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Ministry of Higher Education
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November, 2016 A.D. **Suffer, 1438 A.H**

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ACKNOWLEDGMENTS

First of all I would like to thank Allah (the only God Quran) for helping me and giving me strength, willingness and patience to finish what I have started.

I would like to express my sincere thanks gratefulness and appreciation to my supervisors, Prof. **Dr. Adnan H. Al-Hamadani** and Assist. Prof **Dr. Hassan R. Jallab** for their supervisions, suggestions on the topics of this thesis, their invaluable advice and comments during the course of investigation and writing of this thesis, their endless patience, generousness and kindness .Their efforts and care are highly appreciated and will never be forgotten .

It is only my duty to thank and appreciate the Deanery of College of Medicine, University of Al-Qadissiyah and Department of Microbiology for providing me with the work opportunity to accomplish this rersaerch in their laboratories .

My deep thanks for Assist. Prof **Dr. Aida** and Assist. Prof **Dr. Amin A. Sabbar**, College of Medicine/ University of Baghdad for Help me in the clinical diagnosis and sampling of patients collection.

My deep thanks for **Dr.Thanaa R. Abdulrahman**, College of Medicine University of Al- Nahrain for her valuable, support, guidance.

I would like to express my special thanks to the Dean assistant of College of Medicine /Al-Qadisiya University Assist Prof. **Dr. Hummadi A. Al-Hilaly** and Assist Prof. **Dr. Ibtisam Habeeb** for them valuable, support, and guidance.

My great thanks and deepest gratitude for **Dr. Kifah A. Jassim** consultant in Medical Microbiology and **Dr. Abbas M. Ahmed** consultant in Medical Virology with all him staff of laboratory in the Central Public Health Laboratory in Baghdad city especially in bacteriology department (**Mr. Zaid Saifuldeen,**

Mr. Mudhafar Qadir and Mrs. Suhaila M. Ali) and Virology Department for their cooperation and assistance and providing me important facilities.

My deepest thanks and appreciation to all staff members of Department of Bacteriology and Blood Bank in Al-Yarmouk Teaching Hospital for their great help and valuable advice. Finally special thanks to all my friends especially, Dr. -Mohammed hammed, Mr. Bassim Talib, Mr. Ammar gbbar, Mr. Omar and him family to help and patience and encouragement throughout the whole time.

My great thanks and deepest gratitude for **my close friends Master Ziyad A. Atallah and Master Hassan Ali.**

Dedication

To the most valuable home, the beloved Iraq.

To compassionate my family, my father, mother, my uncles and their sons.

To my brothers, my sisters, all relatives and my lovely Friends.

Summary

P. aeruginosa is a Gram-negative bacillus, straight or slightly curved rods, non-spore forming and capsulate.

The study aimed to identify some phenotypic, genotypic character and antibiotic resistance profile of *Pseudomonas aeruginosa* isolated from inpatient in Baghdad hospitals.

A total of 500 clinical specimens were collected from patients who suffer from wounds 125, burns 75, urinary tract 125, ear 100, pulmonary 50 and diabetic foot 25 infections at Al-Yarmuk Teaching Hospital, Baghdad Teaching Hospital, Special Burn Hospital in Medical City and Children Welfare Hospital in Baghdad city during the period between November 2015 to April 2016.

The *P. aeruginosa* were subjected for phenotypic characterization (morphological cultural, biochemical API 20NE system, biofilm formation, and antibiotic sensitivity tests) and genotypic characterization (*16SrRNA* gene, plasmid profile, *exoA* gene and DNA sequencing).

The results showed that out of 500 specimens, 60 isolates of *P. aeruginosa* were recovered and accounted 12% of hospitalized infection isolates. The diagnosis of *P. aeruginosa* isolates was confirmed genotypically via the amplification of *16SrRNA*gene with 956 bp by using PCR technique.

Sixty isolates of *P. aeruginosa* isolates were tested toward 14 antibiotics by using agar diffusion method, the results showed high resistance against Ticarcillin- clavulanic acid (81.66%), Gentamicin (41.66%), Tobramycin (40%), Mezlocillin (40%), Meropenem, Imipenem (38.33), Amikacin (35%), Cefipime (33.33%), Ofloxacin (31.66%), Ceftazidime (30.%), Levofloxacin (26.66%), Azetreonam (25%), Pipercillin-tazobactam and Ciprofloxacin (20%).

The ability of the *P. aeruginosa* isolates to produce biofilm as a virulence factor was tested using Congo red agar the results revealed that 41/60(68.4%) of isolates produced strong biofilm layer while 19/60(31.7%) of isolates did weak produce biofilm.

On the other hand, Polymerase chain reaction was used to detect *exoA* gene as virulence gene of *P. aeruginosa* tested isolates. The results showed that all 60 tested isolates contained of *exoA* gene.

The results of plasmid analyses of 60 isolates *P. aeruginosa* that extracted and purified using plasmid kit, showed a presence of plasmids in 45/60 (75%) of isolates while 15/60(25%) not detected. The plasmids profile contained 3 patterns based on (A, B, C) molecular size and gave a symbol.

The phylogenetic analysis of local isolates of *P. aeruginosa* showed a close related to NCBI-BLAST *P. aeruginosa* strain (KR81540.1) except the Iraqi isolate No. 2 (burns source) and Iraqi isolate No. 8 from (ear source) which showed genetically differences as unique isolates.

PCR product of *16SrRNA* gene was used for partial sequencing and plotting the phylogenetic tree of local isolates in comparison with some pathogenic standard world strains. Ten local isolates were submitted to GeneBank-NCBI for registration of sequences of*16srRNA* gene: The GeneBank-NCBI gave a code numbers which were: KX963356, KX963357, KX963358, KX963359, KX963360, KX963361, KX963362, KX963363, KX963364 and KX963365.

In conclusion, the present study proved that *P. aeruginosa* isolated had a virulence factors which strongly correlated with antibiotics resistance according to phenotypic and genotypic characterization.

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List of Abbreviations

Abbreviations	Meaning
API20NE	Analytic profile index*20*
ADP	Adenosine diphosphate
AmpC	Ampler type C
ATCC	American type culture collection
AHLS	Acyl homoserine lactones
(α)-hemolysis	Alpha hemolysis
(β)-hemolysis	Beta hemolysis
bp	base pair
CDC	Center for disease control and prevention
˚C	Degree celsius/Centigrade
CFU	Colony forming unit
CLSI	Clinical and laboratory standard institute
CRA	Congo-red -agar
Da	Dalton
DNA	Deoxyribonucleic acid
EF2	Elongation factor 2
EPS	Extracellular substance
ETA	Exotoxins A
ExoS	Exoenzyme S
ESBLs	Extended-spectrum β -lactamase

gm	Gram
GN Card	Gram negative card
GP Card	Gram positive card
LPS	Lipopolysaccharides
ICU	Intensive care unit
ml	Milliliter
mm	Millimeter
µm	Micrometer
µl.	Microliter
MBL	Metallo β-lactamase
MR-VP	Methyl red-Voges Proskauer Reagent
MW	Molecular weight
MDR	Multi-drug resistant
MHA	Mueller-hinton agar
MIC	Minimal inhibitory concentration
Mbp	Mega base pair
NaCl	Sodium chloride
NIH	National institute of health
nm	Nanometer
ng	Nanogram
Las A	Elastase A
las B	Elastase B
PCR	Polymerase chain reaction
P. aeruginosa	*Pseudomonas aeruginosa*

PAO392	*Pseudomonas aeruginosa* O-Antigen392
PA1805	*Pseudomonas aeruginoa* 1805
PA4077	*Pseudomonas aeruginosa* 4077
PEs	Pulmonary exacerbations
QS	Quorum sensing
RAPD	Random amplified polymorphic DNA
RND	Resistance nodulation division
rRNA	ribosomal ribonucleic acid
RFLP	Restriction fragment length polymorphism
IL	Interleukin
UTI	Urinary tract infection
UV	Ultra violate radiation
WHO	World health organization
16SrRNA	16 Svedberg ribosomal ribonucleic acid
RNA	Ribonucleic acid
tRNA	Transfer ribonucleic acid
23SrRNA	23Svedberg ribosomal ribonucleic acid
SNPS	Single nucleotide polymorphisms
TBE	Tris borate EDTA buffer
Zn	Zinc ion
PFGE	Pulsed flied gel electrophoresis
EDTA	Ethylene diaminetetraacetic acid
ATCC	American type culture collection
ORF	Open reading frame

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1. Introduction and literature review:

1.1 Introduction:

Pseudomonas aeruginosa is a Gram's negative opportunistic pathogen has emerged as one of the most problematic of the nosocomial pathogens; considered multi-resistant infections in both community and hospital settings, It causes infections in cancer, burn, urinary tract, surgical wound, eye, blood, ear infection, sepsis cystic fibrosis, and (ICU) (Tille, 2014).

Because of it's an extremely ubiquitous organism and abundantly found in soil, water, plants, humans, animals, and in hospital setting, *P. aeruginosa* is a common pathogen in hospital particularly in ICU although it has ability to colonize healthy subjects, in addition to, bacterial exposure to some antibiotic classes may potentially induce endogenous resistance conferring mutation in bacterial genes that encode drug targets (Sedigli *et al*., 2015).

It has become increasingly clear that resistance development in *P. aeruginosa*is multifactorial with mutations in genes encoding porins, efflux pump, penicillin binding proteins, and chromosomal β-lactamases, all contributing to resistance to β-lactamases, carbapenemes, aminoglycoside, and quinolones (Odumosu *et al*., 2013).

P. aeruginosa is an important pathogen in hospitalized patient's causative to their morbidity and mortality due to its multiple resistance mechanisms. Therefore, as therapeutic option becomes restricted, the search for new agent is priority (Ibrahim *et al*., 2016).

P. aeruginosa is responsible for about 10% - 20% of nosocomial infections as bacteremia and sepsis in ICU, cystic fibrosis, pneumonia, urinary tract infection and wound infection. MDR *P. aeruginosa* phenotype is defined as resistance to one antibiotic in three or more anti-pseudomonas,

antimicrobial classes (carbapenemes, aminoglycoside, and fluroquinolones, and cephalosporin) (Ranjbar *et al*., 2014).

Phenotypic method such as bio-typing, serotyping and molecular method such as plasmid profile analysis and PCR were used for epidemiological purpose (Mahmoud *et al*., 2013).

Knowing of DNA sequences has become indispensible for basic biological research, and in numerous applied fields such as diagnostic biotechnology, forensic biology and biological systematics. The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in sequencing of complete DNA sequences, or genomes of numerous types and species of life including human genome and other complete DNA sequences of many animal species (Pettersson *et al*., 2009).

Accordingly, this study focused was designed according to the proposed project of Ministry of Health for trying to solve the problem of antibiotics resistance by *P. aeruginosa* in hospital and health care centers.

On the causes of the continuous antibiotic resistance by *P .aeruginosa* that prevalent in hospitals especially that caused the hospitalized infections based on the identify the genetic variation between the isolates collected from different clinical samples by using the DNA sequencing and phylogeny analysis.

Aim of the study:

The study aimed to identify some phenotypic, genotypic character and antibiotic resistance profile of *Pseudomonas aeruginosa* isolated from inpatient in Baghdad hospitals. To achieve this aim, the following objectives were conducted:

1- Isolation and identification of *P. aeruginosa* from different samples of nosocomial infection based on the phenotypic and genotypic characters.
2- Determination the susceptibility of isolates to different antibiotics.
3- Typing the *P. aeruginosa* according to the pigment production (pyocyanin and pyoverdin), antibiotics resistance (Antibiogram)**,** biofilm formation, plasmid profile, and *exotoxin A* production.
4- DNA sequencing of *16SrRNA* gene products of ten local isolates for phylogeny and submission these isolates to GeneBank database in NCBI, for registration.

1.2. Literatures review:

1.2.1. *Pseudomonas aeruginosa*:

The origin of the word, Pseudomonas, means false unit, from the Greek pseudo meaning false and Monas, a single unit from Greek. The species name *aeruginosa* is a Latin word meaning verdigris (copper rust), another assertion is that the word may be derived from the Greek prefix ae- meaning "old or aged", and the suffix ruginosa means wrinkled. This also describes the blue-green bacterial pigment seen in laboratory cultures of the species. This blue-green pigment is a combination of two metabolites of *P. aeruginosa,* pyocyanin (blue) and pyoverdine (yellow-green), the derivations of pyocyanin and pyoverdine are of the Greek, with pyo- meaning (pus), cyanin meaning (blue) and verdine meaning (green). Pyoverdine in the absence of pyocyanin is a fluorescent-yellow color (Priya and Anitha, 2013).

P. aeruginosa was first isolated as a pure culture by Gessard in 1882 from wounds with blue-green discoloration and called at that time *Bacillus pyocyaneas* then called *Pseudomonas pyocyaneas* and finally named *P. aeruginosa* (Lister *et al.,* 2009) Shooter *et al.* (1971) were able to isolate *P. aeruginosa* from prepared foods in hospitals entrees and refrigerated meat and vegetables, Also pointed both of Wanderink and Dunn, (1995) to any disruption of local or general in the body's defence factors may cause infection to by those bacteria, so *P. aeruginosa* isolates often from contaminated burns, cutaneous ulcers, wounds of operations, the middle ear and urinary tract.

P. aeruginosa is a Gram-negative bacillus, straight or slightly curved rods, non-spore forming, capsulate; it is usually motile by one polar flagellum (monotrichous), measuring about 1-5 µm long and 0.5-1.0 µm wide. It occurs as single bacteria, in pairs and occasionally in short chains possess mucous layer due to material alginate slime layer as well as, multi-layers of extracellular polysaccharide (Deabreu *et al.*, 2014).

It is strict aerobic, but can grow anaerobically if nitrate or arginine is available as a terminal electron acceptor. The bacterium is capable of utilizing a wide range of organic compounds as food sources; it grows on minimal carbon and nitrogen sources, including simple media and moist surfaces. It was often observed growing in distilled water which was evidence of its minimal nutritional needs (Bhasin *et al*., 2015).

P. *aeruginosa* has a wide growth temperature range, optimum growth at 37°C. Slower growth rates are seen at 4˚C. *P*. *aeruginosa* is distinguishable from other clinically *Pseudomonas* spp. by its capability for growth at 42˚C. They also grow well at pH range 6.6-7.0. It was tolerant to a wide variety of physical conditions, including temperature and pH. Also it was resistant to high concentrations of salts and dyes. It is typically given a positive result to the oxidase test and catalase. It does not ferment carbohydrates, but many strains oxidize glucose (Moore and Flaw, 2011).

The bacterium is ubiquitous in soil, variety of aqueous solutions, including disinfectants, soaps, eye drops, as well as sinks and respiratory equipments, some strains hemolysis blood completely (β-hemolysis) by producing hemolysis toxin. On MacConkey agar, it shows yellow pale color because it is non-fermented lactose. *P. aeruginosa* forms smooth round colonies and irregular edge with a fluorescent greenish to yellow pigment pyoverdine. It also produces the non-fluorescent bluish pigment pyocyanin, which diffuses into the agar. Many types of media may be used for selective isolation of *P. aeruginosa* like cetrimide agar which promotes pyocyanin, pyoverdine, and pyoyrubin red color and pyomelanin black color production. (Brooks, *et al.*, 2013).

According to Bergey's Manual of Systematic Bacteriology *P. aeruginosa* belong to Kingdom; Bacteria; phylum; proteobacteria; Class Gamaproteobacteria; Order; Pseudomonadales; Family; Pseudomonadaceae; Genus: *Pseudomonas*; and species: *aeruginosa*. (Todar, 2011). The family

Pseudomonadaceae is classified into five groups based on rRNA / DNA homology and common culture characteristics, homology groups based on rRNA-DNA homology studies. The first group (rRNA group I) involve three subgroups which include fluorescent group (*P. aeruginosa*, *P. fluorescens* and *P. putida*); stutzeri group (*P. stutzeri* and *P. mendocina*) and alkaligenes group (*P. alkaligenes*, *P. pseudoalcaligenes* and species of group1) (Japoni *et al*., 2009).

1.2.2. Role of *P. aeruginosa* in Nosocomial Infection:

Pseudomonas aeruginosa, is an extremely versatile Gram-negative bacterium capable of thriving in a broad spectrum of environments, and this perform main problems to workers in the field of doctors and nurses (Juhas, 2015).

P. aeruginosa is an opportunistic human pathogen that is a causative agent hard-to-eradicate nosocomial infection. cancer, burn, cystic fibrosis and intensive care unit patients with mechanical ventilation are amongst those with the highest risk of infected by *P. aeruginosa* (Cramer *et al*., 2012), and ability to grow in moist conditions with simple nutrients and because of its ability to resist the antibacterial agents and disinfectants, is commonly found in various places of hospital environment including sinks, drains, taps, food, water, pharmacy preparations, contaminated hospitals equipments, mattresses and cleaning materials (mops, brushes). It grows in liquid and antiseptics such as quaternary ammonium compounds (Yaslianifard *et al*., 2012).

P. aeruginosa forms biofilm with extreme tolerance to antibiotics in nosocomial infections, such as pneumonia and surgical site infections, prompting the Centers for Disease Control and Prevention to classify *P. aeruginosa* under 'serious'-threat level, therefore continuous and careful monitoring of these objects and sites is necessary to control infections in hospitalized patients. Regular practice of environmental survey and suitable

control measures help to reduce hospital acquired infections considerably (Sharma *et al*., 2014).

1.2.2.1. Nosocomial Infections:

Nosocomial infections, also called "hospital-acquired infections", are infections acquired during hospital care by a patient who was admitted for a reason other than that infection or other health care facility in which the infection was not present or incubating at the time of admission, infections occurring more than 48 hours after admission, are usually considered nosocomial infection (Khan, 2009).

Frequency of nosocomial infection occurs world-wide and they are important contributors to morbidity and mortality. They will become even more important as a public health problem with increasing economic and affects both developed and resource-poor countries. But major challenge for low and middle income countries developing which have limited health care resources, infections acquired in health care settings are among the major causes of death and increased morbidity among hospitalized patients (Voirin *et al*,. 2009).

There are two forms of nosocomial infections: 1. Endogenous infections, (self-infections, or auto-infections) can be caused by microorganisms already present in the patient's skin and mucosa present in the patient at the time of admission to hospital but there are no signs of infection. The infection develops during the stay in hospital as a result of the patients altered resistance.

2. Exogenous infections, cross-contamination followed by cross-infection: during staying in hospital the patient comes into contact with new infective agents, by microorganisms transmitted from another patient or from surrounding environment which becomes contaminated and subsequently develops an infection (Percival *et al.*, 2015).

