REPUBLIC OF TURKEY
VAN YUZUNCU YIL UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES
BIOLOGY DEPARTMENT

EPIDEMIOLOGICAL STUDY OF *PSEUDOMONAS AERUGINOSA* USING DIFFERENT MOLECULAR TYPING METHODS

PhD THESIS

PREPARED By: Marwan Khalil QADER
SUPERVISOR : Prof. Dr. Hasan SOLMAZ

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KABUL VE ONAY SAYFASI

Biyoloji Anabilim Dalı'nda Prof. Dr. Hasan SOLMAZ danışmanlığında, Marwan Khalil Qader tarafından sunulan **"Farklı Moleküler Tiplendirme Metotları Kullanılarak *Pseudomonas aeruginosa*'nın Epidemiyolojik Araştırılması"** isimli bu çalışma Lisansüstü Eğitim ve Öğretim Yönetmeliği'nin ilgili hükümleri gereğince 26/01/2018 tarihinde aşağıdaki jüri tarafından oy birliği ile başarılı bulunmuş ve doktora tezi olarak kabul edilmiştir.

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ACCEPTANCE and APPROVAL PAGE

This thesis entitled "**Epidemiological study of *Pseudomonas aeruginosa* Using Different Molecular Typing Methods"** presented by Marwan Khalil Qader under supervision of Prof. Dr. Hasan Solmaz in the department of Biology has been accepted as a Ph.D. thesis according to Legislations of Graduate Higher Education on 26/01/2018 with unanimity of votes of members of jury.

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THESIS STATEMENT

I declare that all information presented in this thesis were obtained within the framework of ethical behavior and academic regulations. Moreover, I inform that I cited properly all expressions and information that does not belong to me in this thesis, which has been written according the rules given in thesis writing guide.

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ABSTRACT

EPIDEMIOLOGICAL STUDY OF *PSEUDOMONAS AERUGINOSA* USING DIFFERENT MOLECULAR TYPING METHODS.

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In the current study, 225 isolates of *P. aeruginosa* burn infection have been collected from major hospitals in Duhok and Erbil / Iraq over a time period from April 2015 until to September 2015. One hundred thirty six of these were isolated from males accounting 60.4 % whereas 89 (39.6 %) of them were recovered from females. One hundred isolates of *P. aeruginosa* burn infection (fifty from each province including; Erbil and Duhok Province) were randomly selected and subjected to antibiotic sensitivity test. the average of concentration of genomic DNA extracted using the commercial kit was 115.25 ng/µl with a purity of 1.8, whereas the average concentration of genomic DNA extracted by the traditional method was found to be 1930.22 ng/µl with a purity of 1.6. The genome all of these isolates were successfully amplified producing a single band of the *16srDNA* locus in all strains with a molecular weight of about 956 bp in order to confirm at molecular level that all these isolates were *P. aeruginosa*. The results of the detection of five virulence related genes including (*opr-1, tox-A,exo-S,las-B,* and *nan-1*) revealed that ten of these isolates accounting (10 %) lacked any tested virulence markers, *oprI* as a marker for presence of pathogenicity island was the most predominant marker among all other virulence markers accounting 90 (90 %) followed by *tox-A,* and *exo-S* accounting 86 (86 %) and 86 (86 %) respectively, while the prevalence of *las-B* gene is found with the rate 82 (82 %) and *nan-1* with 35 (35 %) respectively. The results of the detection of five metalo β-lactamase(MβL) genes including (*vim-1, imp1,spm-1,sim* and *gim*), *vim1* as a marker for presence of pathogenicity island was the most predominant marker among all other antibiotic resistance markers accounting 46 (46 %) followed by *imp1* and *spm-1* accounting 45 (45 %) and 35 (35 %) respectively, while the prevalence of *sim* gene is

found with rate 27 (27 %) and *gim* with 24 (24 %). The results of the detection of three Extended Spectrum β-lactamase (ESβL) genes including (*oxa10, veb1,* and *oxa2*), *oxa10* a marker for presence of pathogenicity island was the most predominant marker among all other antibiotic resistance markers accounting 91 (91 %) followed by *veb1* and *oxa2* accounting 66 (66 %) and 36 (36 %). *P. aeruginos* isolates (100) were also subjected to ERIC-PCR fingerprinting analysis in order assign them into two main clusters Group A accounting 92 %, forty six (46 %) isolates were belonged to subgroup A1 and 46 isolates of the studied strains were in subgroup A2 (46 %), while group B representing only 8each one can be classified into two subgroups 4 (4 %) strains were assigned within each subgroup. Also in this study fifteen isolates of clinical strains *P. aueroginosa* were selected and subjected to single nucleotide polymorphisms (SNPs) analysis using *OprD* gene which encodes *OprD* porine and compared with *P. aureoginosa* Str.PA12 (accession number KJ482587.1) which was used as a reference strain. Nineteen point mutations substitutions (SNPs) type were observed at 19 polymorphic sites accounting 3.77 % of the whole sequenced fragment of *OprD* gene. six of them were (6/19) transversions type accounting 31.57 %, while thirteen 68.42 % (13/19) were transitions. Only one SNPs at 328/503 site due to transversion mutation resulted amino-acid change (sense mutation), all other mutations were silent substitutions. four allelotypes have been identified. It was revealed that nine strains shared the same amino acid replacements G328C (Val 110 Lucien). Dendogram analysis of studied strains revealed that the clinical strains were classified into two main clusters the first cluster divided into two subgroups (*P. aureoginosa* strains1, 3, 4, 8, 13 and 14); (*P. aeruginosa* strains 2 and 12); the other cluster also divided into two subgroups (*P. aeruginosa* strains 5and 15) and *(P. aeruginosa* 6,7, 9,10 and 11). Nearly 53.3 % of studies isolates were assigned with reference strain *P. aureoginosa* Str.PA12, whereas 46.6 % of strains were assigned with other cluster.

