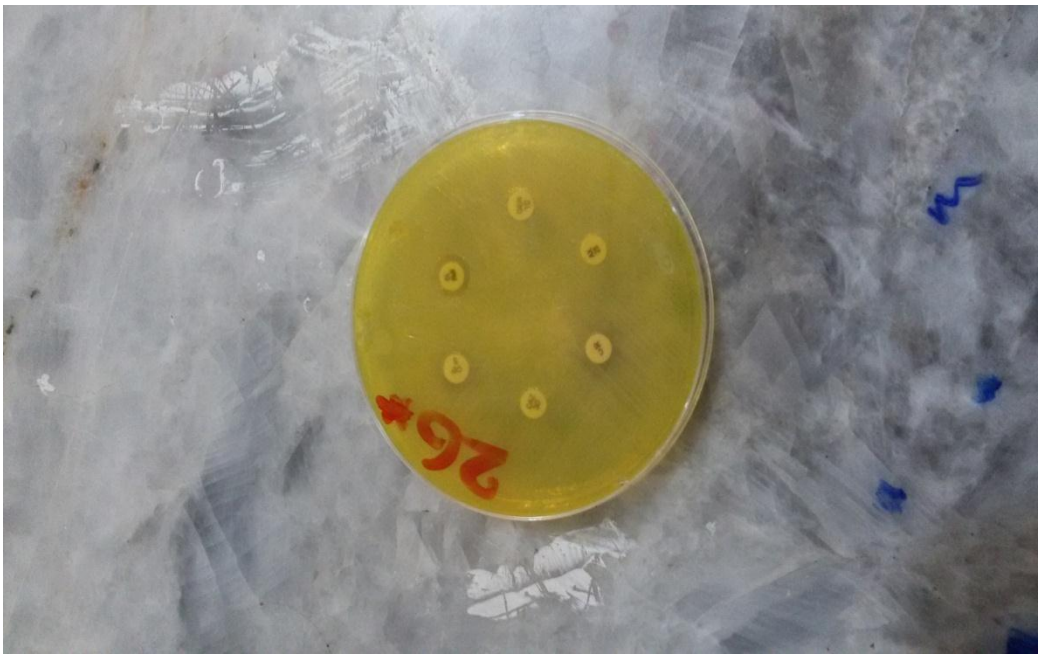
Ingredients	Gms/litter
Magnesium sulphate	0.02

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:

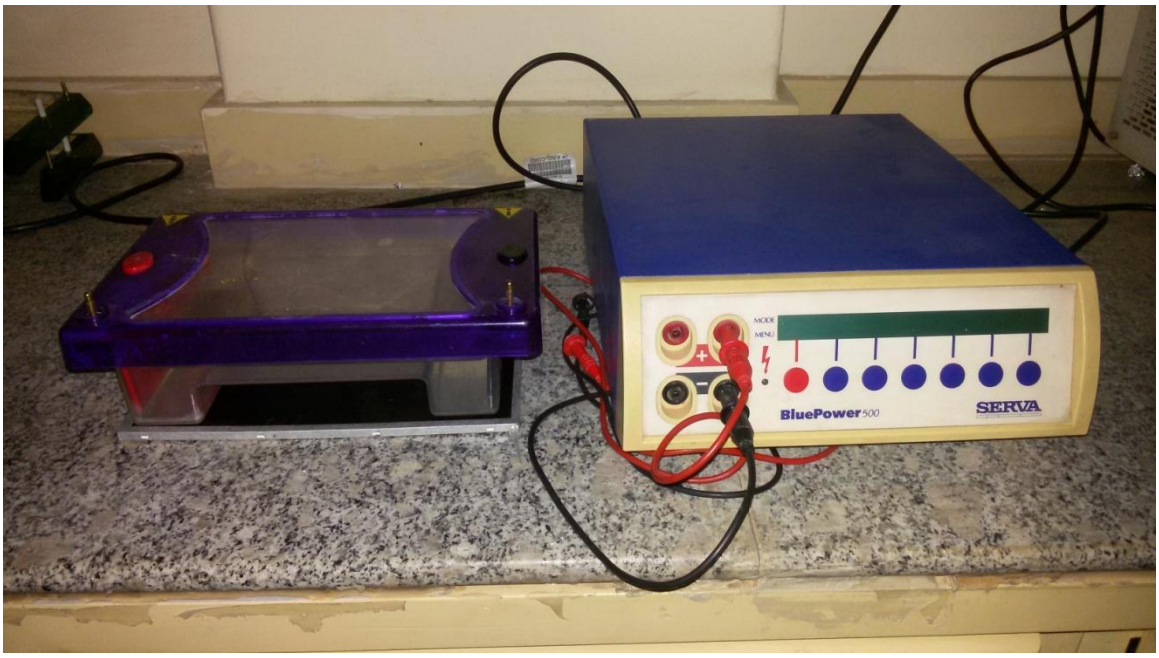
Suspend 24.28 grams in 1000ml distilled water. Heat to boiling to dissolve the medium completely sterilize by autoclaving at 15Ibs pressure (121°C) for 15 minutes .cool to 45°C- 50°C ,mix well pour into sterile tubes.

Appendix No.4: media and instruments picture



Sensitivity of *P.aeruginosa* in Muller Hinton agar, the bellow show pigment

Thermal cycler



Electrophoresis apparatus

UV transilluminater

Sensitive balance