1.2.2.2. Predisposing Factors of *P. aeruginosa* in Nosocomial Infections:

The main factors that effected the development of nosocomial infection involve: firstly patient susceptibility: which is important factors that influencing the acquisition of infection include: age, immune status, underlying disease and diagnostic and therapeutic interventions. The extremes of life-infancy decreased resistance to infection with opportunistic pathogens. Secondly, the bacterial agent which include, the intrinsic virulence and new microorganisms (Sharma *et al*., 2014). And third environmental factor which include health care settings are environment where infected persons are at increased risk of infection congregate. Whereas patients with infections or carriers of pathogenic microorganisms are potential sources of infection for patients and staff. In addition to the increasing numbers and crowded conditions of people in hospitals, with frequent transfers of patients from one unit to another and number of patients highly susceptible to infection in one area, all contribute to the development of nosocomial infection (WHO, 2002).

1.2.2.3. Nosocomial Outbreak of *P. aeruginosa*:

An outbreak is defined as an unusual or unexpected increase of cases of a known nosocomial infection or the emergence be identified and promptly investigated because of cases of a new infection (WHO**,** 2012). Nosocomial outbreaks are a major problem for health care institutions due to increased morbidity and mortality for the affected patients. The containment and control of these outbreaks costs substantial amounts of funds and resources, especially when left unnoticed or untreated (Ibrahim, 2016).

Identifying an outbreak early important to limit transmission among patients by health care workers or through contaminated materials. A potential problem may be initially identified by nurses, physicians, microbiologists, or

any other health care worker, appropriate investigations are required to identify the source of the outbreak, and to implement control measures. The control measures will vary depending on the agent and mode of transmission, but may include isolation procedures or improvements in patient care or environmental cleaning (WHO**,** 2012**).**

Nosocomial outbreaks of *P. aeruginosa* have been reported in surgical wounds, causing post-operative wound infections. Wound infections caused by MDR *P. aeruginosa* have been associated with high morbidity and mortality rates worldwide (Ranjan *et al*., 2010).

In addition, *P. aeruginosa* can disseminate from the initial infection site and enter the blood stream, causing septicemia (Jombo *et al*., 2010).

In cystic fibrosis early infections with *P. aeruginosa* can be transient, and can clear spontaneously, but colonization with *P. aeruginosa* usually occurs by the time when patients reach their teenage year. This important and characteristic shift is associated with more frequent and more severe pulmonary exacerbations (PEs) that result in progressive decrements in lung functions (Sanders *et al*., 2011).

In the later stages of infection, cystic fibrosis with up to 60% of adult patients infected (United Kingdom Annual Data Report 2014). *P. aeruginosa* also dominates chronic infections in a proportion of patients with bronchiectasis (Rogers *et al*., 2014).

Urinary tract infections (UTI) caused by *P. aeruginosa* usually occur secondary to catheterization, instrumentation or surgery. Catheterization of the urinary tract is the major cause of nosocomial acquired-UTI by *P. aeruginosa* catheters are utilized by pathogens as a source of host entry, attaching to the catheter surface (Elkhatib and Noreddin, 2014).

In well-constructed biofilms furthermore, the insertion of the catheter may also disrupt mucosal epithelial layers, promoting bacterial colonization (Mittal *et al*. 2009), the bacterium is regarded as an opportunistic pathogen, primarily

causing nosocomial infections in immunocompromised patients (Brown *et al*., 2012).

However, it is capable of causing wide-spectrum infections when normal physiological function is disrupted, including damaged epithelial barriers depleted neutrophil production, altered mucociliary clearance and the use of medical devices (Engel and Balachandran, 2009).

Keratitis occurs in patients with pre-existing ocular disease, *P. aeruginosa* is the leading cause of bacterial ocular surgery and in individuals who use contact lens develops rapidly that may lead to vision loss (Suryawanshi *et al*., 2013).

P. aeruginosa is a common cause of chronic otitis media and inflammation may also cause otitis externa, including malignant otitis extern (Roland and Stroman, 2002).

Mild skin infections can occur in previously healthy people, associated With *P. aeruginosa* contamination in swimming pools, and other water sources (Guida *et al*., 2009). Follicular dermatitis caused by *P. aeruginosa* as an itchy rash with a red base and white pustules. In addition, nail diseases (e.g. onycholysis) are susceptible to colonization of *P. aeruginosa*, and is commonly referred as "green nail syndrome" (Daniel *et al*., 2011).

Paronychia infection has been associated with prolonged exposure to moist environments (e.g. swimming). McNeil *et al*. (2001) investigated an outbreak of *P. aeruginosa* infections in postsurgical patients, reporting severe onycholysis and onychomycosis in a nurses' thumbnail as the primary source.

The spine, pelvis and sternoclavicular joints are the most common sites affected by *P. aeruginosa*. It reaches in to these sites through direct inoculation of the bacteria or the hematogenous spread of the bacteria from other primary sites of infection. Risk factors include penetrating trauma, peripheral vascular disease, intravenous drug and diabetes mellitus (Todar, 2011).

P. aeruginosa causes gastroenteritis in newborn and young infants in pediatric wards, which results from contaminated milk feeds (Govan, 2007).

P. aeruginosa may infect heart valves in intravenous drug users and also prosthetic heart valves (Yilmaz *et al*., 2013). *P. aeruginosa* may cause meningitis and intracranial abscesses. Most infections result from direct spread from local structures for instance the ear, mastoid and sinuses but blood-borne spread may also occur (Chang *et al*., 2000).

Almost any opportunistic pathogen can infect burns and wounds, but one of the most common and hardest to treat is the Gram negative rod *P. aeruginosa*, which can actually color the burns or damaged tissue with its blue-green fluorescent pigments and may lead later to septicemia (Alinaqvi *et al*., 2011).

1.2.3. Ecology and Epidemiology of *P. aeruginosa:*

Pseudomonas aeruginosa disseminated in different environments such as soil, water and plants as well as, these bacteria located on the skin of humans, animals and part of normal flora in human gut. It can be found in solutions, disinfectant, respiratory equipments, foods, sinks and salts. The bacteria present in different environments because of their ability to utilize the many compounds as sources of energy, such as nitrogenous compounds and carbohydrates as a source of carbon, so do not need a complex growth factors therefore, it is very essential medically (Yitah and Essien, 2008).

P. aeruginosa groups tend to form biofilms, which are complex bacterial communities that stick to variety of surfaces including plastics, medical implant materials and tissue. They are very difficult to destroy (Brown *et al*., 2012). The spread of bacteria *P. aeruginosa* can be controlled through proper isolation of patients, use of sterile techniques and observance conditions of hygiene with careful when using respiratory devices, catheters, food and other machinery (Shahid and Malik, 2004).

Vandeale *et al*. (2005) studied the epidemiology of *P. aeruginosa* in cystic fibrosis rehabilitation center, sixty-seven isolates of *P. aeruginosa* were isolated from patient's sputum, the infection by patient-to-patient-transmission could have happened in the past, the risk of patient-to-patient transmission during the study period (63day) was relatively (10%) and the risk of persisting colonization with a new acquired strain during the study period was (4%).

1.2.4. Role of Virulence Factors in Pathogenesis of *P. aeruginosa:*

1.2.4.1. Flagella:

The first step in *P. aeuroginosa* infection is that adherence to epithelium surface is mediated by pili, flagella and alginate (Cotar *et al*., 2010).The function of flagella is not limited to motility, but also they have a role in activating host inflammatory response through Toll-like receptor 5 and in attaching bacteria to host cells a major protein constituent of the flagellar filament is the flagellin that can be classified into A and B serotypes which serve as target antigens for vaccination (Campodonico *et al*., 2010).

1.2.4.2. Pili:

Like the flagella, pili also play important roles in the bacteria-host cell interactions including the attachment. The pili in *P. aeruginosa* are type IV pili (Mattick, 2002).

1.2.4.3. Lipopolysaccharide (LPS):

Molecules are found in the outer membranes of Gram-negative bacteria with a unique chemical structure, LPS is composed of the polysaccharide O, core antigens and lipid A composed of fatty acid and phosphate groups bonded to a glucosamine disaccharide (Ernst *et al.*, 2006).

1.2.4.4. Quorum Sensing and Biofilm Formation:

Biofilms are highly-structured microbial communities attached to surfaces and enclosed in a matrix of extracellular polymeric substance (EPS). EPS

generally contains polysaccharides, proteins and extracellular DNA (Percival *et al*., 2012).

The presence of EPS allows the biofilms to contain open water channels for transport of nutrients into the biofilm and waste products out of the biofilm (Hentzer *et al*., 2001).

It has been well documented that bacteria in the biofilm are up to 1,000 times more resistant to antimicrobial agents than planktonic cells according to the National Institute of Health; more than 60% of nosocomial infections are associated with biofilms (Li and Zhang, 2007).

Well characterized model organism for biofilm study is *P. aeruginosa* that causes lung infection in patients with cystic fibrosis. The development of *P. aeruginosa* biofilms occurs in the following four stages: 1. initial and reversible attachment of free-floating cells to the surface; 2. irreversible attachment; 3. production of the EPS matrix and 4. early development of biofilm dispersion (Ma, 2014).

Extracellular DNA (eDNA) has been found important to the initial attachment of *P. aeruginosa* biofilms. Additionally, one finding suggested that eDNA is similar to chromosomal DNA based on the (PCR) and southern analysis results (Waters and Bassler, 2005).

Other mechanisms that are thought to play a role in the antimicrobial resistance acquired by certain micro-organisms within biofilms include the presence of efflux pumps, with the expression of several gene-encoding efflux pumps being increased in biofilms (Soto, 2013).

Furthermore, plasmid exchange occurs at a higher rate in biofilms, increasing the chances of developing naturally occurring and antimicrobial-induced resistance (Hausner andWuertz, 1999).

It is thought that an altered micro-environment within a biofilm, such as nutrient depletion and reduced oxygen levels, may also reduce the efficacy of antimicrobials (Francolini and Donelli, 2010).

In the last decade, the knowledge of this feature of bacterial pathogenesis has been improved. Most bacteria to communicate and react coordinated by making use of auto inducers, a system known as QS the cell population density of the developing biofilm, the gene expression of cells within the biofilm is regulated by a process known as QS (Kjelleberg and Molin, 2002).

Through this system, bacteria release chemical signals called auto inducers, which are constitutively produced and increase in concentration as the density of the biofilm increases. As the concentration of these autoinducers reaches a critical threshold, alterations in gene expression occur, leading to an array of physiological processes, including motility, sporulation and release of virulence factors necessary for survival and QS plays a role in biofilm establishment, growth and maintenance (Mangwani *et al*., 2012).

P. aeruginosa are one of the best described bacteria which QS system is linked to biofilm formation. Two systems have been described in *P. aeruginosa*: *las* , *rhl* and QS systems respond to a class of autoinducers named acyl homoserine lactones (AHLs) (Ng and Basler, 2009).

The AHL auto inducer in *las* QS system is synthesized by *LasI* and is regulated by *LasR*, a transcriptional activator protein. The AHL auto inducer in *rhl* QS system is synthesized by *RhlI* and is regulated by RhlR (Fuqua *et al*., 2001). *rhl* QS system is furthermore controlled by *las* QS system (Medina *et al*., 2003).

The study of Melaugh *et al*., (2016) showed new insight into biofilm formation and development, and reveal new factors that may be at play in the social evolution of biofilm communities.

1.2.4.5. Pigments:

1. Pyocyanin from" pyocyaneus" refers to" blue pus" which is a characteristic of supportive infections caused by *P. aeruginosa*. Pyocyanin is a blue-green pigment metabolite of *P. aeruginosa* that has been shown to have numerous

pathogenic effects such as increasing IL-depressing host-response (Brooks *et al*., 2013).

2. Pyoverdin one explanation for this role has emerged when it was found that pyoverdin regulates the secretion of other *P. aeruginosa* virulence factors, exotoxin A and an end protease and its own secretion

3. Some strains produce red pigments (pyorubin)

4. Few produce pyomelanin black pigment (Lamont *et al*., 2002).

1.2.4.6. Enzymes:

Protease IV is other crucial virulence factors; four types have been known to be secreted by *P. aeruginosa*: LasB elastase, LasA elastase or staphylolysin, alkaline protease and protease IV. These proteases are associated with virulence by enhancing the ability of *P. aeruginosa* to invade tissues and interfering with host defense mechanisms. The electrolytic activity of these enzymes is very important in pathogenesis since a number of tissues are composed of elastin (lung, vascular and ocular tissue) (Lyczak *et al*., 2000).

Protease IV causes the destruction of host proteins including fibrinogen, elastin and components of the immune system and is thought to aid in bacterial adhesion.

Also phospholipase C secreted by *P. aeruginosa* into the extracellular space through a type II secretion system, hemolytic phospholipase C targets eukaryotic membrane phospholipids and has been shown to participate in the pathogenesis of *P. aeruginosa* in inflammation and a part of the pathogenic effect of hemolytic phospholipase C may be due to surfactant inactivation, Furthermore, hemolytic phospholipase can suppress the host neutrophil oxidative burst response (Engel and Balachandran, 2009).

1.2.4.7. Toxins:

- **Exoenzyme S (Exo S),** *P. aeruginosa* produce virulence factor Exo S, which has a demonstrated role in pathogenesis (Kenneth, 2009). Exo S has ADP-

ribosylation of many proteins in cell and induces T-cell apoptosis, which is a unique T-cell mitogen and it is powerful immune stimulus that activates a large proportion of T cells, but result in delayed and reduced lymphocyte proliferation (Salva *et al*., 2004).

- **Exotoxin A (ETA),** is one of the most toxic extracellular enzymes produced by this pathogen like diphtheria toxin, it is a heat labile, 613 AA, single polypeptide chain with a MW of 66.583 Da. It catalysis the ADP- ribosylation of elongation factor 2 (EF2) leading to inhibition of protein synthesis and cell death (Yates *et al*., 2005).

ETA is the most potent toxic factor which is responsible for local tissue damage, bacterial invasion and immune suppression. ETA catalyzes ADP-ribosylation and inactivation of EF2, an essential component of the protein synthesis machinery which leads to inhibition of protein biosynthesis and cell death (Todar, 2011).

Structure ETA, is A and B fragments that mediate enzymatic and cell-binding functions, respectively Produced *in - vivo* during *P. aeruginosa* infections (Joseph *et al*., 2001).

The crystallographic structure of exotoxin A revealing tertiary fold has three structural domain and domain I located at N-terminal end of protein is required for binding ETA to target cells responsible for the receptors binding, domain II has been implicated in the translocation of ETA into the cytosol and domain III ADP-ribosyl transferase activities of protein, respectively (Bayat *et al*., 2010).

- **Type III Secretion System (T3SS)**

The bacterial type III secretion system (T3SS) is a conserved injection apparatus, allowing both plant and animal pathogens to deliver its effecter proteins directly into eukaryotic host cells to initiate a sophisticated (biochemical cross-talk) between pathogen and host, T3SSs have been described for many gram negative bacteria species, including pathogens, like *Shigella flexneri*, *Bordetella pertussis*,

P. aeruginosa and *Vibrio Cholerae*. Also (TTSS) of *P. aeruginosa* is a complex pilus-like structure allowing the translocation of effectors proteins from the bacteria, across the bacterial membranes and into the eukaryotic cytoplasm through a needle-like appendage forming a pore in the eukaryotic membrane, there are four known toxins, variably expressed in different strains and isolates, injected into host cells by *P. aeruginosa* through the TTSS: ExoY, ExoS, ExoT and ExoU (Engel and Balachandran, 2009).

1.2.5. Antibiotics Resistance in *P. aeruginosa:*

Antibiotic resistance mechanisms identified in bacteria to date making it a particularly problematic nosocomial pathogen and also one of the biggest therapeutic challenges unfortunately, selection of the most appropriate antibiotic is complicated by the ability of *P. aeruginosa* to develop resistance to multiple classes of antibacterial agents, even during the course of treating an infection (Strateva and Yordanov, 2009).

P. aeruginosa has two main mechanisms of resistance, intrinsic and acquired to antibiotics; contribute to the pathogenicity of *P. aeruginosa* it is intrinsically resistance to anti-microbial agent due to low permeability of its cell wall, efflux pump which pump out the antibiotic from cytoplasm, while acquired resistance is due to the presence metallo β-Lactamases (MBL-Ambler type B) and AmpC (Ampler type C), MBL use metal ion Zn to catalyze hydrolysis of β-lactam (Juhas, 2015). Acquired resistant where *P. aeruginosa* easily develops acquired resistance either by mutation in chromosomally-encoded genes or by the horizontal acquired gene via plasmid, transposons and bacteriophages which are responsible of antibiotic resistance (European Center for Disease Control and Prevention, 2013).

P. aeruginosa is resistant to penicillin, ampicillin, and tetracycline first and second generations of cephalosporin, sulfonamides, neomycin,

streptomycin, kanamycin, chloramphenicol, nitrofurans and trimethoprim-sulfonamide but only a few antibiotics are effective including fluorquinolones, amikacin, gentamicin and certain broad spectrum ß-lactam antibiotics such as imipenem, carbapenem, fourth generation of cephalosporin (cefepime) (Lutz and Lee, 2011).

Resistance has been found particularly high in the family of carbapenemes this has been proved for physicians worldwide, and making the control and treatment of infections (Lagatolla *et al*., 2006). To tackle this growing problem and minimize the chance of these resistance strains of bacteria spreading in hospitals and to the greater global population there is an urgent programs as well as raising awareness amongst clinicians themselves on how to best prescribe these types of drugs knowing their risk and to minimize the chance of antibiotic resistance spreading.

Also, the resistance of MDR strains may be mediated by the active export of the antibiotic out of the bacterial cell by efflux pump. Evidence from diverse bacterial genomes indicated that approximately 5 %- 10 % of genes are involved in transport, with a large proportion of them encoding efflux pump systems that allow it to be resistance to several antibiotics.

Another mechanism present in *P. aeruginosa* in the formation of permeability barriers through the membrane (e.g. imipenem) is due to diminished expression of specific OM protein. It has been shown that OM permeabilizers such as EDTA increase susceptibility to the antibiotic, indicating that the lack of OprD protein leads to a reduction of active antibiotic molecule capable of reaching the target penicillin- binding protein (Kadry, 2003).

Finally, it has been show that cytotoxicity is an important mechanism that contributes to high morbidity and mortality in *P. aeruginosa* infections. Along with mucoidy resultant from the release of alginate *P. aeruginosa* synthesis a secretory apparatus (type III) that allows it to inject toxins from their cytoplasm

into the target cell. The latter mechanism allows mucoid bacteria to lyse the host's macrophages and overcome various defense such as in the case of cystic fibrosis lung infection. (Guespin *et al*., 2008).

In a previous study, Isibor *et al*. (2013) found a high percentage of MDR strains of *P. aeruginosa* associated with diabetic wounds of patients. Resistance rate in *P. aeruginosa*, from 40 to70% of isolates responsible for ICU acquired infections being carbapenem resistant **(**Sader *et al*., 2014).

Also, *P. aeruginosa* have essential genes as antimicrobial targets 'core' essential genes encoding universal cellular functions are good antimicrobial targets due to providing a broad host range, this is not always the case, antibiotics interfering with a 'core' essential gene could also inhibit the function of the essential homologue in human cells recent analyses led to the identification of a number of general and condition-specific *P. aeruginosa* essential genes. A number of these, such as those implicated in asparagine-tRNA biosynthesis, are not essential in mammalian cells and are therefore candidate drug targets (Lee *et al.,* 2015).