Keywords: Burn infection, *ERIC, OprD, P. aeruginosa, PCR*, Single nucleotide sequences, *16srDNA*,

ÖZET

FARKLI MOLEKÜLER TİPLENDİRME METOTLARI KULLANILARAK PSEUDOMONAS AERUGİNOSA'NIN EPİDEMİYOLOJİK OLARAK ARAŞTIRILMASI

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Bu çalışmada kullanılan 225 adet *P. aeruginosa* izolatı Nisan 2015-Eylül 2015 tarihleri arasında Duhok ve Erbil / Irak'taki büyük hastaneden toplandı. Bunların 136'sı (% 60,4) erkeklerden, 89'u (% 39.6) kadınlardan izole edildi. Rasgele seçilen (Erbil ve Duhok bölgesi dahil her ilden 50) 100 adet *P. aeruginosa* izolatı antibiyotik duyarlılığı testine tabi tutuldu. Ticari kit kullanılarak çıkarılan genomik DNA konsantrasyonunun ortalaması 1.8 saflık ile 115.25 ng/µl, geleneksel yöntemle çıkarılan genomik DNA'nın ortalama konsantrasyonunun saflık değeri 1.6 saflık ile 1930.22 ng/µl bulundu. Tüm bu izolatların

P. aeruginosa olduğu moleküler seviyede teyit edilmesi için tüm suşlarda 16srDNA loküsünün yaklaşık 956 bp'lik bir moleküler ağırlığa sahip olan tek bir bandını üreten genom başarıyla amplifiye edildi. Beş virülanla ilişkili genin (Opr-1, tox-A, ekzo-S, las-B ve nan-1) taranması sonucu bu izolatların onda birinin (% 10) herhangi bir test edilmiş virülans belirteçlerinden yoksun olduğunu ortaya koydu. Patojenite varlığı göstergesi için bir belirteç olarak oprI, diğer tüm virülans belirteçleri arasında izolatların 90'ında (% 90) en baskın belirteçti ve bunu takiben toks-A ve ekzo-S her ikisi de 86'sında (% 86) idi. las-B geninin yaygınlığı 82'inde (% 82) ve nan-1 35'inde (% 35) ile bulundu. Beş metalo β-laktamaz (MβL) geninin (Vim-1, imp1, spm-1, sim ve gim) saptanmasının sonuçları patojenite varlığının göstergesi olan Vim1, diğer tüm antibiyotik direnç belirteçleri arasında 46 (% 46) iken en baskın belirteç, bunu takiben imp1and spm-1 miktarları sırasıyla 45 (% 45) ve 35 (% 35) iken, sim geni miktarı 27 (% 27) ve gim 24 (% 24) bulundu. Genişletilmiş Spektrum β-laktamaz (ESβL) genlerini (oxa10, veb1 ve oxa2) içeren, patojenite belirteci olan oksa10 diğer tüm antibiyotik direnç göstergelerinin 91'ini (% 91), takip eden veb1 ve oksa2 sırasıyla

66(% 66) ve 36(% 36) olduğu belirlendi. *P. aeruginosa* izolatları (100) aynı zamanda iki ana kümeye atamak için ERIC-PCR parmak izi analizine tabi tutuldu. İzolatların % 92'si A Grubu ki; 46 izolat (% 46) alt grupA1'e aitti ve incelenen suşların 46'sı (% 46) alt grup A2 idi, grup B sadece %8'i temsil ederken 4'er (% 4) adet iki alt grupta oldukları belirlendi. Bu çalışmada da, *P. aueroginosa* klinik suşlarından onbeş izolat seçildi ve OprD porin kodlayan OPrD geni kullanılarak tek nükleotid polimorfizmlerine (TNPs) tabi tutuldu ve kullanılan *P. aureoginosa* Str.PA12 (erişim numarası KJ482587.1) ile karşılaştırıldı. Bir referans suş olarak. OPrD geninin tüm sıralı fragmanının % 3.77'sini muhafaza eden.19 polimorfik bölgede on dokuz nokta mutasyon ikamesinin (TNP) olduğu gözlemlendi. Bunlardan altısı (6/19) transversiyon tipi muhasebe % 31.57 iken, on üçü % 68.42 (13/19) geçiş idi. Transversiyon mutasyonu nedeniyle 328/503 bölgesinde sadece bir tane SNP sadece amino asit değişikliği (sens mutasyonu), diğer tüm mutasyonlar sessiz yer değiştirmelerle sonuçlandı. Dört allelotip tespit edildi.

Dokuz suşun aynı amino asit değiştirmelerini G328C'yi (Val 110 Lucien) paylaştığı ortaya çıkarıldı. Çalışılan suşların dendogram analizi, klinik soyların iki ana küme halinde sınıflandırıldığını ortaya koydu; ilk küme iki alt gruba ayrıldı; (*P. aureoginosa* suşları1, 3, 4, 8, 13 ve 14); (*P. aureoginosa* suşları 2 ve 12); diğer küme aynı zamanda iki alt gruba (*P. aureoginosa* suşlar 5 ve 15) ve (*P. aureoginosa* 6,7, 9,10 ve *P. aureoginosa* 11) ayrılmıştır. Çalışma izolatlarının yaklaşık 53.3'ü referans suş *P. aureoginosa* Str.PA12 ile ayrılırken, suşların 46.6'sı diğer küme ile görevlendirildi.

Anahtar kelimeler: ERIC, *OprD,* PCR, *P. aeruginosa*, Tek nükleotid dizileri, Yanık enfeksiyonu, *16srDNA*, ,

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2018

Marwan Khalil QADER

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ABBREVIATIONS

Abbreviations used in this study are given below with their corresponding explanations.

Abbreviations	Explanation
LPS	Lipopolysaccharide
IgA	Immunoglobuline A
CF	Cystic Fibrosis
PCR	Polymerase Chain Reaction
DNA	DeoxyriboNucleic Acid
T3SS	Type III Secretion System
ExsA	positive synthesis regulatory protein
ExsD	Negative synthesis regulatory protein
ExsC	Type III secretion gene expression
ExsE	Negative regulator of type III secretion gene expression
VAP	Ventilator-Associated Pneumonia
UTI	Urinary Tract Infections
Mbp	million base pairs
PAPI	*P. aeruginosa* pathogenicity island
ERIC	Enterobacterial repetitive intergenic consensus
exoS	Exoenzyme S
Tox-A	Exotoxin A
Las B	Elastase
nan1	putative neuraminidase
OprI	I lipoprotein
VIM-1	Verona Integron- metallo-B-lactamase
SPM-1	Sao Paulo metallo-β-lactamase
SIM	Seoul Imipenemase
GIM	German Imipenemase
MBL	metallo-β-lactamases

Oxa 10	Oxacillinase group of β-lactamases (Class D)
VEB-1	Vietnamese extended-spectrum beta-lactamase
OPrD	outer membrane protein
ESBL	Extended spectrum B- lactamases
UPGMA	unweighted pair group method with arithmetic mean
EDTA	Ethylenediaminetetraacetic acid
SDS	Sodium dodecyl sulphate
CTA	cetyl trimethlammonium bromid

1. INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that infects organisms and causes nosocomial infections (Vincent *et al.,* 2004). It is one of the major causes of chronic lung infections of cystic fibrosis (CF) patients and a major cause of hospital-acquired infections, thrives in many environments (Stover *et al.,* 2000). *P. aeruginosa* is well suited to survive in a wide variety of environments (water, soil, and animals) and is prevalent in common everyday surroundings (David *et al.,* 2007). *P. aeruginosa* infections may occur in cancer patients, patients suffering from urinary tract infections, and patients suffering from burn wounds (Bodey *et al*., 1983). Infections caused by *P. aeruginosa* are often difficult to treat due to the prominent resistance exhibited by the pathogen to antimicrobial agents (Hancock, 1998). In the context of a breakdown in host defenses; it is capable of infecting a plethora of tissue types, causing both acute and chronic infections. Burn victims as well as immunocompromised, mechanically ventilated, and cystic fibrosis (CF) patients are particularly susceptible to *P. aeruginosa* infection (Sadikot *et al.,* 2005).