Recent genome-wide analyses have also revealed a number of essential genes that are indispensable in *P. aeruginosa*, but not in other bacteria, such as *E. coli*. These include genes involved in central carbon energy metabolism and protection from reactive oxygen species (Lee *et al*., 2015). As the essentiality of these processes stems from the *P. aeruginosa* life style (e.g. the reliance of *P. aeruginosa* on respiration for energy generation), they are considered to be good targets for *P. aeruginosa*-specific antibiotics. Furthermore, the 'accessory' essential genes required for *P. aeruginosa* growth under clinically relevant conditions, such as in cystic fibrosis sputum and in the presence of antibiotics, and those indispensable for pathogenicity appear to be promising targets.

Recently described and validated examples of essential *P. aeruginosa* antimicrobial targets include tobramycin antibiotic resistance-conferring genes, resistance-nodulation-division (RND)-type efflux pumps. Recent Tn-seq circle

method-based analysis led to Identification of 117 genes essential for the growth of *P. aeruginosa* on the aminoglycoside antibiotic tobramycin (Gallagher *et al.*, 2011). In addition to the previously identified tobramycin resistance-conferring genes these include genes for a number of novel tobramycin targets, such as PAO392, PA1805, and PA4077 encode an unknown membrane protein implicated in osmotic stress tolerance, a peptidyl–prolylcis–trans isomerase (ppiD) involved in folding of the membrane and exported proteins, and the transcriptional regulator of the envelope stress response, respectively (Lee *et al*., 2009).

The study of Sheet (2012) on morphological and molecular characterization of multi-drug resistance *Pseudomonas* spp. isolated from clinical and environmental cases of neonatal and infants' nosocomial infections in Mosul showed that *Pseudomonas* spp. is a common pathogen or contaminant in hospital ecology, the *16SrRNA* gene consider as an important confirmative tool for diagnosis of *Pseudomonas* spp., in addition to pigment production and growth on cetrimide as primary diagnosis for this pathogen and The antibiotic susceptibility revealed that *Pseudomonas* isolates showed different levels of resistant to many antibiotics belong to penicillins, cephalosporins, aminogycosides, whereas the most strains were susceptible to imipenem, azithromycin, ciprofloxacin, gentamicin and norfloxacin.

Juhas (2015) provides an update on the investigation of *P. aeruginosa* essential genes special focus is on recently identified *P. aeruginosa* and their exploitation for the development of antimicrobials essential genes.

Mozes (2015) showed the antibiotic consumption may be the main driving force for maintenance of epidemic strains during non out break periods antibiotic resistance in certain situations may be more important than virulence in spread of successful clones.

Detection and determination of the antibiotic resistance patterns in *P. aeruginosa* strains isolated from clinical specimens in hospitals of Isfahan, Iran, showed the prevalence of *P. aeruginosa* strains with MDR was very high amongst the clinical samples in the major hospitals of Isfahan. This is quite a worrisome problem and makes the controlling of the strains more difficult (Sedighi *et al*., 2015).

The study of Juber (2015) on Assessment of Some Virulence Genes of *Pseudomonas aeruginosa* Isolated from Eye Infection, in Al-Qadisiyah University, that proved that *Pseudomonas aeruginosa* was the most common pathogen causes of eye infections, conjunctivitis was the most common infection among children below 10 years, PCR was a reliable technique and sensitive enough for detection of *Pseudomonas aeruginosa* virulence gene such as *exoA* gene and phospholipase C (plC) and Presence of *exoA* and plC genes in pathogenic invader (*Pseudomonas aeruginosa*) could explain the prognosis of eye tissue damages.

1.2.5.1. Efflux Pump and Plasmid Resistance Mechanism in *P. aeruginosa*:

In next two decades, both agent and class specific numerous chromosome and plasmid encoded drug multidrug efflux transporters have been described in a variety of microorganisms associated with antibiotic resistance (Osmon *et al*., 2004).

1. Efflux Pumps Systems:

All of the antibiotics have to cross the cell wall, to reach their targets. Failure of antibiotics to accumulate in the organism is due to the combination of restricted permeability of the outer membrane and the efficient removal of antibiotic molecules by efflux pumps. The outer membrane of *P. aeruginosa* is an important barrier of the penetration of antibiotics (has low permeability) (Hancock and Brinkman, 2002).

The RND-type efflux pumps, such as *MexAB–OprM*, contribute to the intrinsic resistance of *P. aeruginosa* to antibiotics by pumping antimicrobials from the periplasm to the outside of the cell (Opperman and Nguyen, 2015).

A number of efflux pump inhibitors, including phenylalanyl arginyl D13-9001, have been described; however, none has yet been tested clinically (Schuster *et al*., 2014).

2. Plasmids:

Plasmids are defined as double stranded, extra chromosomal genetic elements that replicate independently of the host cell chromosome and are stably inherited. Plasmids capable of integration into the chromosome were earlier called episomes. Plasmids differ from chromosomes in being small and coding for genes that are non-essential for the bacterial survival. Absence of plasmids doesn't kill bacterium, but their presence provides additional benefits to the bacterial cell. Plasmids vary in size; smallest plasmid is only 846 bp long and contains only one gene. Some plasmids carry more than 500 genes. The largest plasmid known carries 1,674 genes. Like chromosomes, plasmids are also closed circular and supercoiled; although certain plasmids (eg. *Borrelia*) are known to be linear, which produce ESBLs and stranded DNA materials; have been found to be useful For Amp C broad-spectrum β-lactamases have been pathogens genetic diversity and prowess as infectious agent (Brinas *et al*., 2003).

Similarly, agents and profiling pathogens for their harbored plasmids articles describing their prevalence in food animals and has been found to be very useful in epidemiological foods of animal origin have been published in recent studies, diagnosis and elucidation of mechanisms of drug years resistance (Bennett, 2008).

On the other hand, study from Iran established by Jafari *et al*. (2013) isolated an antibiotic resistant plasmid in *P. aeruginosa* resistance to third generation of cephalosporin's; cefepime and aminoglycosides. This organism is

a major concern in nosocomial infections and should therefore be monitored in surveillance studies.

1.2.6. Phenotypic and Genotypic Methods Used for Identification of *P. aeruginosa*:

1.2.6.1. Phenotyping Methods:

Successful treatment of a patient with a bacterial infectious disease requires rapid and specific identification of the causative agent, identification of bacterial pathogens by traditional methods is still a crucial element of the diagnostic process, early epidemiological studies used phenotyping methods to characterize microbial isolates, determining antimicrobial susceptibility of the organism, and biochemical testing can be laborious and time-consuming, and may prolong definitive diagnoses and treatment of the patient (Bartlett and Stirling, 2003).

Pseudomonas aeruginosa phenotyping methods include biotyping, pyocin typing, serotyping, and phage typing and antibiobiotic sensitivity profiles. These methods are limited in their ability to differentiate between *P. aeruginosa* strains due to their low discriminatory power (Belkum *et al*., 2007).

P. aeruginosa can undergo phenotypic change during colonization of CF lungs, reducing the capacity of these methods to characterize strains phenotyping limitations (Saiman *et al*., 2003).

1.2.6.2. Genotyping Methods:

1.2.6.2.1. *P. aeruginosa* Genome:

P. aeruginosa is a highly adaptable organism, it has a large genome containing from 5.5 to 7 Mbp (encoding 5567 genes) compared to 4.64 Mbp (4279 genes) in *Escherichia coli* (Juhas, 2012).

Essential genes are often divided into two categories, 'core' and 'accessory' essential genes. 'core' is considered to be those genes that are universally indispensable for all living organisms. Therefore, 'core' essential genes could be exploited as the basic building blocks of the controlled minimal cell factories. 'accessory' essential genes are required for the survival of individual species and cell types or under specific growth conditions. This makes bacterial 'accessory' essential genes promising targets for the development of novel antimicrobials (Juhas, 2015).

Whilst there is the assumption that 'core' essential genes could be exploited as antimicrobial targets due to providing a broad host range, there is a real danger that antimicrobials inhibiting the 'core' essential gene would also target the essential human homologue. The core genome is composed of highly conserved regions including Housekeeping genes that have a low nucleotide divergence of 0.00-0.5, therefore considered to be generally essential in *P. aeruginosa* (Juhas, 2012).

The majority of the general *P. aeruginosa* essential genes play a role in fundamental cellular functions, such as DNA replication, transcription and translation, RNA metabolism, protein export, biosynthesis of cofactors and amino acids, and cell wall biogenesis (Lee *et al*., 2015). The *P. aeruginosa* accessory genome consists of extra chromosomal components such as plasmids, islands and blocks of DNA that are integrated into the chromosome at various sites (Klockgether *et al*., 2011).

These components may be acquired from other species of bacteria through mechanisms such as horizontal gene transfer. The 'mosaic' and plastic genome structure of *P. aeruginosa* provides this bacterium with the ability to modify, cause infection and adapt to a wide range of habitats (Norgaard-Gron, 2010).

A fast and accurate system for the identification of *P. aeruginosa* and their genes are important to isolate patients and prevent further spreading of the

disease. Bacterial culture and smear examination one of the most important techniques which are still used in diagnostic microbiology, because of its ability to quantify the viable bacteria in a sample; as well as obtaining a pure sample for further testing (Minion, 2010).

Therefore, it has become necessary to develop genotype based characterization systems capable of exactly identifying these microorganisms and their virulence genes, DNA marker allow rapid identification of species between DNA markers, the PCR is highly sensitive, specific and rapid method which vastly improved the detection of *P. aeruginosa* especially when using species - specific primer such as *16SrRNA, toxA,* and *16S- 23SrRNA* genes (Yetkin *et al*., 2006).

Molecular typing methods are required to identify the sources and routes of transmission within the health-care setting (Turton *et al*., 2010).

More advanced approaches to identification have been developed, including PCR is a scientific technique used to amplify a specific sequence of a DNA strand generating thousands to millions of copies of a particular DNA sequence developed in 1983 by Kary Mullis (Bartlett and Stirling, 2003).

RFLP, PFGE, RAPD and DNA sequencing initial molecular epidemiological typing were performed. PFGE using restriction enzyme *Spe*, and identified a single outbreak strain among clinical and environmental isolates (Gee, 2004). Molecular tools such as *16SrDNA* gene sequencing provide reliable results although it might have problems to assign at specie level (Fernandez-Olmos *et al*., 2012).

Selective amplification of *Pseudomonas 16SrRNA* gene by PCR has been used to detect differentiates *Pseudomonas* species from clinical and environmental samples (Wolfgang *et al*., 2003). It is also used for genus or species - level identification of *P. aeruginosa* (Spilker *et al*., 2004).

PCR relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the

DNA. Primers containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. The DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq Polymerase, an enzyme originally isolated from the bacterium *Thermos aquaticus*. This DNA polymerase enzymatically assembles a new DNA strand from DNA building-blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (DNA primers), which are required for initiation of DNA synthesis. (Anzai *et al*., 2000)

Al-Marjani *et al*. (2014) used *16SrRNA* sequence data to identify genus- and species-specific *16SrRNA* signature sequences, its account a stable part of the genetic code. Based on these sequences they designed simple, rapid, and accurate PCR assays that allow the differentiation of *P. aeruginosa* from *Pseudomonas* species and other pathogen genus.

1.2.6.2.3. DNA Sequencing in *P. aeruginosa*:

DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule. It includes any method or technology that is used to determine the order of the four bases—adenine, guanine, cytosine, and thymine in a strand of DNA. The advent of rapid DNA sequencing methods has greatly accelerated biological and medical research and discovery knowledge of DNA sequences has become indispensable for basic biological research, and in numerous applied fields such as diagnostic, biotechnology, forensic biology, and biological systematics (Clyde, 2007).

The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of complete DNA

sequences, or genomes of numerous types and species of life, including the human genome and other complete DNA sequences of many animal, plant, and microbial species **(**Pettersson *et al*., 2009**)**. Fifteen years elapsed between the discovery of the DNA double helix in 1953 and the first experimental determination of a DNA sequence ~~(Reinert and Huson, 2007)~~.

Whole genome sequencing, facilitated by the advent of high-through put approaches, brings the promise of single-base-pair resolution between isolates, making it the ultimate molecular typing method for bacteria (Reinert and Huson, 2007).

Snyder *et al*., (2013) Studied of epidemiological investigation of *P. aeruginosa* isolates from a six-year-long hospital outbreak using high –through put whole genome sequencing, In this study, they demonstrated that the single base resolution of whole genome sequencing is a powerful tool in analysis of outbreak isolates that can not only show strain similarity, but also evolution over time and potential adaptation through gene sequence change.

2. Materials and Methods:

2.1. Materials:

2.1.1. Patients:

A total of 500 inpatients from both sexes (300 males and 200 females) with different age, whom are suffering from different clinical infections **(**diabetic foot 25**,** otitis media 100**,** lower respiratory tract 50**,** urinary tract 125, wound 125 and burns 75 infections) in Baghdad Teaching Hospital, Al-Yarmuk Teaching Hospital, Special Burns Hospital in Medical City and Children Welfare Hospital in Baghdad city during the period from November, 2015 to April, 2016 were enrolled in this study. Depending of clinical criteria that diagnosed by physician and laboratory criteria of infections based on diagnosis microbiology mentioned by Brooks *et al*. (2013). A Questionnaire form was done to record the patients' informations (Appendix 2-1).

2.1.2. Laboratory Instruments and Equipments:

The instruments and equipments that used in this study are listed below in Table (2-1):

Table (2-1): Instruments and equipments used with their remarks

Instrument/equipment	Manufacturing company	Origin
Autoclave	Gallenkump	England
Benzene burner	Al baser office	Iraq
Centrifuge	Hermle	Germany
Deep freeze	Marubeni	Japan
Digital camera	Digital	Japan

Disposable latex	Duisburg	Germany
Disposable loop	Fisher brand	USA
Disposable swab stick	AFCO	Gordon
Disposable syringes(5ml)	Changzhou	China
Distal water	K&K	Germany
Dosimeter	Biomerieux	France
Electric oven	Memmert	Germany
Eppendorf tube	Sigma	England
Eppendorf centrifuge	Eppendorf	Germany
Exipin PCR centrifuge	Bioneer	South Korea
Flask (250_500)ml	Oxfords	USA
Forceps	Promega	Promega
Gel electrophoresis	Major science	Taiwan
Graduated glass cylinder	Superior	Germany
Hot plate with magnetic stirrer	Jenway	United kingdom
Incubator	Memmert	Germany
Khan tube	Bioner	South Korea
Laminar-flow cabinet	Memmert	Germany
Light microscope	Olympus	Japan

Micropipettes(0.5-10µl ,2-20µl ,100-1000µl)	Appendof	Germany
Nano drop spectrophotometer 2000	Thermo scientific	USA
PCR thermo cycler	Bio-Rad	USA
Petridis	Himedia	India
pH meter	Orient	USA
Refrigerator	Marubeni	Japan
Screw capped test tube	BBL	USA
Sensitive balance	Promega	USA
Thermo mixer comfort	Appendorf	Germany
Tip PCR(2-20)µl	Promega	USA
Tip(2-20) µl	Promega	USA
Tip(5-10-20-100-1000) µl	Promega	USA
Ultraviolettrans illumination	Major science	Taiwan
Vortex mixer	Stuart	United kingdom
Water bath	GFL	Germany

2.1.3. Chemical and Biological Materials:

The chemical and biological materials used in this study are listed in Table (2-2):

Table (2-2): Chemical and biological materials used with their remarks

Type of material	Manufacturing company	Origin
Agar-agar	Himedia	India
Agarose gel	Promega	USA
Barium Chloride (BaCl)	BDH	UK
Bromophenol blue	Promega	USA
Catalase	Himedia	India
Conga-red stain	Himedia	India
Distel water	Himedia	India
Ethanol (70%)	Himedia	India
Ethanol (96%)	BDH	UK
Ethidium bromide	Promega	USA
Glycerol	Sigma	USA
Gram stain	Himedia	India
Isoproponal (99%)	Himedia	India
Naldixic acids	Himedia	India
Normal saline	Biomerieux	France
Nuclease free water (12.5)ml	Promeda	USA
Oxidase solution	Himedia	India
RNase (3)µl	Promega	USA
Sucrose	Himedia	India
Tris EDTA buffer 10X	Promega	USA

2.1.4. Molecular DNA Markers:

The molecular markers used in this work are listed in Table (2-3):

Table (2-3): Molecular markers sizes as DNA marker for electrophoresis

DNA markers	Description	Manufacturing company Origin state
1500 base pairs ladder	100 base pairs the ladder consist of 11 double strand DNA fragment with size of 100 base pairs and last band 500 base pairs present at triple the intensity of other fragment and serve as reference all other fragments appear with equal intensity on gel.	Promega USA

2.1.5. Culture Media:

The culture media used in this work were listed below in table (2-4):

Table (2-4): Culture media used with their remarks

Medium	Manufacturing company	Origin state
Blood agar	Oxoid	UK
Brain heart infusion agar	Oxoid	UK
Cetremide agar	Himedia	India
Chromogenic agar	Condo	Spain
Congo red agar	Himedia	India
Lauria bertani broth	Himedia	India
MacConkey agar	Oxoid	UK
Muller Hinton agar	Oxoid	UK
Nutrient agar	Oxoid	UK
Nutrient broth	Oxoid	UK

2.1.6. Antibiotic Discs:

The antibiotic discs were used in the antibiotic susceptibility test are provided by Mast Group company United Kingdom is listed in Table (2-5):

Table (2-5): The antibiotic discs used with their remarks

Class	Subclass	Agent	Symbol	Concentration µg/ml.		Diameter inhibition zone (mm).	
					R	I	S
Penicillin	Ureidopencillin	Mezlocillin	MEZ	75	≤15	-	≥16
Penems	Carbapenems	Imipenem	IMI	10	≤13	14-15	≥16
		Meropenem	MEM	10	≤13	14-15	≥16
Cephens	Cephalosporin III	Ceftazidium	CAZ	30	≤14	15-17	≥18
	Cephalosporin IV	Cefepim	CPM	30	≤14	15-17	≥18
Aminoglycosides	Aminoglycosides	Gentamicin	GM	10	≤12	13-14	≥15
		Amikacin	AK	30	≤14	15-16	≥17
		Tobramycin	TOB	10	≤12	13-14	≥15
Quinolones	Fluoroquinolones	Ofloxacin	OFX	5	≤12	13-15	≤16
		Ciprofloxacin	CIP	5	≤15	16-20	≥21
		Levofloxacin	LEV	5	≤13	14-16	≥17
Monobactam	Monobactam	Aztreonam	AZT	30	≤15	16-21	≥22
β -Lactam	Blactamase inhibitor combination	Piperacillin-Tazobactam	PTZ	100-10	≤17	-	≥18
β -lactam	Blactamase inhibitor combination	Ticarcillin-clavulanic acid	TIM	75-10	≤14	-	≥15

Standard values of CLSI (2013): R=Resistant, I=Intermediate, S=Sensitive.

2.1.7. Diagnostic Kit:

2.1.7.1. Mini API 20NE System:

Mini API 20NE system was provided by Biomerieux Company (France), which used in this study.

2.1.7.2. DNA Extraction and Purification Kit:

DNA extraction and purification kit were provided by Promega (Wizard Genomic DNA purification Kit, USA). The kit contents included in Table (2-6) and which stored at 4˚C.

Table (2-6): DNA extraction kit used in this study

Solution	Volume
Cell Lysis Solution	100 ml
DNA Rehydration Solution	50 ml
Nuclei Lysis Solution	50 ml
Protein Precipitation Solution	25 ml
RNase Solution	250 µl

2.1.7.3. Pure Yield Plasmid Mini Presystem:

Pure yield mini prep system Kit was provided by Promega (USA). The kit contents included in Table (2-7) and which stored at 4˚C.

Table (2-7): Pure yield plasmid mini presystem used in this study

Solution	Volume
Cell Lysis buffer (Blue)	11 ml
Column Wash Solution	9 ml
Elution Buffer	10 ml
Endotoxin Removal	20.5 ml
Neutralization solution	36 ml
Pure Yield™ Collection Tubes	2×50
Pure Yield™ Minicolumns	2×50

2.1.7.4. PCR Detection Kit:

PCR detection kit was provided by Promega (USA) which includes GoTaq® Hot start colorless Master Mix, 2× include: Hot start DNA polymerase is supplied in 2 ×colorless GoTaq® Reaction buffer (PH8.5), 400 µm dATP, 400 µM dGTP, 400 dTTP,400 dctp, 4mM Mgcl Nuclease free water biological source for GoTaq® Hot start colorless polymerase: the enzyme is derived from bacteria, the antibody is derived from murine cell culture.

2.1.7.5. Oligonucleotide Primer Sequences Used for PCR Amplification:

1. Specific primer sequences of *16SrRNA* gene were used to confirm the identification of *P .aeruginosa* by PCR according to Spilker *et al*. (2004) that provided by Alpha DNA company (Canada) and prepared according to the instructions of supplied company, as shown in Table (2-8):

Table (2-8): Oligonucleotide primers sequences of *16SrRNA* gene in *P. aeruginosa* used for confirmatory identification

Name of primer	Primer sequences (5́ → 3́)		References	Product size
16SrRNA	F	GGGGGATCTTCGGACCTCA	(Spilker *et al*., 2004)	956 bp
	R	TTA GCT CCA CCT CGC GGC		

2. Specific primer sequences of *exoA* gene of *P. aeruginosas* were used to detect this gene according to Rosario *et al*. (2012) which provided by Alpha DNA(Canada) prepared according to the information of supplied company, as show in Table (2-9):

Table (2-9): Oligonucleotide primer of *exoA* gene in *P. aeruginosa* used as virulence gene

Name of primer	Primer sequences (5́→3́)		References	Product size
toxA	F	TCAGGGCGCACGAGAGCAACGAGA	(Rosario *et al.*, 2012)	454 bp
	R	GACAGCCGCGCCGCCAGGTAGAGG		

3. Specific primer sequences of *16SrRNA* gene sequence in *P. aeruginosa* was used according to Jiang *et al*. (2006) for partial DNA sequencing to detect the phylogeny of tested isolates which are provided by Alpha DNA company (Canada) and prepared according to the information of supplied company, shown in Table (2-10):

Table (2-10): Oligonucleotide primer sequences *16SrRNA* gene of *P. aeruginosa* used for phylogeny.

Name of primer	Primer sequences (5́→3́)		References	Product size
16SrRNA	F	AGA GTT TGA TCM TGG CTC AG	(Jiang *et al.*, 2006)	1504 bp
	R	CGG TTA CCT TGT TAC GAC TT		

2.2. Methods:

2.2.1. Specimens Collection:

Five hundred specimens from urine 125, wounds 125, burns 75, sputum 50, ear swab 100 and diabetic foot 25 were collected from inpatients of many hospitals during November, 2015 to April, 2016 in Baghdad city. The types and the numbers of clinical specimens were distributed as shown in Table (2-11). All specimens were collected by clean sterilized cotton swabs or containers under supervision of clinical consultant physicians. Burn and wound swabs were taken after three and six days of burn or wound patients from the pus of the burned or wound area in the morning before the bathing of affected area (before hydrotherapy). Each swab was placed in a sterile tube with transport media till reaching the laboratory to be inoculated on culture media (blood agar, MacConkey agar, cetremide agar, nutrient agar and chromogenic agar) and incubated aerobically for (24-48) hours at 37C˚. Urine specimens were taken (by standard mid-stream clean catch method) from patients with UTI. Culture result of urine samples were interpreted as being significant and insignificant bacteriuria, according to the standard microbiology procedure. A growth of ≥10 colony forming units/ ml was considered as significant bacteriuria. Sputum specimens was taken in the early morning from patient and examined the pus cell which must be more than 20 in high power flied and the number of epithelial cells must be lower than 15 under high power flied microscopically. Ear swabs were taken from the patient with chronic otitis media and transferred with transport media and immeadiely culture for isolation. (Collee *et al*., 1996).

Table (2-11): Distribution of clinical collected specimens

Clinical specimens	No. of specimens
Diabetic foot	25
Sputum swabs	50
Ear swabs	100
Urine specimens	125
Burns swabs	75
Wounds swabs	125
Total	500

2.2.2. Preparation of Buffers, Solutions, Reagents and Stains:

2.2.2.1. Buffers and Solutions:

All the buffers and solutions were prepared according to Sambrook and Russell, (2001).

● **Tris-borate EDTA buffer (TBE-10X):**

This buffer was prepared by dissolving 3.8 gm Tris–HCl, 2.7gm boric acid and 2ml EDTA (0.5M) in 50 ml of distilled water, the pH was adjusted to 8, autoclaved, and then kept at 4˚C until used in electrophoresis.

● **TBE (1X)**:

This solution was prepared by mixing 10ml of stock TBE-10x with 90ml of distilled water, then kept at 4˚C until used in electrophoresis.

2.2.2.2. Reagents:

● **Oxidase and Catalase**:

Those reagents were provided by Himedia Company which used in this study.

2.2.3. Preparation of Media:

2.2.3.1. Ready-Prepared Media:

Ready-made media including Brain heart infusion broth, MacConkey agar, Mueller Hinton agar, Nutrient agar, Nutrient broth, Pseudomonas chromogenic agar and Lauria bertani broth were prepared according to the instructions of the manufacturing companies, which are usually fixed on the container of the media. They were sterilized according to the procedures that mentioned in Section 2.2.4 they were kept at 4°C until being used.

2.2.3.2. Laboratory Prepared Media:

2.2.3.2.1. Blood Agar:

Blood agar base was dissolved 21.25 gm. in 500 ml distilled water and sterilized. Then cooled to 45°C and 5% of sterile defibrinated blood was added. After, mixing well, it was poured into sterile petridishes, cooled to 37°C and left to solidify at 25°C. This medium is suitable for isolation and cultivation of bacteria, as well as it was used for the detection of hemolytic bacterial activity (Brook *et al*., 2013).

2.2.3.2.2. Cetrimide Agar Base:

Cetrimide agar was prepared according to instruction of company by dissolving 46.7 gm. in 1000 ml of distilled water containing 10 ml glycerol and then heat to boiling until the constituent is completely dissolved. The media was sterilized by autoclaving at 15 bar/in² pressures, 121°C for 15 min. The media was cooled to 50°C and aseptically add sterile rehydrated content of one vial of naldixic selective supplement which make this media selective isolation of *P. aeruginosa,* then the media was mix well and poured into sterile petridish (Juber, 2015).

2.2.3.2.3. Congo Red Agar:

It was prepared by dissolving of 37g brain-heart infusion broth, 50g sucrose, and 15g agar-agar in 900 ml of distilled water then sterilized by autoclaving. Congo red stain was prepared by dissolving 0.8g in 100 ml of distilled water and sterilized by autoclaving then added to the above media cooling it to 55°C . The medium was poured in a sterile petridish. This media used to detect on the biofilm production (Al-Musawi, 2014).

2.2.4. Sterilization Methods:

The solutions which were affected by high temperature were sterilized by filtrations through millipore filters (0.22 µl.), the sterilization of glasses depended on dry heat by electric oven at 180˚C for 2 hours and micropipettes tips and eppendorf tubes were all sterilized by UV light **(**Russell**,** 2004).

2.2.5. Reference Strain:

Table (2-12) shows the type of reference strain of *P. aeruginosa* that used for comparison in all lab tests.

Table (2-12): Reference strain of *P. aeruginosa* that used in this study.

Strain name	Laboratory identification	Key characteristics	Source
Pseudomonas aeruginosa	American type culture collection (ATCC ®27853)	Susceptible to all antipseudomonal agent	Central Public Health Laboratory in Baghdad

2.2.6. Maintenance of bacterial isolates:

Short-Term Preservation:

Pure isolates of bacteria were maintained for short period (maximum two weeks) by growing them on nutrient agar tube. The plates were tightly wrapped with parafilm and stored at 4˚C. (Benson, 2001).

2.2.7. Isolation and Identification of *P. aeruginosa*:

Pseudomonas aeruginosa were diagnosed according to procedures recommended by MacFaddin (2000). The identification of bacteria from clinical samples was performed as following:

2.2.7.1. Sample Culturing:

All specimens were cultured on MacConkey agar, blood agar, *Pseudomonas* chromogenic agar and cetrimide agar as selective media for. *P. aeruginosa* and all culture media were incubated aerobically at 37°C for 18-24 hour. The colonies were identified according to their staining ability, shape, color, size, odor, and edge, production of pigments, swarming movement transparency and hemolysis pattern (McFaddin, 2000).

2.2.7.2. Microscopic Examination:

One isolated colony from selective media was transported to a microscopic slide, fixed then stained with Gram stain to determine the cell shape, Gram reaction and arrangement by loopful of isolated colony was mixed in the water and spread out, the smear was air dried then heat-fixed by passing the slid rapidly through the burner flames three times. The smear was stained with crystal violet, treated with iodine to fix the stain, decolorized with mix (70% alcohol+30% acetone), and counter stained with safranine, then examined under light microscope (Morello *et al*., 2006).

2.2.7.3. Biochemical Tests:

- **Oxidase test**:

A piece of filter paper placed in a clean Petridish and 2-3 drops of freshly prepared of a solution 1.0% tetra methyl-p-phenylenediamine dihydrochloride were added to the filter paper. A single colony from tested bacteria was transferred to the filter paper and rubbed onto the reagent with an applicator wooden stick. The positive result was indicated by blue purple color formation within 10-15 seconds (Harley and Prescott, 2002).

- **Catalase tests:**

A drop of catalase reagent 3.0% H2O2 was placed on a glass slide. A single colony of the tested bacteria was mixed with the reagent on the slide, and positive results were indicated by air bubbles formation (Harley and Prescott, 2002).

- **Growth at 42°C:**

Single colony of each isolate was streaked separately on nutrient agar medium and incubated at 42°C for 24 hrs. *P. aeuroginosa* colonies have the ability to grow at 42°C (MacFaddin, 2000).

2.2.8. Confirmatory Identification by Using API 20 NE System:

Strip system is a standardized identification system for identification of non-fastidious, enteric Gram negative rods one of these *P. aeruginosa*. The system consists of 20 micro tubes containing dehydrated substrates packaged together. These micro tubes are inoculated with a suspension of a single pure microorganism and incubated for 24 hour at 37°C. During incubation, metabolism produces color changes that are either spontaneous or by the addition of reagents, the results of the tests are read and the identity of the organism is determined. The positive and negative results obtained for each

test are used to generate a seven-digit code number. The company's database then uses this number to determine the identity of the microorganism. The biochemical tests included in this system are show in appendix (2-2).

API 20 E reagent composed of the following reagents:

1. Kovac's reagent (IND).
2. Voges-Proskauer reagent, which is composed of the following reagent:

a. Vp1 (40.0 % Potassium hydroxide).

b. Vp2 (6.0 % alpha-naphthol).

3. Ferric chloride 10.0 % (TDA).

Identification steps of *P. aeruginosa* were performed through the following:

1. The strip was prepared by dispensing five-milliliter of water into the wells of the tray in order to provide humidity during incubation.
2. The inoculum was prepared by picking a single pure isolated colony up from selective media. This colony was suspended in a test tube containing 5 mL of 0.85 % NaCl and mixed to obtain 0.5 McFarland (1.5×10^8 CFU/mL).
3. Inoculation of the strip was performed according to the manufacturer instructions, where both the tube and cupule part of CIT, VP and GEL tests were filled with the bacterial suspension. Other tests, only the tubes were filled. The tests LCD, ODC, URE and H_2S were overlaid with mineral oil to create anaerobic conditions.
4. After inoculations, the plastic lid was placed on the tray and was incubated at 37°C for 24 hour.
5. After incubation, the above mentioned reagents were added to the corresponding micro tubes:

A. One drop of VP1 and VP2 reagents to (VP) micro tube and after 10 minute, the result was recorded immediately.

B. One drop of 10% ferric chloride to (TDA) micro tube then the result was recorded immediately.

C. One drop of Kovac's reagent to the (IND) micro tube.

6. Finally, the results were recorded and compared to that of identification table included with kit, and the identification of the isolates was performed using Analytical Profile Index (API).

2.2.9. Antibiotics Susceptibility Test:

The test was done according to Kirby-Bauer method as follows:

(a) Preparation of Mueller-Hinton agar: Mueller-Hinton agar was prepared by following the procedure mentioned in section (2.2.4.1)

(b) Preparation of inoculums: to prepare the inoculums, pure colonies from overnight culture on nutrient broth and isolates were transferred to a tube contain 5.0 mL of normal saline to obtain suspension with 1.5×10^8 CFU/mL by adjusting to Dosimeter system to give equivalent suspension. The antimicrobial susceptibility testing was done by the agar discs diffusion method depending on the (CLSI, 2013)

(c) Streaking of test plates**:** A sterile cotton swab was dipped into the adjusted suspension, then rotated several times firmly on the inside wall of the tube an above the fluid level to remove excess inoculums from the swab. The surface of a Muller-Hinton agar plates was streaked with the swab. The streaking was repeated two more times and the plates was rotated approximately 60 (degree) each time to ensure an even distribution of inoculums. As a final step the rim of the agar was swabbed. The Petridishes were allowed to dry for 15-20 min. at room temperature before the application of the discs.

(d) Application of discs: The antimicrobial discs were dispensed onto the surface of the inoculated agar plates; the discs were pressed gently to ensure complete contact with the agar surface by helping flame and cooled forceps while the plates were inverted and placed in an incubator at 37˚C form 16-18 hours incubation clear, inhibition zones were calculated in mm by using transparent ruler, the diameter of the inhibition zones for individual

antimicrobial agent was translated in terms of Sensitive (S), Intermediate (I) and Resistant (R) categories by comparison with the standard inhibition zone as shown in Table (2-5) (CLSI, 2013).

2.2.10. P CR Assay:

2.2.10.1. Isolating Genomic DNA from *P. aeruginosa*:

The following procedure was done according to the instructions of manufacturing company:

- Avolume 1ml of an overnight culture was added to a 1.5ml micro-centrifuge tube, centrifuged at 16,000 × *g* for 2 min to pellet the cells and removed the supernatant.

- Avolume 600µl of nuclei lysis solution was added. Gently pipet until the cells are suspended incubated at 80°C for 5 min to lyse the cells and then cooled to room temperature.

- Avolume of 3µl RNase solution was added to the cell lysate. Invert the tube 2–5 times to mix. Incubated at 37°C for 30 minutes. Cooled the sample to room temperature, A volume 200µl of protein precipitation solution was added to the RNase-treated cell lysate vortex vigorously at high speed for 20 seconds to mixed the protein precipitation solution with the cell lysate.

- The sample was incubated on ice for 5 minutes, centrifuged at 13,000–16,000 × *g* for 3 min and then transferred the supernatant containing the DNA to a clean 1.5ml micro-centrifuge tube which contained on 600µl of isopropanol at room temperature then gently mixed by inversion until the thread-like strands of DNA form a visible mass then centrifuge at 13,000–16,000 × *g* for 2 min.

- Carefully the supernatant was poured off and drained the tube on clean absorbent paper. A volume 600µl of room temperature 70% ethanol was

added and gently inverted the tube several times to wash the DNA pellet then centrifuged at 13,000–16,000 × *g* for 2 minutes and drained the tube on clean absorbent paper and allow the pellet to air-dry for10–15 minutes.

- A volume 100µl of DNA rehydration solutions was added to the tube and rehydrates the DNA by incubating at 65°C for 1 hour was periodically mixed the solution by gently tapping the tube. Alternatively, rehydrate the DNA by incubating the solution overnight at room temperature or at 4°C and then stored the DNA at 2–8°C.

2.2.10.2. Isolation of Plasmid DNA from *P. aeruginosa*:

The following procedure was performed at room temperature according to instructions of manufactory Company:

- A total of 600µl of bacterial culture grown in Lauria bertani broth medium was transferred to a 1.5 ml microcentrifuge tube. A 100µl of cell lysis buffer was added, and mixed by inverting the tube 6 times. The solutions should change from opaque to lysis clear blue, indicating complete.

- A volume 350µl of cold (4–8°C) neutralization solution was used and mixed thoroughly by inverting the tube the sample will turn yellow when neutralization is completed, and will form a yellow precipitate, inverted the sample an additional 3 times to ensure completed neutralization, centrifuged at maximum speed in a micro-centrifuge for 3 minutes and transferred the supernatant (~900µl) to a pure yield™ mini- column, do not disturb the cell debris pellet. For maximum yield, transfer the supernatant with a pipette.

- The mini-column was placed into a pure yield™ collection tube, and centrifuged at maximum speed in a micro-centrifuge for 15 seconds then discarded the flow through, and placed the mini-column into the same pure

yield™ collection tube and A volume 200µl of endotoxin removal washed to the mini-column centrifuged at maximum speed in a micro-centrifuge for 15 seconds. It is not necessary to empty the Pure Yield™ Collection Tube.

- A volume of 400 µl of column wash solution was added to the mini-column, centrifuged at maximum speed in a micro centrifuge for 30 seconds then transferred the mini-column to a clean 1.5ml microcentrifuge tube, then added 30µl of elution buffer directly to the mini-column matrix, let stand for1 minute at room temperature. Then centrifuged at maximum speed in a micro centrifuge for 15 seconds to elude the plasmid DNA. Sealed the micro centrifuge tube, and stored eluted plasmid DNA at –20°C

2.2.11. Estimation of DNA Yield and Purity

The extracted genomic DNA was checked by using Nanodrop spectrophotometer to estimate the concentration and purity of extracted DNA through reading the absorbance at (260 /280 nm).