P. aeruginosa is found as major colonizer of the burn infection because it thrives on moist burn wound surface and usually gains access to burn patients through cross contamination. It persists as a major nosocomial infection threat to burn patients. Arising of resistance against multiple antimicrobial drugs frequently complicates the treatment of *P. aeruginosa* infection. This may lead to serious infection and thus mortality rate in these patients becomes high (Holder *et al.,* 1995). The emergence of multi-resistance *P. aeruginosa* in burn wound is becoming a challenging problem in infection control programmes (Douglas, 2001). It is almost always predominant in monobacterial as well as polybacterial infection (Nagoba *et al.,* 1999). Therefore, the development of rapid and sensitive technique for early detection of *P. aeruginosa* is very important for effective treatment of those diseases. The development and application of molecular diagnostic techniques has initiated a revolution in the diagnosis and monitoring of infectious diseases. Molecular tools have been introduced and found to be very helpful in accurately diagnosing diseases with greater sensitivity and specificity than conventional methods. The use of such tools, especially the techniques

based on polymerase chain reaction (PCR), had a huge impact on the characterization, detection, diagnosis and taxonomy of the infectious disease agents (Tang *et al*., 1997; Siripattanapipong *et al.,* 2010). In this context, many different DNA markers based on PCR technologies have been developed over the last quarter century. PCR-based technique has been used for the identification of phylogenetic groups of the *P. aeruginosa* species (Clermont *et al.*, 2000).

DNA Sequence is determining the order of the nucleotides bases, the rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of the human genome, in the human genome project(Tang *et al*., 1997). Therefore, the main aims of this study were;

- Characterization of *P. aeruginosa* collected from the major hospitals in Erbil and Duhok /Iraq.
- Screening of antimicrobial sensitivity profile of *P. aeruginosa*.
- Application of Specific-species PCR technique for the confirmation of *P. aeruginosa* isolates at molecular level.
- Studying the Phylogenetic analysis of *P. aeruginosa* based on PCR-technique.
- Detection of the prevalence of virulence and antibiotic resistance genes among *P. aeruginosa* and their distribution among phylogenetic groups.
- Study the distribution and patterns resistance to antimicrobial agents of these *P. aeruginosa* strains by enterobacterial repetitive intergenic consensus ERIC-PCR
- Determine the relationship between *P.aeruginosa* isolated strains using enterobacterial repetitive intergenic consensus ERIC-PCR based on genomic fingerprinting.
- Applications of sequences technique on the *P. aeruginosa* collected from different province and place them into different phylogenetic groups to study the most prevalent sequence types and their genetic relatedness in this region.

2. REVIEW OF LITERATURE

2.1. Characteristics of *Pseudomonas aeruginosa*

This bacterium was first mentioned in literature by Schroeter in 1872; it was called *Bacterium aeruginosum* (Schroeter, 1872). The name has been changed several times until it finally set to be *Pseudomonas aeruginosa* which is currently in use (Skerman *et al*., 1980).

P. aeruginosa is an opportunistic pathogen characterized by aerobic growth, non-fermentative, and stained negative by gram staining technique. It is a highly versatile microorganism can to tolerate low oxygen tension. It can survive with decreasing levels of nutrients and has a wide range of temperatures to grow in ranging from 4-42°C. (Lewenza *et al.*, 2005). It measures from 0.5 to 0.8 μm by 1.5 to 3.0 μm. Nearly all strains are motile by means of a single polar flagellum. It normally multiplies in moist environments, and uses a variety of organic compounds for growth, thus it has an exceptional advantage to colonize ecological niches where nutrients are scarce, from water and soil to plant and animal tissues (Gilligan, 1991; Filiatrault *et al.*, 2006). *P. aeruginosa* is considered to be a common cause of nosocomial infections, typing techniques are mandatory to verify a clonal relationship between individual strains in the hospital settings, to recognize outbreaks, and to determine the origin of infection (Corona *et al.*, 2001).

P. aeruginosa is oxidase positive, hydrolyze arginine and gelatine, and reduce nitrate to nitrite. The bacterium is also capable of producing two types of soluble pigments, pyoverdin and pyocyanin. Blue pigment is produced abundantly in media containing low level of iron and has an essential role in iron metabolism by the bacteria. Pyocyanin (from "pyocyaneus") refers to "blue pus", which is characteristic for suppurative infections caused by *P. aeruginosa* (Gilligan, 1991).

P. aeruginosa causes diseases in a variety of unrelated creatures, humans and animals are known to be highly susceptible, non-mammalian eukaryotes such as insects,

nematodes are also infected and plants can also be considered as a distinctive host (Rahme *et al.*, 1995; Jander *et al.*, 2000; Park *et al.*, 2005).

2.2. Virulence factors of *P. aeruginosa*

P. aeruginosa exhibit a variety of virulence factors to overcome host defenses and establish infection. These factors include the production of hemolysin, pyocyanin, gelatinase, and the formation biofilm, which act by increasing tissue damage and helping the bacteria to evade the immune system and to avoid the action of antibiotics (Cevahir *et al.,* 2008). The pathogenesis of infections is multifactorial, as suggested by the number and broad range of virulence determinants expressed by the bacterium (Todar, 2009). *P. aeruginosa* is notorious for its multiple virulence factors such as adhesins, biofilm formation, elastase production, surface hemagglutinin, motility, synthesis and production of pyocyanin, rhamnolipid, type III secretion system, colonization Pili, lipopolysaccharide (LPS), flagella, alkaline protease, siderophore uptake systems and extracellular protein toxins (exoenzyme S and exotoxin A) (Gallagher and Manoil, 2001; Holder *et al.*, 2001).