2.2.12. Detection of Specific Genes for *P. aeruginosa* by PCR Method:

2.2.13.1. Preparing the Primers:

An oligonucleotide primer was prepared depending on manufacturer's instruction by dissolving the lyophilized sample with nuclease free water after rotating down briefly. Working primer tube was prepared by diluting with nuclease - free water. The final pico- moles depended on the procedure of each primer.

2.2.13.2. PCR Programs for *16SrRNA* and *exoA* Genes Detection:

PCR was used for detecting *P. aeruginosa*, the mixture 40µl consisted of 20µl.of GoTaq Hot Star master mix (which contains Taq DNA polymerase,

dNTPs, $mgcl_2$ and reaction buffer at optimal concentration for efficient amplification of DNA templates by PCR), 5µl DNA template (20ng.), 1.5µl of each forward and reverse primer (10 pmol.) 12 µl, of nuclease free water to complete the amplification mixture volume, The PCR tubes containing the mixture were transferred to preheated thermo cycler and started the program as in the Table (2-13):

Table (2-13): PCR amplification program of *16SrRNA* gene used for confirmatory identification of *P. aeruginosa* (Alornaaouti, 2015).

Stage		Steps	Temperature (Cº)	Time	No. of cycles
First		Initial Denaturation	95ºC	120sec	1
Second	I	Denaturation	95ºC	20sec	25
	II	Annealing	58ºC	20sec	
	III	Extension	72ºC	40sec	
Third		Final Extension	72ºC	60sec	1

Table (2-14): PCR amplification program of *16SrRNA* gene used for only DNA sequencing in *P. aeruginosa* (Tamura *et al*., 2013)**.**

Stage		Steps	Temperature (C˚)	Time (sec)	No. of cycles
First		Initial Denaturation	95˚C	4min	1
Second	I	Denaturation	95˚C	30sec	30
	II	Annealing	55˚C	30sec	
	III	Extension	72˚C	90sec	
Third		Final Extension	72˚C	9min	1

Table (2-15): PCR amplification program of *exoA* genes detection in *P. aeruginosa* as virulence gene (Juber, 2015).

Stage		Steps	Temperature (C˚)	Time (min)	No. of cycles
First		Initial Denaturation	95˚C	4 min	1
Second	I	Denaturation	95˚C	30 sec	30
	II	Annealing	60˚C	30 sec	
	III	Extension	72˚C	105 sec	
Third		Final Extension	72˚C	9 min	1

2.2.14. Agarose Gel Electrophoresis:

Agarose gel was prepared by dissolving 1 g for chromosomal DNA or 1.5 g for plasmid DNA of agarose powder in 100 ml of (1X) TBE buffer (pH 8) on hot plate with magnetic stirrer and magnetic capsule was added, left until boiling and becoming clear, allowed to cool to 50˚C, and 5 µl ethidium bromide was added, (Sambrook and Russell, 2001).

2.2.14.1 Casting of the Horizontal Agarose gel:

The tape was placed across the end of the gel tray. The comb was fixed at one end of the tray for making wells used for loading DNA samples. The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 minutes. Then, the comb was removed gently from the tray and the tape was also removed from the ends of the tray. The agarose gel was fixed in electrophoresis chamber which was filled with TBE buffer (1X) that had covered the surface of the gel (Sambrook and Russell, 2001).

2.2.14.2. Loading and Running DNA in Agarose Gel:

Five µl of each DNA template was transferred to eppendorf tube, 3µl of loading dye was added to the tube and the mixture was loaded into the wells in agarose gel with the addition of loading buffer and DNA Ladder (100bp) as standard in electrophoresis. The electric current was allowed at 100 volt for 10 min and then 80 volt for 1 hour to detected the *16srRNA*gene (956 base pairs amplicon), DNA extraction and plasmid extraction while used 90 min at 100 volt/50 mAmp to detected, *16srRNA*gene (1504 base pairs amplicon) and *exoA.* After complete time read the results on UV transilluminater was used for the observation of DNA bands, and the gel was photographed.

2.2.15. DNA sequencing and Phylogenetic Analysis by *16SrRNA* Gene:

Partial *16SrRNA* gene sequences were obtained from the PCR amplicon generated with the same primers mentioned in Table (2-10) for *P. aeruginosa*. The PCR products of *16SrRNA* (1504bp) were sent to Micro gene company in South Korea for sequencing by their ABI 3730 genetic analyzer. DNA sequencing compared with those available in the Gene Bank data base by using basic alignment search tool, BLAST of NCBI to identify wither they aligned with closely related organisms.

Percentage nucleotide identity and pairwise uncorrected (p-distances) were calculated for *16SrRNA* sequences of clinical strains of the *P. aeruginosa* examined by using sequence an alignments explorer CLUSTAL (Felsenstein, 2006).

Phylogenetic analysis was carried out using the software MEGA version 6, using the neighbour joining method to reconstruct the phylogenetic tree with option of complete detection of gaps (Tamura *et al*., 2013).

2.2.16. Statistical Analysis

The results were analyzed statistically by mean, standard deviation and percentage of frequencies obtained Percentages of similarities for partial *16SrRNA* sequences obtained in this study were calculated by the FAST method. Multi alignments were obtained with alignments Explorer CLUSTAI (Felsenstein, 2006).

1.2. Literatures review:

1.2.1. *Pseudomonas aeruginosa*:

The origin of the word, Pseudomonas, means false unit, from the Greek pseudo meaning false and Monas, a single unit from Greek. The species name *aeruginosa* is a Latin word meaning verdigris (copper rust), another assertion is that the word may be derived from the Greek prefix ae- meaning "old or aged", and the suffix ruginosa means wrinkled. This also describes the blue-green bacterial pigment seen in laboratory cultures of the species. This blue-green pigment is a combination of two metabolites of *P. aeruginosa,* pyocyanin (blue) and pyoverdine (yellow-green), the derivations of pyocyanin and pyoverdine are of the Greek, with pyo- meaning (pus), cyanin meaning (blue) and verdine meaning (green). Pyoverdine in the absence of pyocyanin is a fluorescent-yellow color (Priya and Anitha, 2013).

P. aeruginosa was first isolated as a pure culture by Gessard in 1882 from wounds with blue-green discoloration and called at that time *Bacillus pyocyaneas* then called *Pseudomonas pyocyaneas* and finally named *P. aeruginosa* (Lister *et al.,* 2009) Shooter *et al.* (1971) were able to isolate *P. aeruginosa* from prepared foods in hospitals entrees and refrigerated meat and vegetables, Also pointed both of Wanderink and Dunn, (1995) to any disruption of local or general in the body's defence factors may cause infection to by those bacteria, so *P. aeruginosa* isolates often from contaminated burns, cutaneous ulcers, wounds of operations, the middle ear and urinary tract.

P. aeruginosa is a Gram-negative bacillus, straight or slightly curved rods, non-spore forming, capsulate; it is usually motile by one polar flagellum (monotrichous), measuring about 1-5 µm long and 0.5-1.0 µm wide. It occurs as single bacteria, in pairs and occasionally in short chains possess mucous layer due to material alginate slime layer as well as, multi-layers of extracellular polysaccharide (Deabreu *et al.*, 2014).

It is strict aerobic, but can grow anaerobically if nitrate or arginine is available as a terminal electron acceptor. The bacterium is capable of utilizing a wide range of organic compounds as food sources; it grows on minimal carbon and nitrogen sources, including simple media and moist surfaces. It was often observed growing in distilled water which was evidence of its minimal nutritional needs (Bhasin *et al*., 2015).

P. *aeruginosa* has a wide growth temperature range, optimum growth at 37°C. Slower growth rates are seen at 4˚C. *P*. *aeruginosa* is distinguishable from other clinically *Pseudomonas* spp. by its capability for growth at 42˚C. They also grow well at pH range 6.6-7.0. It was tolerant to a wide variety of physical conditions, including temperature and pH. Also it was resistant to high concentrations of salts and dyes. It is typically given a positive result to the oxidase test and catalase. It does not ferment carbohydrates, but many strains oxidize glucose (Moore and Flaw, 2011).

The bacterium is ubiquitous in soil, variety of aqueous solutions, including disinfectants, soaps, eye drops, as well as sinks and respiratory equipments, some strains hemolysis blood completely (β-hemolysis) by producing hemolysis toxin. On MacConkey agar, it shows yellow pale color because it is non-fermented lactose. *P. aeruginosa* forms smooth round colonies and irregular edge with a fluorescent greenish to yellow pigment pyoverdine. It also produces the non-fluorescent bluish pigment pyocyanin, which diffuses into the agar. Many types of media may be used for selective isolation of *P. aeruginosa* like cetrimide agar which promotes pyocyanin, pyoverdine, and pyoyrubin red color and pyomelanin black color production. (Brooks, *et al.*, 2013).

According to Bergey's Manual of Systematic Bacteriology *P. aeruginosa* belong to Kingdom; Bacteria; phylum; proteobacteria; Class Gamaproteobacteria; Order; Pseudomonadales; Family; Pseudomonadaceae; Genus: *Pseudomonas*; and species: *aeruginosa*. (Todar, 2011). The family

Pseudomonadaceae is classified into five groups based on rRNA / DNA homology and common culture characteristics, homology groups based on rRNA-DNA homology studies. The first group (rRNA group I) involve three subgroups which include fluorescent group (*P. aeruginosa*, *P. fluorescens* and *P. putida*); stutzeri group (*P. stutzeri* and *P. mendocina*) and alkaligenes group (*P. alkaligenes*, *P. pseudoalcaligenes* and species of group1) (Japoni *et al*., 2009).

1.2.2. Role of *P. aeruginosa* in Nosocomial Infection:

Pseudomonas aeruginosa, is an extremely versatile Gram-negative bacterium capable of thriving in a broad spectrum of environments, and this perform main problems to workers in the field of doctors and nurses (Juhas, 2015).

P. aeruginosa is an opportunistic human pathogen that is a causative agent hard-to-eradicate nosocomial infection. cancer, burn, cystic fibrosis and intensive care unit patients with mechanical ventilation are amongst those with the highest risk of infected by *P. aeruginosa* (Cramer *et al*., 2012), and ability to grow in moist conditions with simple nutrients and because of its ability to resist the antibacterial agents and disinfectants, is commonly found in various places of hospital environment including sinks, drains, taps, food, water, pharmacy preparations, contaminated hospitals equipments, mattresses and cleaning materials (mops, brushes). It grows in liquid and antiseptics such as quaternary ammonium compounds (Yaslianifard *et al*., 2012).

P. aeruginosa forms biofilm with extreme tolerance to antibiotics in nosocomial infections, such as pneumonia and surgical site infections, prompting the Centers for Disease Control and Prevention to classify *P. aeruginosa* under 'serious'-threat level, therefore continuous and careful monitoring of these objects and sites is necessary to control infections in hospitalized patients. Regular practice of environmental survey and suitable

control measures help to reduce hospital acquired infections considerably (Sharma *et al*., 2014).

1.2.2.1. Nosocomial Infections:

Nosocomial infections, also called "hospital-acquired infections", are infections acquired during hospital care by a patient who was admitted for a reason other than that infection or other health care facility in which the infection was not present or incubating at the time of admission, infections occurring more than 48 hours after admission, are usually considered nosocomial infection (Khan, 2009).

Frequency of nosocomial infection occurs world-wide and they are important contributors to morbidity and mortality. They will become even more important as a public health problem with increasing economic and affects both developed and resource-poor countries. But major challenge for low and middle income countries developing which have limited health care resources, infections acquired in health care settings are among the major causes of death and increased morbidity among hospitalized patients (Voirin *et al*,. 2009).

There are two forms of nosocomial infections: 1. Endogenous infections, (self-infections, or auto-infections) can be caused by microorganisms already present in the patient's skin and mucosa present in the patient at the time of admission to hospital but there are no signs of infection. The infection develops during the stay in hospital as a result of the patients altered resistance.

2. Exogenous infections, cross-contamination followed by cross-infection: during staying in hospital the patient comes into contact with new infective agents, by microorganisms transmitted from another patient or from surrounding environment which becomes contaminated and subsequently develops an infection (Percival *et al.*, 2015).

1.2.2.2. Predisposing Factors of *P. aeruginosa* in Nosocomial Infections:

The main factors that effected the development of nosocomial infection involve: firstly patient susceptibility: which is important factors that influencing the acquisition of infection include: age, immune status, underlying disease and diagnostic and therapeutic interventions. The extremes of life-infancy decreased resistance to infection with opportunistic pathogens. Secondly, the bacterial agent which include, the intrinsic virulence and new microorganisms (Sharma *et al*., 2014). And third environmental factor which include health care settings are environment where infected persons are at increased risk of infection congregate. Whereas patients with infections or carriers of pathogenic microorganisms are potential sources of infection for patients and staff. In addition to the increasing numbers and crowded conditions of people in hospitals, with frequent transfers of patients from one unit to another and number of patients highly susceptible to infection in one area, all contribute to the development of nosocomial infection (WHO, 2002).

1.2.2.3. Nosocomial Outbreak of *P. aeruginosa*:

An outbreak is defined as an unusual or unexpected increase of cases of a known nosocomial infection or the emergence be identified and promptly investigated because of cases of a new infection (WHO**,** 2012). Nosocomial outbreaks are a major problem for health care institutions due to increased morbidity and mortality for the affected patients. The containment and control of these outbreaks costs substantial amounts of funds and resources, especially when left unnoticed or untreated (Ibrahim, 2016).

Identifying an outbreak early important to limit transmission among patients by health care workers or through contaminated materials. A potential problem may be initially identified by nurses, physicians, microbiologists, or

any other health care worker, appropriate investigations are required to identify the source of the outbreak, and to implement control measures. The control measures will vary depending on the agent and mode of transmission, but may include isolation procedures or improvements in patient care or environmental cleaning (WHO**,** 2012**).**

Nosocomial outbreaks of *P. aeruginosa* have been reported in surgical wounds, causing post-operative wound infections. Wound infections caused by MDR *P. aeruginosa* have been associated with high morbidity and mortality rates worldwide (Ranjan *et al*., 2010).

In addition, *P. aeruginosa* can disseminate from the initial infection site and enter the blood stream, causing septicemia (Jombo *et al*., 2010).

In cystic fibrosis early infections with *P. aeruginosa* can be transient, and can clear spontaneously, but colonization with *P. aeruginosa* usually occurs by the time when patients reach their teenage year. This important and characteristic shift is associated with more frequent and more severe pulmonary exacerbations (PEs) that result in progressive decrements in lung functions (Sanders *et al*., 2011).

In the later stages of infection, cystic fibrosis with up to 60% of adult patients infected (United Kingdom Annual Data Report 2014). *P. aeruginosa* also dominates chronic infections in a proportion of patients with bronchiectasis (Rogers *et al*., 2014).

Urinary tract infections (UTI) caused by *P. aeruginosa* usually occur secondary to catheterization, instrumentation or surgery. Catheterization of the urinary tract is the major cause of nosocomial acquired-UTI by *P. aeruginosa* catheters are utilized by pathogens as a source of host entry, attaching to the catheter surface (Elkhatib and Noreddin, 2014).

In well-constructed biofilms furthermore, the insertion of the catheter may also disrupt mucosal epithelial layers, promoting bacterial colonization (Mittal *et al*. 2009), the bacterium is regarded as an opportunistic pathogen, primarily

causing nosocomial infections in immunocompromised patients (Brown *et al*., 2012).

However, it is capable of causing wide-spectrum infections when normal physiological function is disrupted, including damaged epithelial barriers depleted neutrophil production, altered mucociliary clearance and the use of medical devices (Engel and Balachandran, 2009).

Keratitis occurs in patients with pre-existing ocular disease, *P. aeruginosa* is the leading cause of bacterial ocular surgery and in individuals who use contact lens develops rapidly that may lead to vision loss (Suryawanshi *et al*., 2013).

P. aeruginosa is a common cause of chronic otitis media and inflammation may also cause otitis externa, including malignant otitis extern (Roland and Stroman, 2002).

Mild skin infections can occur in previously healthy people, associated With *P. aeruginosa* contamination in swimming pools, and other water sources (Guida *et al*., 2009). Follicular dermatitis caused by *P. aeruginosa* as an itchy rash with a red base and white pustules. In addition, nail diseases (e.g. onycholysis) are susceptible to colonization of *P. aeruginosa*, and is commonly referred as "green nail syndrome" (Daniel *et al*., 2011).

Paronychia infection has been associated with prolonged exposure to moist environments (e.g. swimming). McNeil *et al*. (2001) investigated an outbreak of *P. aeruginosa* infections in postsurgical patients, reporting severe onycholysis and onychomycosis in a nurses' thumbnail as the primary source.

The spine, pelvis and sternoclavicular joints are the most common sites affected by *P. aeruginosa*. It reaches in to these sites through direct inoculation of the bacteria or the hematogenous spread of the bacteria from other primary sites of infection. Risk factors include penetrating trauma, peripheral vascular disease, intravenous drug and diabetes mellitus (Todar, 2011).

P. aeruginosa causes gastroenteritis in newborn and young infants in pediatric wards, which results from contaminated milk feeds (Govan, 2007).

P. aeruginosa may infect heart valves in intravenous drug users and also prosthetic heart valves (Yilmaz *et al*., 2013). *P. aeruginosa* may cause meningitis and intracranial abscesses. Most infections result from direct spread from local structures for instance the ear, mastoid and sinuses but blood-borne spread may also occur (Chang *et al*., 2000).

Almost any opportunistic pathogen can infect burns and wounds, but one of the most common and hardest to treat is the Gram negative rod *P. aeruginosa*, which can actually color the burns or damaged tissue with its blue-green fluorescent pigments and may lead later to septicemia (Alinaqvi *et al*., 2011).

1.2.3. Ecology and Epidemiology of *P. aeruginosa:*

Pseudomonas aeruginosa disseminated in different environments such as soil, water and plants as well as, these bacteria located on the skin of humans, animals and part of normal flora in human gut. It can be found in solutions, disinfectant, respiratory equipments, foods, sinks and salts. The bacteria present in different environments because of their ability to utilize the many compounds as sources of energy, such as nitrogenous compounds and carbohydrates as a source of carbon, so do not need a complex growth factors therefore, it is very essential medically (Yitah and Essien, 2008).

P. aeruginosa groups tend to form biofilms, which are complex bacterial communities that stick to variety of surfaces including plastics, medical implant materials and tissue. They are very difficult to destroy (Brown *et al*., 2012). The spread of bacteria *P. aeruginosa* can be controlled through proper isolation of patients, use of sterile techniques and observance conditions of hygiene with careful when using respiratory devices, catheters, food and other machinery (Shahid and Malik, 2004).

Vandeale *et al*. (2005) studied the epidemiology of *P. aeruginosa* in cystic fibrosis rehabilitation center, sixty-seven isolates of *P. aeruginosa* were isolated from patient's sputum, the infection by patient-to-patient-transmission could have happened in the past, the risk of patient-to-patient transmission during the study period (63day) was relatively (10%) and the risk of persisting colonization with a new acquired strain during the study period was (4%).