2.2.1. Elastase

Elastases are considered important virulence factors which damage host tissues and affect host defense systems (Hoštacka and Karelova, 1997). Elastase is a metalloproteinase group enzyme which increases the invasiveness of *P. aeruginosa* in eyes, burns, and pulmonary infections and degrades the elastin of the human lung (Bejarano *et al.,* 1989). Elastase is responsible for the destruction of normal basement membranes extracted from bovine lungs and lenses (Bainbridge and Fick, 1989). The elastic lamina of arteries and arterioles are dissolved by elastase (Kon *et al.,* 1999) and the enzymes also destroy the extracellular matrices at epithelial cells junctions (Bejarano *et al.,* 1989). Lymphocytes aggregation in the lungs of mice is induced by the action of elastase and they are also responsible for the interaction with the proteins of the human defense system (Yanagihara *et al.,* 2003). Heck and coworkers were able to

show that elastase can destruct immunoglobuline A (IgA) which is a prominent serum immunoglobulin that bath mucosal surfaces and is predominant antibody class of the respiratory, gastrointestinal tract, and the eye which plays a key role in the clearing of invading organisms (Bainbridge and Fick, 1989).

2.2.2. Siderophores

Virulence of *P. aeruginosa* is also aided by siderophores which chelate bound tissue iron during infections (Gabisoniia *et al.,* 1992). Importance of siderophores has been proven in the pathogenesis of corneal, respiratory, burn wound and urinary tract infections (Lamont *et al.,* 2002). In most situations the level of soluble iron is very low for sufficient iron to be gained by passive diffusion of ions into the cell (Visca *et al.*, 1992). Bacteria have developed a number of different strategies to counteract this problem and one of the most common of these is the production of iron-chelating compounds named siderophores (Lamont *et al.,* 2002). These chelateferric ions in the surroundings and the ferrisiderophore complexes are taken up by the bacteria through specific cell-membrane receptor proteins. The iron is then freed from the ferrisiderophore complex for integration into cellular proteins. Five hundred siderophores have been defined and characterized and most bacterial families contain siderophore producers (Drechsel, and Winkelmann, 1997). Pyochelin and pyoverdine are two siderophores produced by *P. aeruginosa* as secondary metabolites to gather iron and more than thirty genes are responsible for encoding iron receptors (Kaneko *et al.*, 2007). Pyochelin is released into the nearby environment where it binds with iron and transfers it to the bacterial cell through a specific outer membrane receptor (Ankenbauer and Quan, 1994). A number of reports have revealed an association between the virulence of *P. aeruginosa* and Pyochelin production. Pyochelin stimulates bacterial growth in murine infections and efficiently removes iron from transferring (Cox, 1982; Ankenbauer *et al*., 1985). Pyochelin has a lower affinity than pyoverdine for iron but is still constitutes an importance for the virulence of *P. aeruginosa*, and two operons control the synthesis of Pyochelin (Poole and McKay, 2003).

Pyoverdine is a water-soluble, yellow-green, fluorescent hydroxamate-catecholate pigment synthysized by *P. aeruginosa*. It binds to iron thus acts as a sidrophore (Cunliffe *et al.,* 1995). Pyoverdine is the main siderophore of *P. aeruginosa*, studies had shown that mutant lacked pyoverdine is less virulent in mice models (Meyer *et al.,* 1996). In *P. aeruginosa,* pyoverdine synthesis is mainly controlled by the *pvd* operon, while PvdS acts as a sigma factor for initiation of pyoverdine production (Cunliffe *et al.,* 1995). Pyoverdine controls three virulence proteins, endotoxin A, elastase, and itself. This fact pointed to the ultimate importance of pyoverdine for the pathogenicity of *P. aeruginosa*. (Lamont *et al.,* 2002).

2.2.3. Pyocyanin

Pyocyanin is a water soluble blue green phenazine compound (Pigment) produced in large quantities by *P. aeruginosa*. This pigment has antimicrobial activity against a wide variety of microorganisms (Mavrodi *et al.,* 2001). This characteristic can be utilized by *P. aeruginosa* for the elimination of competing microorganisms (Baron and Rowe, 1981). Pyocyanin is a redox-active molecule which is unique for *P. aeruginosa,* it inhibits epidermal cells growth, lymphocyte proliferation and bacterial cells through the generation of reactive oxygen componds (Sorenson *et al.,* 1983; Yahr and Parsek, 2006). Pyocyanin plays critical role as an electron transfer molecule and catalyst in the phosphorylation reactions associated with photosynthetic pathways of bacteria and green plants; it could also function as an extra-cellular respiratory pigment (Zaugg, 1964). In nature, pyocyanin production is affected by environmental factors such as salinity (Mavrodi *et al.,* 2006).

2.2.4. Motility

Motility is critical for biofilm formation and infection caused by *P. aeruginosa* (Van Alst *et al.,* 2007). Non-motile mutants of *pseudomonas* are severely defected in colonization of alfalfa (Martínez-Granero *et al.,* 2006). Three types of motility have been adopted by *P. aeruginosa* namely, swimming, swarming, and twitching motility.

Outside a host, the agar concentration determines the type of motility. Swarming motility is a multicellular behaviour involving the coordinated and rapid movement of a bacterial population across a semisolid media (Fraser and Hughes, 1999). Flagella are required for swarming and swimming motility, while, type IV pili are essential for twitching motility (Rashid and Kornberg, 2000). Impairment of bacterial motility necessary for proper biofilm formation follows a concentration-dependent manner (Samuel *et al.,* 2011). The virulence of *P. aeruginosa* and its ability to colonize different locations depends on this motility; it is capable of swimming in aqueous surroundings (low-agar conditions), and twitching on dry environments (high agar conditions) (Yeung *et al.,* 2009).

2.2.5. Rhamnolipid

P. aeruginosa produce the glycol lipidic biosurfactants (Rhamnolipids) as exoproducts of the opportunistic pathogen, these molecules have been defined as virulence factors contributing to the pathogenesis of these bacteria (Dimitrios *et al.*, 2013). Rhamnolipids have been used for the remediation of oil contaminated water and soil, they are produced during late log and stationary phases of growth of *P. aeruginosa* (Chen *et al.*, 2007). In fact, these compounds can be described with dual function, one is their application in industrial biotechnology and second, their distinctive virulence potential. Prieto *et al.,* 2008 showed that rhamnolipids help bacteria to infiltrate the respiratory epithelium and rhamnolipids exacerbate the invasion of rhamnolipid-deficient *P. aeruginosa* by disrupting the epithelia. It has also been stated that rhamnolipids are essential for swarming motility and biofilm formation by *P. aeruginosa* (Davey *et al.,* 2003). Rhamnolipid is composed of a gluconic part usually with one or two rhamnoses and a lipid part made of one or two hydroxy-fatty acids (Abdel-Mawgoud *et al*., 2011). The invasion of respiratory epithelia by *P. aeruginosa* is mainly attributed to rhamnolipid production, the mechanism underlies this phenomena is primarily starts with the incorporation of rhamnolipid within the host cell membrane which is then followed by tight-junction changes and the junction-dependent barrier of the respiratory epithelium is selectively manipulated by rhamnolipid (Zulianello *et al.*,

2006). A positive correlation was reported between elevated levels of rhamnolipid in the bronchial epithelium of patients with concomitant cystic fibrosis and infection caused by *P. aeruginosa* and the worsening of the clinical outcomes of the affected individuals (Kownatzki *et al.,* 1987). Rhamnolipid inhibits the mucociliary function of the epithelium and causes damage to the bronchus and it also varies the ion transport by decreasing the absorption of sodium and single direction chlorine through bronchial epithelium (Stutts *et al.,* 1986).

2.2.6. Type III secretion system

Gram-negative bacteria utilize type III secretion system to inject their virulence effectors proteins (ExoS or ExoU, ExoT and ExoY) into the cytosolle of the target host cells where they mediate their pathogenic effects (Hauser *et al.,*1998 ; Hueck, 1998). Toxins secreted by *P. aeruginosa* follow a two-step secretion process initiated by translocation across the inner membrane and then an additional step of translocation through the outer membrane (Tommassen *et al.,* 1992). There are approximately 40 coordinated genes arranged into five operons which are responsible for encoding type III secretion systems. They encode the needle complex, translocation apparatus and regulator proteins; the effectors and chaperones are encoded elsewhere on the genome (Frank, 1997). Transcription of the regular is entirely related to secretion through a cascade of interactions containing ExsC, ExsD and ExsE, which finally impinge upon the master transcriptional activator of T3SS gene expression, ExsA (Urbanowski *et al.*, 2005). The expression the type III is found to be affected by oxygen level, the expression of the T3SS in oxygen-deficient conditions is strongly related to the glyoxylate shunt enzyme, isocitrate lyase (Jade *et al.,* 2013). It had been previously documented that type III secretion is responsible for apoptosis of epithelial cells (Hauser and Engel, 1999) and plays a role in the virulence of *P. aeruginosa* in cystic fibrosis patients (Roy-Burman *et al.*, 2001).

2.3. Biofilm formation in *P. aeruginosa*

Biofilms are highly-architecture; matrix-closed bacterial communities within exopolysaccharide envelopes (Costerton *et al.,* 1995) contained in a matrix of mainly polysaccharide nature allowing them to mutiply and survive in various target environments (Hall-Stoodley *et al.*, 2004). Clinically, persistent and chronic infections can be attributed to biofilms due to their inherent resistance to antimicrobial drugs and the selection for phenotypic variants. Understanding of the genetic and molecular bases of biofilm formation may have critical role in the control of chronic infections and problems associated with biofilm formation (Branda *et al.,* 2005). Biofilm formation occurs when microbes are put under stress such as antibiotic treatments or limited nutrients to enhance their survival (Singh *et al.,* 2000). The biofilm matrix is comprised of bacterial cells, DNA, secreted cell products, polysaccharides, proteins, and water (Allesen-Holm *et al*., 2006). The capability to adhere to solid surfaces and the resultant formation of an organized bacterial biofilm are also essential steps in the establishment of chronic microbial infections and persistence in host tissues (Costerton *et al.,* 1999). *P. aeruginosa* has shown a dramatic ability to create biofilms on different surfaces such as lungs of patients with cystic fibrosis, contact lenses and catheters (Sankaridurg *et al.*, 2000; Govan and Deretic, 1996).

2.4. Pathogencity of *Pseudomonas aeruginosa*

P. aeruginosa is considered an essentially opportunistic pathogen and a bacterium of environmental origin. It is can cause a large set of distinct infections, both acute and chronic; it can invade any part of the body and commonly infects the respiratory tract of immunocompromised individuals and people with the cystic fibrosis disease (CF) (Pitt and Simpson, 2006). In intravenous drug abusers, native heart valves might be infected with *P. aeruginosa* (Bicanic & Eykyn, 2002). It can also cause meningitis and brain abscess when invading the central nervous system (Davidson *et al.*, 1982). Individuals with uncontrolled diabetes mellitus are at high risk for getting chronic otitis media moreover extension of this otitis can result in osteomyelitis and

more seriously can culminate in cranial nerve palsies (Sander, 2001). Bacterial keratitis and scleral abscess can also be a manifestation of *P. aeruginosa* (Hobden *et al.*, 1999). Vertebral osteomyelitis involving the areas of vertebral column pelvis and sternoclavicular joint might accompany infections caused by *P. aeruginosa* (Watanakunakorn, 1975). Following surgery catheterization and instrumentation an infection of urinary tract is frequently observed and it's almost hospital acquired (Shigemura *et al.*, 2006). *P. aeruginosa* is one of the curses of the burn units because it is the most frequent source of burn wound sepsis (Baltch, 1994).

2.5. Diseases caused by *Pseudomonas aeruginosa*

2.5.1. Pneumonia

Pneumonia is a common disease of the respiratory tract that constitutes a major problem and lead to considerable morbidity and mortality in developed world. The percentage of patients suffering from pneumonia and requiring hospitalization is not well estimated and may differ from state to state, reflecting different criteria for hospitalization and different available resources (Ewig *el al.*, 2009). *P. aeruginosa* is one of the most common gram-negative bacteria causing pneumonia in immunocompromised patients (Di Pasquale *et al.*, 2014) and it is almost associated with Ventilator-Associated Pneumonia (VAP) (McClure *et al.*, 2009). Ventilated patients are especially at high risk of developing pneumonia and *P. aeruginosa* is almost responsible for approximately 20of the cases and it also imposes considerable threat as it is difficult to treat (Fujitani *et al.*, 2011). The diminished susceptibility of these bacteria to antibiotics has become a major health issue worldwide. Few antimicrobials are in use to treat Gram-negative multidrug-resistant VAP and often Colistin remains the only effective drug (Kallel *et al.*, 2006).