1.2.4. Role of Virulence Factors in Pathogenesis of *P. aeruginosa:*

1.2.4.1. Flagella:

The first step in *P. aeuroginosa* infection is that adherence to epithelium surface is mediated by pili, flagella and alginate (Cotar *et al*., 2010).The function of flagella is not limited to motility, but also they have a role in activating host inflammatory response through Toll-like receptor 5 and in attaching bacteria to host cells a major protein constituent of the flagellar filament is the flagellin that can be classified into A and B serotypes which serve as target antigens for vaccination (Campodonico *et al*., 2010).

1.2.4.2. Pili:

Like the flagella, pili also play important roles in the bacteria-host cell interactions including the attachment. The pili in *P. aeruginosa* are type IV pili (Mattick, 2002).

1.2.4.3. Lipopolysaccharide (LPS):

Molecules are found in the outer membranes of Gram-negative bacteria with a unique chemical structure, LPS is composed of the polysaccharide O, core antigens and lipid A composed of fatty acid and phosphate groups bonded to a glucosamine disaccharide (Ernst *et al.*, 2006).

1.2.4.4. Quorum Sensing and Biofilm Formation:

Biofilms are highly-structured microbial communities attached to surfaces and enclosed in a matrix of extracellular polymeric substance (EPS). EPS

generally contains polysaccharides, proteins and extracellular DNA (Percival *et al*., 2012).

The presence of EPS allows the biofilms to contain open water channels for transport of nutrients into the biofilm and waste products out of the biofilm (Hentzer *et al*., 2001).

It has been well documented that bacteria in the biofilm are up to 1,000 times more resistant to antimicrobial agents than planktonic cells according to the National Institute of Health; more than 60% of nosocomial infections are associated with biofilms (Li and Zhang, 2007).

Well characterized model organism for biofilm study is *P. aeruginosa* that causes lung infection in patients with cystic fibrosis. The development of *P. aeruginosa* biofilms occurs in the following four stages: 1. initial and reversible attachment of free-floating cells to the surface; 2. irreversible attachment; 3. production of the EPS matrix and 4. early development of biofilm dispersion (Ma, 2014).

Extracellular DNA (eDNA) has been found important to the initial attachment of *P. aeruginosa* biofilms. Additionally, one finding suggested that eDNA is similar to chromosomal DNA based on the (PCR) and southern analysis results (Waters and Bassler, 2005).

Other mechanisms that are thought to play a role in the antimicrobial resistance acquired by certain micro-organisms within biofilms include the presence of efflux pumps, with the expression of several gene-encoding efflux pumps being increased in biofilms (Soto, 2013).

Furthermore, plasmid exchange occurs at a higher rate in biofilms, increasing the chances of developing naturally occurring and antimicrobial-induced resistance (Hausner andWuertz, 1999).

It is thought that an altered micro-environment within a biofilm, such as nutrient depletion and reduced oxygen levels, may also reduce the efficacy of antimicrobials (Francolini and Donelli, 2010).

In the last decade, the knowledge of this feature of bacterial pathogenesis has been improved. Most bacteria to communicate and react coordinated by making use of auto inducers, a system known as QS the cell population density of the developing biofilm, the gene expression of cells within the biofilm is regulated by a process known as QS (Kjelleberg and Molin, 2002).

Through this system, bacteria release chemical signals called auto inducers, which are constitutively produced and increase in concentration as the density of the biofilm increases. As the concentration of these autoinducers reaches a critical threshold, alterations in gene expression occur, leading to an array of physiological processes, including motility, sporulation and release of virulence factors necessary for survival and QS plays a role in biofilm establishment, growth and maintenance (Mangwani *et al*., 2012).

P. aeruginosa are one of the best described bacteria which QS system is linked to biofilm formation. Two systems have been described in *P. aeruginosa*: *las* , *rhl* and QS systems respond to a class of autoinducers named acyl homoserine lactones (AHLs) (Ng and Basler, 2009).

The AHL auto inducer in *las* QS system is synthesized by *LasI* and is regulated by *LasR*, a transcriptional activator protein. The AHL auto inducer in *rhl* QS system is synthesized by *RhlI* and is regulated by RhlR (Fuqua *et al*., 2001). *rhl* QS system is furthermore controlled by *las* QS system (Medina *et al*., 2003).

The study of Melaugh *et al*., (2016) showed new insight into biofilm formation and development, and reveal new factors that may be at play in the social evolution of biofilm communities.

1.2.4.5. Pigments:

1. Pyocyanin from" pyocyaneus" refers to" blue pus" which is a characteristic of supportive infections caused by *P. aeruginosa*. Pyocyanin is a blue-green pigment metabolite of *P. aeruginosa* that has been shown to have numerous

pathogenic effects such as increasing IL-depressing host-response (Brooks *et al*., 2013).

2. Pyoverdin one explanation for this role has emerged when it was found that pyoverdin regulates the secretion of other *P. aeruginosa* virulence factors, exotoxin A and an end protease and its own secretion

3. Some strains produce red pigments (pyorubin)

4. Few produce pyomelanin black pigment (Lamont *et al*., 2002).

1.2.4.6. Enzymes:

Protease IV is other crucial virulence factors; four types have been known to be secreted by *P. aeruginosa*: LasB elastase, LasA elastase or staphylolysin, alkaline protease and protease IV. These proteases are associated with virulence by enhancing the ability of *P. aeruginosa* to invade tissues and interfering with host defense mechanisms. The electrolytic activity of these enzymes is very important in pathogenesis since a number of tissues are composed of elastin (lung, vascular and ocular tissue) (Lyczak *et al*., 2000).

Protease IV causes the destruction of host proteins including fibrinogen, elastin and components of the immune system and is thought to aid in bacterial adhesion.

Also phospholipase C secreted by *P. aeruginosa* into the extracellular space through a type II secretion system, hemolytic phospholipase C targets eukaryotic membrane phospholipids and has been shown to participate in the pathogenesis of *P. aeruginosa* in inflammation and a part of the pathogenic effect of hemolytic phospholipase C may be due to surfactant inactivation, Furthermore, hemolytic phospholipase can suppress the host neutrophil oxidative burst response (Engel and Balachandran, 2009).

1.2.4.7. Toxins:

- **Exoenzyme S (Exo S),** *P. aeruginosa* produce virulence factor Exo S, which has a demonstrated role in pathogenesis (Kenneth, 2009). Exo S has ADP-

ribosylation of many proteins in cell and induces T-cell apoptosis, which is a unique T-cell mitogen and it is powerful immune stimulus that activates a large proportion of T cells, but result in delayed and reduced lymphocyte proliferation (Salva *et al*., 2004).

- **Exotoxin A (ETA),** is one of the most toxic extracellular enzymes produced by this pathogen like diphtheria toxin, it is a heat labile, 613 AA, single polypeptide chain with a MW of 66.583 Da. It catalysis the ADP- ribosylation of elongation factor 2 (EF2) leading to inhibition of protein synthesis and cell death (Yates *et al*., 2005).

ETA is the most potent toxic factor which is responsible for local tissue damage, bacterial invasion and immune suppression. ETA catalyzes ADP-ribosylation and inactivation of EF2, an essential component of the protein synthesis machinery which leads to inhibition of protein biosynthesis and cell death (Todar, 2011).

Structure ETA, is A and B fragments that mediate enzymatic and cell-binding functions, respectively Produced *in - vivo* during *P. aeruginosa* infections (Joseph *et al*., 2001).

The crystallographic structure of exotoxin A revealing tertiary fold has three structural domain and domain I located at N-terminal end of protein is required for binding ETA to target cells responsible for the receptors binding, domain II has been implicated in the translocation of ETA into the cytosol and domain III ADP-ribosyl transferase activities of protein, respectively (Bayat *et al*., 2010).

- **Type III Secretion System (T3SS)**

The bacterial type III secretion system (T3SS) is a conserved injection apparatus, allowing both plant and animal pathogens to deliver its effecter proteins directly into eukaryotic host cells to initiate a sophisticated (biochemical cross-talk) between pathogen and host, T3SSs have been described for many gram negative bacteria species, including pathogens, like *Shigella flexneri*, *Bordetella pertussis*,

P. aeruginosa and *Vibrio Cholerae*. Also (TTSS) of *P. aeruginosa* is a complex pilus-like structure allowing the translocation of effectors proteins from the bacteria, across the bacterial membranes and into the eukaryotic cytoplasm through a needle-like appendage forming a pore in the eukaryotic membrane, there are four known toxins, variably expressed in different strains and isolates, injected into host cells by *P. aeruginosa* through the TTSS: ExoY, ExoS, ExoT and ExoU (Engel and Balachandran, 2009).

1.2.5. Antibiotics Resistance in *P. aeruginosa:*

Antibiotic resistance mechanisms identified in bacteria to date making it a particularly problematic nosocomial pathogen and also one of the biggest therapeutic challenges unfortunately, selection of the most appropriate antibiotic is complicated by the ability of *P. aeruginosa* to develop resistance to multiple classes of antibacterial agents, even during the course of treating an infection (Strateva and Yordanov, 2009).

P. aeruginosa has two main mechanisms of resistance, intrinsic and acquired to antibiotics; contribute to the pathogenicity of *P. aeruginosa* it is intrinsically resistance to anti-microbial agent due to low permeability of its cell wall, efflux pump which pump out the antibiotic from cytoplasm, while acquired resistance is due to the presence metallo β-Lactamases (MBL-Ambler type B) and AmpC (Ampler type C), MBL use metal ion Zn to catalyze hydrolysis of β-lactam (Juhas, 2015). Acquired resistant where *P. aeruginosa* easily develops acquired resistance either by mutation in chromosomally-encoded genes or by the horizontal acquired gene via plasmid, transposons and bacteriophages which are responsible of antibiotic resistance (European Center for Disease Control and Prevention, 2013).

P. aeruginosa is resistant to penicillin, ampicillin, and tetracycline first and second generations of cephalosporin, sulfonamides, neomycin,

streptomycin, kanamycin, chloramphenicol, nitrofurans and trimethoprim-sulfonamide but only a few antibiotics are effective including fluorquinolones, amikacin, gentamicin and certain broad spectrum ß-lactam antibiotics such as imipenem, carbapenem, fourth generation of cephalosporin (cefepime) (Lutz and Lee, 2011).

Resistance has been found particularly high in the family of carbapenemes this has been proved for physicians worldwide, and making the control and treatment of infections (Lagatolla *et al*., 2006). To tackle this growing problem and minimize the chance of these resistance strains of bacteria spreading in hospitals and to the greater global population there is an urgent programs as well as raising awareness amongst clinicians themselves on how to best prescribe these types of drugs knowing their risk and to minimize the chance of antibiotic resistance spreading.

Also, the resistance of MDR strains may be mediated by the active export of the antibiotic out of the bacterial cell by efflux pump. Evidence from diverse bacterial genomes indicated that approximately 5 %- 10 % of genes are involved in transport, with a large proportion of them encoding efflux pump systems that allow it to be resistance to several antibiotics.

Another mechanism present in *P. aeruginosa* in the formation of permeability barriers through the membrane (e.g. imipenem) is due to diminished expression of specific OM protein. It has been shown that OM permeabilizers such as EDTA increase susceptibility to the antibiotic, indicating that the lack of OprD protein leads to a reduction of active antibiotic molecule capable of reaching the target penicillin- binding protein (Kadry, 2003).

Finally, it has been show that cytotoxicity is an important mechanism that contributes to high morbidity and mortality in *P. aeruginosa* infections. Along with mucoidy resultant from the release of alginate *P. aeruginosa* synthesis a secretory apparatus (type III) that allows it to inject toxins from their cytoplasm

into the target cell. The latter mechanism allows mucoid bacteria to lyse the host's macrophages and overcome various defense such as in the case of cystic fibrosis lung infection. (Guespin *et al*., 2008).

In a previous study, Isibor *et al*. (2013) found a high percentage of MDR strains of *P. aeruginosa* associated with diabetic wounds of patients. Resistance rate in *P. aeruginosa*, from 40 to70% of isolates responsible for ICU acquired infections being carbapenem resistant **(**Sader *et al*., 2014).

Also, *P. aeruginosa* have essential genes as antimicrobial targets 'core' essential genes encoding universal cellular functions are good antimicrobial targets due to providing a broad host range, this is not always the case, antibiotics interfering with a 'core' essential gene could also inhibit the function of the essential homologue in human cells recent analyses led to the identification of a number of general and condition-specific *P. aeruginosa* essential genes. A number of these, such as those implicated in asparagine-tRNA biosynthesis, are not essential in mammalian cells and are therefore candidate drug targets (Lee *et al.,* 2015).

Recent genome-wide analyses have also revealed a number of essential genes that are indispensable in *P. aeruginosa*, but not in other bacteria, such as *E. coli*. These include genes involved in central carbon energy metabolism and protection from reactive oxygen species (Lee *et al*., 2015). As the essentiality of these processes stems from the *P. aeruginosa* life style (e.g. the reliance of *P. aeruginosa* on respiration for energy generation), they are considered to be good targets for *P. aeruginosa*-specific antibiotics. Furthermore, the 'accessory' essential genes required for *P. aeruginosa* growth under clinically relevant conditions, such as in cystic fibrosis sputum and in the presence of antibiotics, and those indispensable for pathogenicity appear to be promising targets.

Recently described and validated examples of essential *P. aeruginosa* antimicrobial targets include tobramycin antibiotic resistance-conferring genes, resistance-nodulation-division (RND)-type efflux pumps. Recent Tn-seq circle

method-based analysis led to Identification of 117 genes essential for the growth of *P. aeruginosa* on the aminoglycoside antibiotic tobramycin (Gallagher *et al.*, 2011). In addition to the previously identified tobramycin resistance-conferring genes these include genes for a number of novel tobramycin targets, such as PAO392, PA1805, and PA4077 encode an unknown membrane protein implicated in osmotic stress tolerance, a peptidyl–prolylcis–trans isomerase (ppiD) involved in folding of the membrane and exported proteins, and the transcriptional regulator of the envelope stress response, respectively (Lee *et al*., 2009).

The study of Sheet (2012) on morphological and molecular characterization of multi-drug resistance *Pseudomonas* spp. isolated from clinical and environmental cases of neonatal and infants' nosocomial infections in Mosul showed that *Pseudomonas* spp. is a common pathogen or contaminant in hospital ecology, the *16SrRNA* gene consider as an important confirmative tool for diagnosis of *Pseudomonas* spp., in addition to pigment production and growth on cetrimide as primary diagnosis for this pathogen and The antibiotic susceptibility revealed that *Pseudomonas* isolates showed different levels of resistant to many antibiotics belong to penicillins, cephalosporins, aminogycosides, whereas the most strains were susceptible to imipenem, azithromycin, ciprofloxacin, gentamicin and norfloxacin.

Juhas (2015) provides an update on the investigation of *P. aeruginosa* essential genes special focus is on recently identified *P. aeruginosa* and their exploitation for the development of antimicrobials essential genes.

Mozes (2015) showed the antibiotic consumption may be the main driving force for maintenance of epidemic strains during non out break periods antibiotic resistance in certain situations may be more important than virulence in spread of successful clones.

Detection and determination of the antibiotic resistance patterns in *P. aeruginosa* strains isolated from clinical specimens in hospitals of Isfahan, Iran, showed the prevalence of *P. aeruginosa* strains with MDR was very high amongst the clinical samples in the major hospitals of Isfahan. This is quite a worrisome problem and makes the controlling of the strains more difficult (Sedighi *et al*., 2015).

The study of Juber (2015) on Assessment of Some Virulence Genes of *Pseudomonas aeruginosa* Isolated from Eye Infection, in Al-Qadisiyah University, that proved that *Pseudomonas aeruginosa* was the most common pathogen causes of eye infections, conjunctivitis was the most common infection among children below 10 years, PCR was a reliable technique and sensitive enough for detection of *Pseudomonas aeruginosa* virulence gene such as *exoA* gene and phospholipase C (plC) and Presence of *exoA* and plC genes in pathogenic invader (*Pseudomonas aeruginosa*) could explain the prognosis of eye tissue damages.

1.2.5.1. Efflux Pump and Plasmid Resistance Mechanism in *P. aeruginosa*:

In next two decades, both agent and class specific numerous chromosome and plasmid encoded drug multidrug efflux transporters have been described in a variety of microorganisms associated with antibiotic resistance (Osmon *et al*., 2004).

1. Efflux Pumps Systems:

All of the antibiotics have to cross the cell wall, to reach their targets. Failure of antibiotics to accumulate in the organism is due to the combination of restricted permeability of the outer membrane and the efficient removal of antibiotic molecules by efflux pumps. The outer membrane of *P. aeruginosa* is an important barrier of the penetration of antibiotics (has low permeability) (Hancock and Brinkman, 2002).

The RND-type efflux pumps, such as *MexAB–OprM*, contribute to the intrinsic resistance of *P. aeruginosa* to antibiotics by pumping antimicrobials from the periplasm to the outside of the cell (Opperman and Nguyen, 2015).

A number of efflux pump inhibitors, including phenylalanyl arginyl D13-9001, have been described; however, none has yet been tested clinically (Schuster *et al*., 2014).

2. Plasmids:

Plasmids are defined as double stranded, extra chromosomal genetic elements that replicate independently of the host cell chromosome and are stably inherited. Plasmids capable of integration into the chromosome were earlier called episomes. Plasmids differ from chromosomes in being small and coding for genes that are non-essential for the bacterial survival. Absence of plasmids doesn't kill bacterium, but their presence provides additional benefits to the bacterial cell. Plasmids vary in size; smallest plasmid is only 846 bp long and contains only one gene. Some plasmids carry more than 500 genes. The largest plasmid known carries 1,674 genes. Like chromosomes, plasmids are also closed circular and supercoiled; although certain plasmids (eg. *Borrelia*) are known to be linear, which produce ESBLs and stranded DNA materials; have been found to be useful For Amp C broad-spectrum β-lactamases have been pathogens genetic diversity and prowess as infectious agent (Brinas *et al*., 2003).

Similarly, agents and profiling pathogens for their harbored plasmids articles describing their prevalence in food animals and has been found to be very useful in epidemiological foods of animal origin have been published in recent studies, diagnosis and elucidation of mechanisms of drug years resistance (Bennett, 2008).

On the other hand, study from Iran established by Jafari *et al*. (2013) isolated an antibiotic resistant plasmid in *P. aeruginosa* resistance to third generation of cephalosporin's; cefepime and aminoglycosides. This organism is

a major concern in nosocomial infections and should therefore be monitored in surveillance studies.

1.2.6. Phenotypic and Genotypic Methods Used for Identification of *P. aeruginosa*:

1.2.6.1. Phenotyping Methods:

Successful treatment of a patient with a bacterial infectious disease requires rapid and specific identification of the causative agent, identification of bacterial pathogens by traditional methods is still a crucial element of the diagnostic process, early epidemiological studies used phenotyping methods to characterize microbial isolates, determining antimicrobial susceptibility of the organism, and biochemical testing can be laborious and time-consuming, and may prolong definitive diagnoses and treatment of the patient (Bartlett and Stirling, 2003).