2.5.2. Cystic fibrosis (CF)

P. aeruginosa is the most prevalent and significant pathogen responsible for chronic infection in individuals suffering from cystic fibrosis lung infection (Lyczak *et al.,* 2002). In lungs, the bacteria grow to high densities (107 –109 cfu/mL) within airway sputum, which likely provides the nutritional supplements during infection (Ohman and Chakrabarty, 1982). The generation time *P. aeruginosa* in CF sputum is highly variable but can be as short as forty minutes, indicating that sputum offers a robust growth conditions for *P. aeruginosa* (Palmer *et al.,* 2005). Long-term colonization of the lungs in CF patients may lead to the development of numerous adaptive phenotypes including mucoidy, amino acid auxotrophy, loss of antibiotic resistance and acute virulence factors (Barth and Pitt, 1995). In healthy persons, when inhaled bacteria are directed onto a mucus matrix lining the respiratory airways, moved upward by mucociliary action, and then swallowed and killed by gastric acids whereas the opportunistic pathogens able to combat mucociliary clearance are attacked by phagocytic cells aided by immunological mechanisms including specific opsonizing antibodies (Govan and Deretic, 1996). Bacterial colonization in the CF patients stands for a biological jigsaw puzzle including the combined pathophysiological outcomes of the CF gene mutation and pulmonary damage from previous microbial invasion with the ability of a relatively narrow spectrum of opportunistic pathogens to counteract normally highly efficient lung defenses (Butler *et al.,* 1994). CF is the most common inherited lethal genetic defect of Caucasian populations, with an incidence of nearly 1 in 2,500 live births and a carrier frequency of 1 in 25, 95 % of patients with CF will die from respiratory failure (Govan and Deretic, 1996; Ballmann *et al.,* 1998). Infection and inflammation are intimately linked early in the course of CF lung disease (Khan *et al.,* 1995). The observation of airway inflammation with no evident infection led to the suggestion that CF is linked with an intrinsic abnormality of immune regulation (Chmiel *et al.,* 2002). Non mucoid phenotype usually of *P. aeruginosa* usually accompany colonization during early stages of infection associated with CF, while mucoid colonies appear when the condition becomes chronic and the bacteria tend to form biofilms (Mena *et al.,* 2008).

2.5.3. Urinary tract infections (UTI)

Urinary tract infections are one of the most common bacterial infections affecting males and more frequently females through their life time (Mittal *et al.,* 2009). *P. aeruginosa* is an opportunistic human pathogen responsible for chronic infections especially medical device-associated infections as well as urinary tract infections (Ghafoor *et al.,* 2011). UTI is an inflammation results from the growth and multiplication of microorganisms anywhere in the urinary tract. It is perhaps the single most common bacterial infection of human (Ebie *et al.,* 2001). Urinary tract includes the organs that collect, store and release urine from the body namely: kidneys, ureters, bladder and urethra. Urinary tract infection is one of the most common bacterial infections in humans, both in the community and hospital environment and has been reported in both sexes and all age groups (Hooton *et al.,* 1995). UTI inssues as a result of interactions between the pathogen and host, the pathogenicity of uropathogens involves many processes, first the uropathogen attaches to the epithelial surface; it then multiplies and disseminates throughout the mucosa causing nearby tissue destruction. After the primary colonization stage, pathogens can ascend into the urinary bladder resulting in symptomatic or asymptomatic bacteriuria; further progression may result in pyelonephritis and renal dysfunction (Abrutyn *et al.,* 1988). UTI caused by *P. aeruginosa* is a serious health condition affecting millions of people worldwide each year and catheterization of the urinary tract is a predisposing factor to such infection (Mittal *et al.,* 2009). Pathogenicity of *P. aeruginosa* is mediated by the elaboration of a number of virulence factors including exotoxin A, alginate, exoenzyme S, elastase, phospholipase C, haemolysin, alkaline protease and siderophores (Woods *et al.,* 1986). Uncomplicated UTIs occur in sexually active healthy females with structurally and functionally normal urinary system. Complicated UTIs are those that occur in conjunction with certain conditions that prolong the need for treatment or increase the opportunity for therapeutic failure. These conditions include abnormalities of the urinary tract that impair urine flow, the presence of foreign bodies (e.g., indwelling catheter, stone), or infection with multidrug resistant organisms (Stapleton AE, 2008).

2.6. Antimicrobial drug resistance in *P. aeruginosa*

Antibiotic resistance is a worldwide issue of major; *P. aeruginosa* is responsible for a wide variety of infections that may present high rates of antibiotic resistance (Tumbarello *et al.,* 2011). This can be traced to the large genome of this microorganism which allows for great genetic capacity and high adaptability to environmental impacts (Lambert, 2002). Several factors may contribute the development and spread of antibiotic resistance including; improper and empirical application of wide spectrum antibiotics, inadequate hygiene, immunosuppression and extended periods of hospitalization (Khan and Zaman, 2006). In addition to the use of antibiotics in animal nutrition practices and overcrowded living conditions (Erb *et al.,* 2007). Furthermore, the ability of bacterial pathogens to adapt and to overcome the challenges of antibiotics in their environment has been nothing short of impressive (Lister *et al.,* 2009). Selection of an effective antibiotic is complicated by the ability of *P. aeruginosa* to develop resistance to different groups of antibiotics, even during the course of treating an infection (Aloush *et al.,* 2006). *P. aeruginosa* can develop resistance to antibiotic either through gaining of resistance genes on mobile genetic elements (plasmids) or through mutational changes that alter the expression and function of chromosomally encoded mechanisms. Both strategies for developing antibiotic resistance can severely decrease the therapeutic choices for the treatment of serious conditions (Lister *et al.*, 2009). However, the problem of antibiotic resistance is not limited to pathogenic bacteria. It also affects the commensal bacterial flora, which may become a major reservoir of resistant microbes (Erb *et al.,* 2007). *P. aeruginosa* shows the highest rates of resistance to fluoroquinolones (Ciprofloxacin, Levofloxacin) whereas lowest rates of resistance to the β-lactams (Cefepime, Ceftazidime, Piperacillin-tazobactam, Aztreonam, Imipenem and Meropenem) and Aminoglycosides (Amikacin, Tobramycin and Gentamicin) were recorded (Lister *et al.,* 2009). Resistance to the most frequently prescribed antibiotics, β -lactam antibiotics, for the management of UTI is mostly associated with bacterial production of different β -lactamase enzymes that destroy the β -lactam ring and inactivate the antibiotics (Bush and Macielag, 2010). Resistance to fluoroquinolones typically occurs as a result of a multi-steps mutational event in the

target enzymes (DNA gyrase and topoisomerase IV) and alterations in the drug entry and efflux. Aminoglycosides are broad spectrum bactericidal drugs such as gentamicin, amikacin and streptomycin. They act by inhibiting bacterial protein synthesis through inactivation of the ribosomes. Because of their remarkable toxicity, the use of these antibiotics has been restricted to serious infections (Mingeot-Leclercq *et al.,* 1999). In addition to the variety of aminoglycoside-modifying enzymes, methylation of the 16S rRNA can cause high-level resistance to multiple aminoglycosides. The later mechanism was first pointed to in 1993, and the methylase encoding gene was designated *rmtA* (Yokoyama *et al.,* 2003). Different resistance mechanisms have been noticed in *P. aeruginosa*, such as decreased permeability of antimicrobials through outer membranes, antimicrobial efflux mechanisms, lipopolysaccharide change, modification of DNA gyrase protein and inactivation or alteration of the antibiotic structure through enzymes release (Bert and Lambert-Zechovsky, 1996; Esparragón *et al.,* 1999).

2.7. Genomic of *P. aeruginosa*

P. aeruginosa genome (G + C content 65–67%, size 5.5–7 Mbp) is made up of a single circular chromosome and a variable number of plasmids. Sequencing of *P. aeruginosa* complete genomes has showed that the genome encodes for a large repertoire of transporters, transcriptional regulators, and two-component systems proteins which reflect its metabolic diversity to make use of a broad range of nutrients (Spencer *et al.,* 2003). *P. aeruginosa* has one of the largest genomes among bacterial pathogens, averaging 6.6 Mbp in size. As opposed to other bacterial species with extended genomes standing for gene duplication events (Serres *et al.,* 2009). Genome diversity is primarily due to accessory DNA elements located in 79 regions of genome plasticity that are distributed around the genome and reveal an anomalous usage of mono- to tetradeca nucleotides (Cramer *et al.,* 2011). The genetic makeup of *P. aeruginosa* reflects the lifestyle of this ubiquitous bacterial pathogen. This bacterium is found in various environmental habitats as well as in human and animal hosts, where they can act as opportunistic pathogens (Ramos, 2004). The genome of *P. aeruginosa*

strains is larger than those of most sequenced bacterial genomes. It is nearly made up of 6.3 million base pairs (Mbp) thus it is markedly larger than most of the 25 sequenced bacterial genomes. Moreover, genome size varies within the species between 5.5 and 7 Mbp (Schmidt *et al.*, 1996). The main part of the genome, the core genome, is present in all *P. aeruginosa* strains with the respective DNA generally collinearly arranged (Römling *et al.,* 1995). The core genome, with a few exceptions of regions that are subject to diversifying selection, is highly conserved among clonal complexes and reveals sequence diversities of 0.5–0.7 % (Spencer *et al.,* 2003; Cramer *et al.,* 2011). The accessory genome elements have obviously been acquired by horizontal gene transfer from different sources, including other species or genera. Therefore, a *P. aeruginosa* chromosome is often described as a mosaic structure of a conserved core genome frequently interrupted by the inserted portions of the accessory genome (Wiehlmann *et al.*, 2007).

2.8. Genomic islands and pathogenicity islands

Genomes of most strains of *P. aeruginosa* include a condiderable number of large and small genomic islands, involving those having virulence determinants (pathogenicity islands) (He *et al.,* 2004). Pathogenicity islands are functionally specialized genomic regions that encode virulence factors. The characterization of these pathogenicity islands in a wide range of pathogenic microorganisms has led to the identification and determination of virulence factors used to infect their respective hosts (Parkhill *et al.,* 2001). *P. aeruginosa* have the ability to survive in a diversity of niches due to the presence of various genomic islands that confer adaptive traits upon individual strains (Battle *et al.,* 2009). *P. aeruginosa* harbors a large amount of genetic material necessary for environmental versatility. Consistent with its capability to inhabit different niches, *P. aeruginosa's* large genome has one of the highest proportions of expected regulatory genes noticed among bacterial genomes 8.4 %of all predicted genes (Stover, *et al.,* 2000). Despite that *P. aeruginosa* genome harbors a large number of genomic islands. About 90 % of the *P. aeruginosa* chromosome is conserved (Wolfgang, *et al.*, 2003), but the core genome is interpreted with genomic islands,

which are not universally distributed among strains (Schmidt *et al.,* 1996). Genomic islands of *P. aeruginosa* constitute an accessory genome that may represent 10of an individual isolate's genetic makeup and are thought to participate to the survival potential of some *P. aeruginosa* strains to inhabit severe environments (Spencer, *et al*., 2003, Shen, *et al.,* 2006). The pathogenic multiplicity of certain strains of *P. aeruginosa* is further introduced via factors carried by pathogenicity islands (He *et al.,* 2004). *P. aeruginosa* Pathogenicity Island in PAPI-1 of strain PA14 for example is an accumulation of 108 genes that encode a number of virulence determinants (Qiu *et al.,* 2006). *P. aeruginosa* Pathogenicity Island PAPI-1can be mobilized from a donor chromosome and transferred into a recipient lacking this island (He *et al.,* 2004; Qiu *et al.,* 2006).

2.9. Typing methods

Strain typing is a completing part of epidemiological survey of bacterial infections in both health cares' as well as in the community settings. In general, typing methods fall into two main categories including; phenotypic typing methods, those that detect traits expressed by microorganisms, and genotyping techniques, and those that involve direct or indirect nucleic acid detection approaches of chromosomal or extra-chromosomal genetic elements (Leeuwen, 2009).

2.9.1. Phenotypic methods

Phenotypic methods are those that describe the outcomes of gene expression to identify the species level or to discriminate strains, such as biochemical test result pictures, susceptibility to bacteriophages, the different cell surface proteins, whole protein analysis and antimicrobial susceptibility profile (Gaston *et al.,* 1988). Since these characteristics are mainly based on gene expressions, they will eventually change (Leeuwen, 2009).

2.9.1.1 Biotyping

Biotyping is one of the most widely used techniques. It relies on the differentiation of microorganism according to their features such as differences in biochemical properties, morphology, and environmental stress tolerances. Biotyping is routinely adopted in laboratories uses automated systems designed for species identification and differentiation (Singh *et al.,* 2006). The main advantageous potential of the system occurs in its incredible ability to distinguish among strains within a species (Bochner *et al.,* 2001). The methods of biotyping are usually easy and inexpensive, plus the resulted data are simple to score and interpret, and all tests can be accomplished, even in the smallest laboratories with limited capabilities, on large numbers of isolates (Belkum *et al.,* 2007). Biotyping typically has poor discriminatory ability and cannot distinguish among some of the newly discovered nosocomial pathogens, where biochemical diversity is uncommon (Singh *et al.,* 2006).