Pseudomonas aeruginosa phenotyping methods include biotyping, pyocin typing, serotyping, and phage typing and antibiobiotic sensitivity profiles. These methods are limited in their ability to differentiate between *P. aeruginosa* strains due to their low discriminatory power (Belkum *et al*., 2007).

P. aeruginosa can undergo phenotypic change during colonization of CF lungs, reducing the capacity of these methods to characterize strains phenotyping limitations (Saiman *et al*., 2003).

1.2.6.2. Genotyping Methods:

1.2.6.2.1. *P. aeruginosa* Genome:

P. aeruginosa is a highly adaptable organism, it has a large genome containing from 5.5 to 7 Mbp (encoding 5567 genes) compared to 4.64 Mbp (4279 genes) in *Escherichia coli* (Juhas, 2012).

Essential genes are often divided into two categories, 'core' and 'accessory' essential genes. 'core' is considered to be those genes that are universally indispensable for all living organisms. Therefore, 'core' essential genes could be exploited as the basic building blocks of the controlled minimal cell factories. 'accessory' essential genes are required for the survival of individual species and cell types or under specific growth conditions. This makes bacterial 'accessory' essential genes promising targets for the development of novel antimicrobials (Juhas, 2015).

Whilst there is the assumption that 'core' essential genes could be exploited as antimicrobial targets due to providing a broad host range, there is a real danger that antimicrobials inhibiting the 'core' essential gene would also target the essential human homologue. The core genome is composed of highly conserved regions including Housekeeping genes that have a low nucleotide divergence of 0.00-0.5, therefore considered to be generally essential in *P. aeruginosa* (Juhas, 2012).

The majority of the general *P. aeruginosa* essential genes play a role in fundamental cellular functions, such as DNA replication, transcription and translation, RNA metabolism, protein export, biosynthesis of cofactors and amino acids, and cell wall biogenesis (Lee *et al*., 2015). The *P. aeruginosa* accessory genome consists of extra chromosomal components such as plasmids, islands and blocks of DNA that are integrated into the chromosome at various sites (Klockgether *et al*., 2011).

These components may be acquired from other species of bacteria through mechanisms such as horizontal gene transfer. The 'mosaic' and plastic genome structure of *P. aeruginosa* provides this bacterium with the ability to modify, cause infection and adapt to a wide range of habitats (Norgaard-Gron, 2010).

A fast and accurate system for the identification of *P. aeruginosa* and their genes are important to isolate patients and prevent further spreading of the

disease. Bacterial culture and smear examination one of the most important techniques which are still used in diagnostic microbiology, because of its ability to quantify the viable bacteria in a sample; as well as obtaining a pure sample for further testing (Minion, 2010).

Therefore, it has become necessary to develop genotype based characterization systems capable of exactly identifying these microorganisms and their virulence genes, DNA marker allow rapid identification of species between DNA markers, the PCR is highly sensitive, specific and rapid method which vastly improved the detection of *P. aeruginosa* especially when using species - specific primer such as *16SrRNA, toxA,* and *16S- 23SrRNA* genes (Yetkin *et al*., 2006).

Molecular typing methods are required to identify the sources and routes of transmission within the health-care setting (Turton *et al*., 2010).

More advanced approaches to identification have been developed, including PCR is a scientific technique used to amplify a specific sequence of a DNA strand generating thousands to millions of copies of a particular DNA sequence developed in 1983 by Kary Mullis (Bartlett and Stirling, 2003).

RFLP, PFGE, RAPD and DNA sequencing initial molecular epidemiological typing were performed. PFGE using restriction enzyme *Spe*, and identified a single outbreak strain among clinical and environmental isolates (Gee, 2004). Molecular tools such as *16SrDNA* gene sequencing provide reliable results although it might have problems to assign at specie level (Fernandez-Olmos *et al*., 2012).

Selective amplification of *Pseudomonas 16SrRNA* gene by PCR has been used to detect differentiates *Pseudomonas* species from clinical and environmental samples (Wolfgang *et al*., 2003). It is also used for genus or species - level identification of *P. aeruginosa* (Spilker *et al*., 2004).

PCR relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the

DNA. Primers containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. The DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq Polymerase, an enzyme originally isolated from the bacterium *Thermos aquaticus*. This DNA polymerase enzymatically assembles a new DNA strand from DNA building-blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (DNA primers), which are required for initiation of DNA synthesis. (Anzai *et al*., 2000)

Al-Marjani *et al*. (2014) used *16SrRNA* sequence data to identify genus- and species-specific *16SrRNA* signature sequences, its account a stable part of the genetic code. Based on these sequences they designed simple, rapid, and accurate PCR assays that allow the differentiation of *P. aeruginosa* from *Pseudomonas* species and other pathogen genus.

1.2.6.2.3. DNA Sequencing in *P. aeruginosa*:

DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule. It includes any method or technology that is used to determine the order of the four bases—adenine, guanine, cytosine, and thymine in a strand of DNA. The advent of rapid DNA sequencing methods has greatly accelerated biological and medical research and discovery knowledge of DNA sequences has become indispensable for basic biological research, and in numerous applied fields such as diagnostic, biotechnology, forensic biology, and biological systematics (Clyde, 2007).

The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of complete DNA

sequences, or genomes of numerous types and species of life, including the human genome and other complete DNA sequences of many animal, plant, and microbial species **(**Pettersson *et al*., 2009**)**. Fifteen years elapsed between the discovery of the DNA double helix in 1953 and the first experimental determination of a DNA sequence ~~(Reinert and Huson, 2007)~~.

Whole genome sequencing, facilitated by the advent of high-through put approaches, brings the promise of single-base-pair resolution between isolates, making it the ultimate molecular typing method for bacteria (Reinert and Huson, 2007).

Snyder *et al*., (2013) Studied of epidemiological investigation of *P. aeruginosa* isolates from a six-year-long hospital outbreak using high –through put whole genome sequencing, In this study, they demonstrated that the single base resolution of whole genome sequencing is a powerful tool in analysis of outbreak isolates that can not only show strain similarity, but also evolution over time and potential adaptation through gene sequence change.

2. Materials and Methods:

2.1. Materials:

2.1.1. Patients:

A total of 500 inpatients from both sexes (300 males and 200 females) with different age, whom are suffering from different clinical infections **(**diabetic foot 25**,** otitis media 100**,** lower respiratory tract 50**,** urinary tract 125, wound 125 and burns 75 infections) in Baghdad Teaching Hospital, Al-Yarmuk Teaching Hospital, Special Burns Hospital in Medical City and Children Welfare Hospital in Baghdad city during the period from November, 2015 to April, 2016 were enrolled in this study. Depending of clinical criteria that diagnosed by physician and laboratory criteria of infections based on diagnosis microbiology mentioned by Brooks *et al*. (2013). A Questionnaire form was done to record the patients' informations (Appendix 2-1).

2.1.2. Laboratory Instruments and Equipments:

The instruments and equipments that used in this study are listed below in Table (2-1):

Table (2-1): Instruments and equipments used with their remarks

Instrument/equipment	Manufacturing company	Origin
Autoclave	Gallenkump	England
Benzene burner	Al baser office	Iraq
Centrifuge	Hermle	Germany
Deep freeze	Marubeni	Japan
Digital camera	Digital	Japan

Disposable latex	Duisburg	Germany
Disposable loop	Fisher brand	USA
Disposable swab stick	AFCO	Gordon
Disposable syringes(5ml)	Changzhou	China
Distal water	K&K	Germany
Dosimeter	Biomerieux	France
Electric oven	Memmert	Germany
Eppendorf tube	Sigma	England
Eppendorf centrifuge	Eppendorf	Germany
Exipin PCR centrifuge	Bioneer	South Korea
Flask (250_500)ml	Oxfords	USA
Forceps	Promega	Promega
Gel electrophoresis	Major science	Taiwan
Graduated glass cylinder	Superior	Germany
Hot plate with magnetic stirrer	Jenway	United kingdom
Incubator	Memmert	Germany
Khan tube	Bioner	South Korea
Laminar-flow cabinet	Memmert	Germany
Light microscope	Olympus	Japan

Micropipettes(0.5-10µl ,2-20µl ,100-1000µl)	Appendof	Germany
Nano drop spectrophotometer 2000	Thermo scientific	USA
PCR thermo cycler	Bio-Rad	USA
Petridis	Himedia	India
pH meter	Orient	USA
Refrigerator	Marubeni	Japan
Screw capped test tube	BBL	USA
Sensitive balance	Promega	USA
Thermo mixer comfort	Appendorf	Germany
Tip PCR(2-20)µl	Promega	USA
Tip(2-20) µl	Promega	USA
Tip(5-10-20-100-1000) µl	Promega	USA
Ultraviolettrans illumination	Major science	Taiwan
Vortex mixer	Stuart	United kingdom
Water bath	GFL	Germany

2.1.3. Chemical and Biological Materials:

The chemical and biological materials used in this study are listed in Table (2-2):

Table (2-2): Chemical and biological materials used with their remarks

Type of material	Manufacturing company	Origin
Agar-agar	Himedia	India
Agarose gel	Promega	USA
Barium Chloride (BaCl)	BDH	UK
Bromophenol blue	Promega	USA
Catalase	Himedia	India
Conga-red stain	Himedia	India
Distel water	Himedia	India
Ethanol (70%)	Himedia	India
Ethanol (96%)	BDH	UK
Ethidium bromide	Promega	USA
Glycerol	Sigma	USA
Gram stain	Himedia	India
Isoproponal (99%)	Himedia	India
Naldixic acids	Himedia	India
Normal saline	Biomerieux	France
Nuclease free water (12.5)ml	Promeda	USA
Oxidase solution	Himedia	India
RNase (3)µl	Promega	USA
Sucrose	Himedia	India
Tris EDTA buffer 10X	Promega	USA

2.1.4. Molecular DNA Markers:

The molecular markers used in this work are listed in Table (2-3):

Table (2-3): Molecular markers sizes as DNA marker for electrophoresis

DNA markers	Description	Manufacturing company Origin state
1500 base pairs ladder	100 base pairs the ladder consist of 11 double strand DNA fragment with size of 100 base pairs and last band 500 base pairs present at triple the intensity of other fragment and serve as reference all other fragments appear with equal intensity on gel.	Promega USA

2.1.5. Culture Media:

The culture media used in this work were listed below in table (2-4):

Table (2-4): Culture media used with their remarks

Medium	Manufacturing company	Origin state
Blood agar	Oxoid	UK
Brain heart infusion agar	Oxoid	UK
Cetremide agar	Himedia	India
Chromogenic agar	Condo	Spain
Congo red agar	Himedia	India
Lauria bertani broth	Himedia	India
MacConkey agar	Oxoid	UK
Muller Hinton agar	Oxoid	UK
Nutrient agar	Oxoid	UK
Nutrient broth	Oxoid	UK

2.1.6. Antibiotic Discs:

The antibiotic discs were used in the antibiotic susceptibility test are provided by Mast Group company United Kingdom is listed in Table (2-5):

Table (2-5): The antibiotic discs used with their remarks

Class	Subclass	Agent	Symbol	Concentration µg/ml.		Diameter inhibition zone (mm).	
					R	I	S
Penicillin	Ureidopencillin	Mezlocillin	MEZ	75	≤15	-	≥16
Penems	Carbapenems	Imipenem	IMI	10	≤13	14-15	≥16
		Meropenem	MEM	10	≤13	14-15	≥16
Cephens	Cephalosporin III	Ceftazidium	CAZ	30	≤14	15-17	≥18
	Cephalosporin IV	Cefepim	CPM	30	≤14	15-17	≥18
Aminoglycosides	Aminoglycosides	Gentamicin	GM	10	≤12	13-14	≥15
		Amikacin	AK	30	≤14	15-16	≥17
		Tobramycin	TOB	10	≤12	13-14	≥15
Quinolones	Fluoroquinolones	Ofloxacin	OFX	5	≤12	13-15	≤16
		Ciprofloxacin	CIP	5	≤15	16-20	≥21
		Levofloxacin	LEV	5	≤13	14-16	≥17
Monobactam	Monobactam	Aztreonam	AZT	30	≤15	16-21	≥22
β -Lactam	Blactamase inhibitor combination	Piperacillin-Tazobactam	PTZ	100-10	≤17	-	≥18
β -lactam	Blactamase inhibitor combination	Ticarcillin-clavulanic acid	TIM	75-10	≤14	-	≥15

Standard values of CLSI (2013): R=Resistant, I=Intermediate, S=Sensitive.

2.1.7. Diagnostic Kit:

2.1.7.1. Mini API 20NE System:

Mini API 20NE system was provided by Biomerieux Company (France), which used in this study.

2.1.7.2. DNA Extraction and Purification Kit:

DNA extraction and purification kit were provided by Promega (Wizard Genomic DNA purification Kit, USA). The kit contents included in Table (2-6) and which stored at 4˚C.

Table (2-6): DNA extraction kit used in this study

Solution	Volume
Cell Lysis Solution	100 ml
DNA Rehydration Solution	50 ml
Nuclei Lysis Solution	50 ml
Protein Precipitation Solution	25 ml
RNase Solution	250 µl

2.1.7.3. Pure Yield Plasmid Mini Presystem:

Pure yield mini prep system Kit was provided by Promega (USA). The kit contents included in Table (2-7) and which stored at 4˚C.

Table (2-7): Pure yield plasmid mini presystem used in this study

Solution	Volume
Cell Lysis buffer (Blue)	11 ml
Column Wash Solution	9 ml
Elution Buffer	10 ml
Endotoxin Removal	20.5 ml
Neutralization solution	36 ml
Pure Yield™ Collection Tubes	2×50
Pure Yield™ Minicolumns	2×50

2.1.7.4. PCR Detection Kit:

PCR detection kit was provided by Promega (USA) which includes GoTaq® Hot start colorless Master Mix, 2× include: Hot start DNA polymerase is supplied in 2 ×colorless GoTaq® Reaction buffer (PH8.5), 400 µm dATP, 400 µM dGTP, 400 dTTP,400 dctp, 4mM Mgcl Nuclease free water biological source for GoTaq® Hot start colorless polymerase: the enzyme is derived from bacteria, the antibody is derived from murine cell culture.

2.1.7.5. Oligonucleotide Primer Sequences Used for PCR Amplification:

1. Specific primer sequences of *16SrRNA* gene were used to confirm the identification of *P .aeruginosa* by PCR according to Spilker *et al*. (2004) that provided by Alpha DNA company (Canada) and prepared according to the instructions of supplied company, as shown in Table (2-8):

Table (2-8): Oligonucleotide primers sequences of *16SrRNA* gene in *P. aeruginosa* used for confirmatory identification

Name of primer	Primer sequences (5́ → 3́)		References	Product size
16SrRNA	F	GGGGGATCTTCGGACCTCA	(Spilker *et al*., 2004)	956 bp
	R	TTA GCT CCA CCT CGC GGC		

2. Specific primer sequences of *exoA* gene of *P. aeruginosas* were used to detect this gene according to Rosario *et al*. (2012) which provided by Alpha DNA(Canada) prepared according to the information of supplied company, as show in Table (2-9):

Table (2-9): Oligonucleotide primer of *exoA* gene in *P. aeruginosa* used as virulence gene

Name of primer	Primer sequences (5́→3́)		References	Product size
toxA	F	TCAGGGCGCACGAGAGCAACGAGA	(Rosario *et al.*, 2012)	454 bp
	R	GACAGCCGCGCCGCCAGGTAGAGG		

3. Specific primer sequences of *16SrRNA* gene sequence in *P. aeruginosa* was used according to Jiang *et al*. (2006) for partial DNA sequencing to detect the phylogeny of tested isolates which are provided by Alpha DNA company (Canada) and prepared according to the information of supplied company, shown in Table (2-10):

Table (2-10): Oligonucleotide primer sequences *16SrRNA* gene of *P. aeruginosa* used for phylogeny.

Name of primer	Primer sequences (5́→3́)		References	Product size
16SrRNA	F	AGA GTT TGA TCM TGG CTC AG	(Jiang *et al.*, 2006)	1504 bp
	R	CGG TTA CCT TGT TAC GAC TT		

2.2. Methods:

2.2.1. Specimens Collection:

Five hundred specimens from urine 125, wounds 125, burns 75, sputum 50, ear swab 100 and diabetic foot 25 were collected from inpatients of many hospitals during November, 2015 to April, 2016 in Baghdad city. The types and the numbers of clinical specimens were distributed as shown in Table (2-11). All specimens were collected by clean sterilized cotton swabs or containers under supervision of clinical consultant physicians. Burn and wound swabs were taken after three and six days of burn or wound patients from the pus of the burned or wound area in the morning before the bathing of affected area (before hydrotherapy). Each swab was placed in a sterile tube with transport media till reaching the laboratory to be inoculated on culture media (blood agar, MacConkey agar, cetremide agar, nutrient agar and chromogenic agar) and incubated aerobically for (24-48) hours at 37C˚. Urine specimens were taken (by standard mid-stream clean catch method) from patients with UTI. Culture result of urine samples were interpreted as being significant and insignificant bacteriuria, according to the standard microbiology procedure. A growth of ≥10 colony forming units/ ml was considered as significant bacteriuria. Sputum specimens was taken in the early morning from patient and examined the pus cell which must be more than 20 in high power flied and the number of epithelial cells must be lower than 15 under high power flied microscopically. Ear swabs were taken from the patient with chronic otitis media and transferred with transport media and immeadiely culture for isolation. (Collee *et al*., 1996).

Table (2-11): Distribution of clinical collected specimens

Clinical specimens	No. of specimens
Diabetic foot	25
Sputum swabs	50
Ear swabs	100
Urine specimens	125
Burns swabs	75
Wounds swabs	125
Total	500

2.2.2. Preparation of Buffers, Solutions, Reagents and Stains:

2.2.2.1. Buffers and Solutions:

All the buffers and solutions were prepared according to Sambrook and Russell, (2001).

● **Tris-borate EDTA buffer (TBE-10X):**

This buffer was prepared by dissolving 3.8 gm Tris–HCl, 2.7gm boric acid and 2ml EDTA (0.5M) in 50 ml of distilled water, the pH was adjusted to 8, autoclaved, and then kept at 4˚C until used in electrophoresis.

● **TBE (1X)**:

This solution was prepared by mixing 10ml of stock TBE-10x with 90ml of distilled water, then kept at 4˚C until used in electrophoresis.

2.2.2.2. Reagents:

● **Oxidase and Catalase**:

Those reagents were provided by Himedia Company which used in this study.

2.2.3. Preparation of Media:

2.2.3.1. Ready-Prepared Media:

Ready-made media including Brain heart infusion broth, MacConkey agar, Mueller Hinton agar, Nutrient agar, Nutrient broth, Pseudomonas chromogenic agar and Lauria bertani broth were prepared according to the instructions of the manufacturing companies, which are usually fixed on the container of the media. They were sterilized according to the procedures that mentioned in Section 2.2.4 they were kept at 4°C until being used.

2.2.3.2. Laboratory Prepared Media:

2.2.3.2.1. Blood Agar:

Blood agar base was dissolved 21.25 gm. in 500 ml distilled water and sterilized. Then cooled to 45°C and 5% of sterile defibrinated blood was added. After, mixing well, it was poured into sterile petridishes, cooled to 37°C and left to solidify at 25°C. This medium is suitable for isolation and cultivation of bacteria, as well as it was used for the detection of hemolytic bacterial activity (Brook *et al*., 2013).

2.2.3.2.2. Cetrimide Agar Base:

Cetrimide agar was prepared according to instruction of company by dissolving 46.7 gm. in 1000 ml of distilled water containing 10 ml glycerol and then heat to boiling until the constituent is completely dissolved. The media was sterilized by autoclaving at 15 bar/in² pressures, 121°C for 15 min. The media was cooled to 50°C and aseptically add sterile rehydrated content of one vial of naldixic selective supplement which make this media selective isolation of *P. aeruginosa,* then the media was mix well and poured into sterile petridish (Juber, 2015).

2.2.3.2.3. Congo Red Agar:

It was prepared by dissolving of 37g brain-heart infusion broth, 50g sucrose, and 15g agar-agar in 900 ml of distilled water then sterilized by autoclaving. Congo red stain was prepared by dissolving 0.8g in 100 ml of distilled water and sterilized by autoclaving then added to the above media cooling it to 55°C . The medium was poured in a sterile petridish. This media used to detect on the biofilm production (Al-Musawi, 2014).

2.2.4. Sterilization Methods:

The solutions which were affected by high temperature were sterilized by filtrations through millipore filters (0.22 µl.), the sterilization of glasses depended on dry heat by electric oven at 180˚C for 2 hours and micropipettes tips and eppendorf tubes were all sterilized by UV light **(**Russell**,** 2004).

2.2.5. Reference Strain:

Table (2-12) shows the type of reference strain of *P. aeruginosa* that used for comparison in all lab tests.

Table (2-12): Reference strain of *P. aeruginosa* that used in this study.

Strain name	Laboratory identification	Key characteristics	Source
Pseudomonas aeruginosa	American type culture collection (ATCC ®27853)	Susceptible to all antipseudomonal agent	Central Public Health Laboratory in Baghdad

2.2.6. Maintenance of bacterial isolates:

Short-Term Preservation:

Pure isolates of bacteria were maintained for short period (maximum two weeks) by growing them on nutrient agar tube. The plates were tightly wrapped with parafilm and stored at 4˚C. (Benson, 2001).

2.2.7. Isolation and Identification of *P. aeruginosa*:

Pseudomonas aeruginosa were diagnosed according to procedures recommended by MacFaddin (2000). The identification of bacteria from clinical samples was performed as following:

2.2.7.1. Sample Culturing:

All specimens were cultured on MacConkey agar, blood agar, *Pseudomonas* chromogenic agar and cetrimide agar as selective media for. *P. aeruginosa* and all culture media were incubated aerobically at 37°C for 18-24 hour. The colonies were identified according to their staining ability, shape, color, size, odor, and edge, production of pigments, swarming movement transparency and hemolysis pattern (McFaddin, 2000).

2.2.7.2. Microscopic Examination:

One isolated colony from selective media was transported to a microscopic slide, fixed then stained with Gram stain to determine the cell shape, Gram reaction and arrangement by loopful of isolated colony was mixed in the water and spread out, the smear was air dried then heat-fixed by passing the slid rapidly through the burner flames three times. The smear was stained with crystal violet, treated with iodine to fix the stain, decolorized with mix (70% alcohol+30% acetone), and counter stained with safranine, then examined under light microscope (Morello *et al*., 2006).

2.2.7.3. Biochemical Tests:

- **Oxidase test**:

A piece of filter paper placed in a clean Petridish and 2-3 drops of freshly prepared of a solution 1.0% tetra methyl-p-phenylenediamine dihydrochloride were added to the filter paper. A single colony from tested bacteria was transferred to the filter paper and rubbed onto the reagent with an applicator wooden stick. The positive result was indicated by blue purple color formation within 10-15 seconds (Harley and Prescott, 2002).

- **Catalase tests:**

A drop of catalase reagent 3.0% H2O2 was placed on a glass slide. A single colony of the tested bacteria was mixed with the reagent on the slide, and positive results were indicated by air bubbles formation (Harley and Prescott, 2002).

- **Growth at 42°C:**

Single colony of each isolate was streaked separately on nutrient agar medium and incubated at 42°C for 24 hrs. *P. aeuroginosa* colonies have the ability to grow at 42°C (MacFaddin, 2000).

2.2.8. Confirmatory Identification by Using API 20 NE System:

Strip system is a standardized identification system for identification of non-fastidious, enteric Gram negative rods one of these *P. aeruginosa*. The system consists of 20 micro tubes containing dehydrated substrates packaged together. These micro tubes are inoculated with a suspension of a single pure microorganism and incubated for 24 hour at 37°C. During incubation, metabolism produces color changes that are either spontaneous or by the addition of reagents, the results of the tests are read and the identity of the organism is determined. The positive and negative results obtained for each

test are used to generate a seven-digit code number. The company's database then uses this number to determine the identity of the microorganism. The biochemical tests included in this system are show in appendix (2-2).

API 20 E reagent composed of the following reagents:

1. Kovac's reagent (IND).
2. Voges-Proskauer reagent, which is composed of the following reagent:

a. Vp1 (40.0 % Potassium hydroxide).

b. Vp2 (6.0 % alpha-naphthol).

3. Ferric chloride 10.0 % (TDA).

Identification steps of *P. aeruginosa* were performed through the following:

1. The strip was prepared by dispensing five-milliliter of water into the wells of the tray in order to provide humidity during incubation.
2. The inoculum was prepared by picking a single pure isolated colony up from selective media. This colony was suspended in a test tube containing 5 mL of 0.85 % NaCl and mixed to obtain 0.5 McFarland (1.5×10^8 CFU/mL).
3. Inoculation of the strip was performed according to the manufacturer instructions, where both the tube and cupule part of CIT, VP and GEL tests were filled with the bacterial suspension. Other tests, only the tubes were filled. The tests LCD, ODC, URE and H_2S were overlaid with mineral oil to create anaerobic conditions.
4. After inoculations, the plastic lid was placed on the tray and was incubated at 37°C for 24 hour.
5. After incubation, the above mentioned reagents were added to the corresponding micro tubes:

A. One drop of VP1 and VP2 reagents to (VP) micro tube and after 10 minute, the result was recorded immediately.

B. One drop of 10% ferric chloride to (TDA) micro tube then the result was recorded immediately.

C. One drop of Kovac's reagent to the (IND) micro tube.

6. Finally, the results were recorded and compared to that of identification table included with kit, and the identification of the isolates was performed using Analytical Profile Index (API).

2.2.9. Antibiotics Susceptibility Test:

The test was done according to Kirby-Bauer method as follows:

(a) Preparation of Mueller-Hinton agar: Mueller-Hinton agar was prepared by following the procedure mentioned in section (2.2.4.1)

(b) Preparation of inoculums: to prepare the inoculums, pure colonies from overnight culture on nutrient broth and isolates were transferred to a tube contain 5.0 mL of normal saline to obtain suspension with 1.5×10^8 CFU/mL by adjusting to Dosimeter system to give equivalent suspension. The antimicrobial susceptibility testing was done by the agar discs diffusion method depending on the (CLSI, 2013)

(c) Streaking of test plates**:** A sterile cotton swab was dipped into the adjusted suspension, then rotated several times firmly on the inside wall of the tube an above the fluid level to remove excess inoculums from the swab. The surface of a Muller-Hinton agar plates was streaked with the swab. The streaking was repeated two more times and the plates was rotated approximately 60 (degree) each time to ensure an even distribution of inoculums. As a final step the rim of the agar was swabbed. The Petridishes were allowed to dry for 15-20 min. at room temperature before the application of the discs.

(d) Application of discs: The antimicrobial discs were dispensed onto the surface of the inoculated agar plates; the discs were pressed gently to ensure complete contact with the agar surface by helping flame and cooled forceps while the plates were inverted and placed in an incubator at 37˚C form 16-18 hours incubation clear, inhibition zones were calculated in mm by using transparent ruler, the diameter of the inhibition zones for individual

antimicrobial agent was translated in terms of Sensitive (S), Intermediate (I) and Resistant (R) categories by comparison with the standard inhibition zone as shown in Table (2-5) (CLSI, 2013).

2.2.10. P CR Assay:

2.2.10.1. Isolating Genomic DNA from *P. aeruginosa*:

The following procedure was done according to the instructions of manufacturing company:

- Avolume 1ml of an overnight culture was added to a 1.5ml micro-centrifuge tube, centrifuged at 16,000 × *g* for 2 min to pellet the cells and removed the supernatant.

- Avolume 600µl of nuclei lysis solution was added. Gently pipet until the cells are suspended incubated at 80°C for 5 min to lyse the cells and then cooled to room temperature.

- Avolume of 3µl RNase solution was added to the cell lysate. Invert the tube 2–5 times to mix. Incubated at 37°C for 30 minutes. Cooled the sample to room temperature, A volume 200µl of protein precipitation solution was added to the RNase-treated cell lysate vortex vigorously at high speed for 20 seconds to mixed the protein precipitation solution with the cell lysate.

- The sample was incubated on ice for 5 minutes, centrifuged at 13,000–16,000 × *g* for 3 min and then transferred the supernatant containing the DNA to a clean 1.5ml micro-centrifuge tube which contained on 600µl of isopropanol at room temperature then gently mixed by inversion until the thread-like strands of DNA form a visible mass then centrifuge at 13,000–16,000 × *g* for 2 min.

- Carefully the supernatant was poured off and drained the tube on clean absorbent paper. A volume 600µl of room temperature 70% ethanol was

added and gently inverted the tube several times to wash the DNA pellet then centrifuged at 13,000–16,000 × *g* for 2 minutes and drained the tube on clean absorbent paper and allow the pellet to air-dry for10–15 minutes.

- A volume 100µl of DNA rehydration solutions was added to the tube and rehydrates the DNA by incubating at 65°C for 1 hour was periodically mixed the solution by gently tapping the tube. Alternatively, rehydrate the DNA by incubating the solution overnight at room temperature or at 4°C and then stored the DNA at 2–8°C.

2.2.10.2. Isolation of Plasmid DNA from *P. aeruginosa*:

The following procedure was performed at room temperature according to instructions of manufactory Company:

- A total of 600µl of bacterial culture grown in Lauria bertani broth medium was transferred to a 1.5 ml microcentrifuge tube. A 100µl of cell lysis buffer was added, and mixed by inverting the tube 6 times. The solutions should change from opaque to lysis clear blue, indicating complete.

- A volume 350µl of cold (4–8°C) neutralization solution was used and mixed thoroughly by inverting the tube the sample will turn yellow when neutralization is completed, and will form a yellow precipitate, inverted the sample an additional 3 times to ensure completed neutralization, centrifuged at maximum speed in a micro-centrifuge for 3 minutes and transferred the supernatant (~900µl) to a pure yield™ mini- column, do not disturb the cell debris pellet. For maximum yield, transfer the supernatant with a pipette.

- The mini-column was placed into a pure yield™ collection tube, and centrifuged at maximum speed in a micro-centrifuge for 15 seconds then discarded the flow through, and placed the mini-column into the same pure

yield™ collection tube and A volume 200µl of endotoxin removal washed to the mini-column centrifuged at maximum speed in a micro-centrifuge for 15 seconds. It is not necessary to empty the Pure Yield™ Collection Tube.

- A volume of 400 µl of column wash solution was added to the mini-column, centrifuged at maximum speed in a micro centrifuge for 30 seconds then transferred the mini-column to a clean 1.5ml microcentrifuge tube, then added 30µl of elution buffer directly to the mini-column matrix, let stand for1 minute at room temperature. Then centrifuged at maximum speed in a micro centrifuge for 15 seconds to elude the plasmid DNA. Sealed the micro centrifuge tube, and stored eluted plasmid DNA at –20°C

2.2.11. Estimation of DNA Yield and Purity

The extracted genomic DNA was checked by using Nanodrop spectrophotometer to estimate the concentration and purity of extracted DNA through reading the absorbance at (260 /280 nm).

2.2.12. Detection of Specific Genes for *P. aeruginosa* by PCR Method:

2.2.13.1. Preparing the Primers:

An oligonucleotide primer was prepared depending on manufacturer's instruction by dissolving the lyophilized sample with nuclease free water after rotating down briefly. Working primer tube was prepared by diluting with nuclease - free water. The final pico- moles depended on the procedure of each primer.

2.2.13.2. PCR Programs for *16SrRNA* and *exoA* Genes Detection:

PCR was used for detecting *P. aeruginosa*, the mixture 40µl consisted of 20µl.of GoTaq Hot Star master mix (which contains Taq DNA polymerase,

dNTPs, $mgcl_2$ and reaction buffer at optimal concentration for efficient amplification of DNA templates by PCR), 5µl DNA template (20ng.), 1.5µl of each forward and reverse primer (10 pmol.) 12 µl, of nuclease free water to complete the amplification mixture volume, The PCR tubes containing the mixture were transferred to preheated thermo cycler and started the program as in the Table (2-13):

Table (2-13): PCR amplification program of *16SrRNA* gene used for confirmatory identification of *P. aeruginosa* (Alornaaouti, 2015).

Stage		Steps	Temperature (Cº)	Time	No. of cycles
First		Initial Denaturation	95ºC	120sec	1
Second	I	Denaturation	95ºC	20sec	25
	II	Annealing	58ºC	20sec	
	III	Extension	72ºC	40sec	
Third		Final Extension	72ºC	60sec	1

Table (2-14): PCR amplification program of *16SrRNA* gene used for only DNA sequencing in *P. aeruginosa* (Tamura *et al*., 2013)**.**

Stage		Steps	Temperature (C˚)	Time (sec)	No. of cycles
First		Initial Denaturation	95˚C	4min	1
Second	I	Denaturation	95˚C	30sec	30
	II	Annealing	55˚C	30sec	
	III	Extension	72˚C	90sec	
Third		Final Extension	72˚C	9min	1

Table (2-15): PCR amplification program of *exoA* genes detection in *P. aeruginosa* as virulence gene (Juber, 2015).

Stage		Steps	Temperature (C˚)	Time (min)	No. of cycles
First		Initial Denaturation	95˚C	4 min	1
Second	I	Denaturation	95˚C	30 sec	30
	II	Annealing	60˚C	30 sec	
	III	Extension	72˚C	105 sec	
Third		Final Extension	72˚C	9 min	1

2.2.14. Agarose Gel Electrophoresis:

Agarose gel was prepared by dissolving 1 g for chromosomal DNA or 1.5 g for plasmid DNA of agarose powder in 100 ml of (1X) TBE buffer (pH 8) on hot plate with magnetic stirrer and magnetic capsule was added, left until boiling and becoming clear, allowed to cool to 50˚C, and 5 µl ethidium bromide was added, (Sambrook and Russell, 2001).

2.2.14.1 Casting of the Horizontal Agarose gel:

The tape was placed across the end of the gel tray. The comb was fixed at one end of the tray for making wells used for loading DNA samples. The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 minutes. Then, the comb was removed gently from the tray and the tape was also removed from the ends of the tray. The agarose gel was fixed in electrophoresis chamber which was filled with TBE buffer (1X) that had covered the surface of the gel (Sambrook and Russell, 2001).

2.2.14.2. Loading and Running DNA in Agarose Gel:

Five µl of each DNA template was transferred to eppendorf tube, 3µl of loading dye was added to the tube and the mixture was loaded into the wells in agarose gel with the addition of loading buffer and DNA Ladder (100bp) as standard in electrophoresis. The electric current was allowed at 100 volt for 10 min and then 80 volt for 1 hour to detected the *16srRNA*gene (956 base pairs amplicon), DNA extraction and plasmid extraction while used 90 min at 100 volt/50 mAmp to detected, *16srRNA*gene (1504 base pairs amplicon) and *exoA.* After complete time read the results on UV transilluminater was used for the observation of DNA bands, and the gel was photographed.

2.2.15. DNA sequencing and Phylogenetic Analysis by *16SrRNA* Gene:

Partial *16SrRNA* gene sequences were obtained from the PCR amplicon generated with the same primers mentioned in Table (2-10) for *P. aeruginosa*. The PCR products of *16SrRNA* (1504bp) were sent to Micro gene company in South Korea for sequencing by their ABI 3730 genetic analyzer. DNA sequencing compared with those available in the Gene Bank data base by using basic alignment search tool, BLAST of NCBI to identify wither they aligned with closely related organisms.

Percentage nucleotide identity and pairwise uncorrected (p-distances) were calculated for *16SrRNA* sequences of clinical strains of the *P. aeruginosa* examined by using sequence an alignments explorer CLUSTAL (Felsenstein, 2006).

Phylogenetic analysis was carried out using the software MEGA version 6, using the neighbour joining method to reconstruct the phylogenetic tree with option of complete detection of gaps (Tamura *et al*., 2013).

2.2.16. Statistical Analysis

The results were analyzed statistically by mean, standard deviation and percentage of frequencies obtained Percentages of similarities for partial *16SrRNA* sequences obtained in this study were calculated by the FAST method. Multi alignments were obtained with alignments Explorer CLUSTAI (Felsenstein, 2006).

Conclusions:

1) *P. aeruginosa* essential genes sequencing have recently been better defied by a number of studies employing more sensitive genomic approach.
2) It can concluded that threats of MDR *P. aeruginosa* become of major concern in our hospital and implementation of infection control strategies are major concerns to avoid the spread of this threats.
3) Detection of three patterns of plasmid profile (A, B, C) with different sizes according to antibiotyping and source of clinical samples.
4) The present data proved recording of two new strains or mutants of local isolates in Gene Bank and NCBI locus. In addition to, that the first recording of 10 isolates of *P. aeruginosa* in NCBI data.

Recommendations:

1) Finding general policy strategies should be made for minimize the inappropriate use of antibiotics and prevent the emergence of drug resistance.
2) For diagnostic efficiency of hospital microbiologist as well as treatment should be based on basis laboratory susceptibility test result.
3) Encouraging good hygienic measures could help crucial possible transmission of MDR *P. aeruginosa* infections within and outside the hospital environment; also suggest that plasmids should be characterized in all MDR *P. aeruginosa* strains.
4) Due to their promising antimicrobial properties, Nano materials and Nano-particle are currently being studied as potential, highly potent antimicrobial agents for a variety of medical applications in order to enhance

antimicrobial activity and overcome the various resistance mechanisms in *P. aeruginosa*

5) Further investigation is a required in particular; the performance of Nano materials and Nano particles is an interdisciplinary endeavor.
6) Further analyses of essential cellular functions, including the unknown *P. aeruginosa* essential genes, will lead to the developing of novel antibiotics against this hard to eradicate pathogen.
7) It seems necessary for physicians and health related personal to be aware of the status of the drug resistance and prevent from the uncontrolled prescriptions of drug.