2.9.1.2. Antibiogram-based typing

Antibiogram typing depends on comparison of susceptibilities of microbial strains to a range of antibiotics (Mehndiratta and Bhalla, 2012). It can be done either by antibiotic diffusion in solid growth media or by making serial drug dilutions in liquid media using different measurement systems (Belkum *et al.,* 2007). This method is easy to accomplish, inexpensive, non-time consuming and is readily available in ordinary microbiology laboratory; and has been frequently used for screening of epidemic strains. However, in most instances; antibiogram typing cannot be used solely because of its poor discriminatory power. Antibiotic resistance profiles are affected by the surrounding environment, selective antibiotic pressure, loss and acquisition of extra-chromosomal elements carrying resistance genes and various other genetic mechanisms (Mehndiratta and Bhalla, 2012).

2.9.1.3. Serotyping

Serotyping is traditionally considered as the most important phenotypic technique applied in epidemiological investigations in tracing the origin of infection and defining the various pathogenic strains of *P. aeruginosa*. Serotyping for *P. aeruginosa* has a long and conflicting history because microbiologists from different institutions are willing to utilize different serotyping schemata for this microorganism and the results obtained and declared were often not directly related (LIU, 1987).

2.9.2. Genotyping methods

Since the late 1980, various genetic methods have been used for genotyping of bacteria and become frequently applied for bacterial identification (Yıldırım *et al.*, 2011). The invention and extensive application of these methods which relied upon the direct analysis of genomic polymorphisms have revolutionized the understanding of the evolutionary history, population movements and patterns of distribution of bacteria (Miragaia *et al*., 2008). The main characteristics of the DNA molecule make it as an extremely useful tool for species identification on molecular bases, DNA is a very stable and long-lived biochemical molecule, it can be found in all body tissues or fluids with nucleated cells or non-nucleated cells and can also be found in plastids and/or mitochondria. Finally, DNA can provide more valuable information than proteins due to the degeneracy of the genetic code and the existence of large non-coding regions (Pereira *et al.,* 2008). Genotyping methods can be categorized into three major methods including; Restriction based methods, Polymerase chain reaction and DNA Sequence based methods (Bonofiglio *et al.,* 2012).

2.9.2.1. Polymerase chain reaction (PCR)

The development of polymerase chain reaction (PCR) by K. Mullis and co-workers in 1985 has opened a new era in the field of molecular biology (Saiki *et al.*, 1985). It was practiced as a promising *in vitro* tool for enzymatic amplification of the

intended nucleic acid sequences using short oligonucleotide primers and thermal resistant DNA polymerase. The basic technique of PCR involves repeated cycles of amplification for a selected nucleic acid sequences (Mullis and Faloona, 1987; Rijpens and Herman, 2002). Each cycle consists of three steps; a DNA unwinding step, in which the two strands of the DNA double helix are separated; a primer annealing step, in which primers anneal to their complementary target sequences and it's often performed at a lower temperature; and an extension reaction step, it starts when DNA polymerase extends the sequences between the primers, at the end of each cycle (each consisting of the above three steps). The quantities of PCR products are theoretically doubled. The whole procedure is accomplished in a thermal cycler. In general, the performance of 30 to 50 thermal cycles results in an exponential increase in the total number of the synthesized DNA copies (Eisenstein, 1990; Tang *et al.*, 1997). The polymerase chain reaction is widely accepted as a convincing molecular technique that has been popularly utilized in the investigation and diagnosis of an elongated list of diseases, molecular fingerprinting and detection of infectious agents (Joshi and Deshpande, 2010; Rahman *et al.*, 2013).

2.9.2.2. Species-specific PCR

The introduction of extremely rapid molecular based technologies has dramatically influenced the diagnosis and surveillance of infectious diseases (Gillespie and Hawkey, 2006). Identification and confirmation of specific DNA region associated with known pathogenicity-related genes or conserved sequences unique to a particular pathotype can be used to aid confirmation of the identity of a specific bacterial species (Samadpour *et al.*, 1994). PCR is a widely accepted technique used for the detection and identification of a microbial strain in a complex sample like clinical, environmental and food matrices because of its high versatility, specificity and sensitivity (Beneduce *et al.*, 2007). The PCR is the most sensitive of the whole available rapid methods to identify microbial pathogens in clinical specimens. Especially, for the specific pathogen that is difficult to culture *in vitro* or require a long cultivation period (Yamamoto, 2002).

2.9.2.3. ERIC-PCR assay

Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) methods could differentiate the genotypes of the large number of O antigen serotypes, thus enabling determination of an appropriate immunogenic strain (Guay, 2009; Flores and Okhuysen, 2009). Some researchers determined the sequence distribution and copy numbers on chromosomes for ERICs and revealed the presence of interspecies specificity. ERIC sequences, also described as intergenic repetitive units, are characterized by a higher distribution rate across a wider range of species than any other repeat. ERIC sequences were first described in *Escherichia coli*, *Salmonella typhimurium* (now Salmonella enterica serovar Typhimurium), and other members of the Enterobacteriaceae, as well as *Vibrio cholera* (Sharples and Lloyd 1990; Hulton, Higgins, and Sharp 1991).The ERIC-PCR method is rapid, sensitive, repeatable and reliable; therefore, it could be widely applied in the molecular differentiation of microbes (Guimarãesa *et al.,* 2011). ERIC-PCR involves the use of oligonucleotides directed to short repetitive sequences scattered throughout various bacterial genomes. ERIC sequences are distributed throughout the genome of enterobacteriaceae in different orientations thus allowing discrimination at genus and serovars level based on their electrophoretic amplification products (Yoke-Kqueen *et al.,* 2008). ERIC-PCR uses any combination of primers designed to the conserved ERIC sequences in order to obtain an electrophoretic banding profile based on the frequency and orientation of ERIC sequences in a bacterial genome. Molecular genotyping by ERIC-PCR is faster and more cost-effective than pulsed-field gel electrophoresis or multilocus sequencing for generating information about the genetic similarity of bacterial strains (Puente-Redondo *et al.,* 2000; Saxena *et al.,* 2002). ERIC sequences have been discovered only in intergenic regions, apparently only within transcribed regions and the number of copies of the ERIC sequence varies among species (Hulton and Sharp, 1991).

2.9.2.4. DNA Sequence based methods

DNA sequencing-based techniques are considered the best approach used for subtyping and characterizing bacterial isolates. In these methods, complete or partial nucleotide sequences are determined for one or more bacterial genes or chromosomal regions, thus providing clear and discrete data (Cai *et al.*, 2002). The rapidly increasing list of sequenced microbial genomes has provided catalyst for the development of a variety of molecular typing approaches that focus on either single or multiple chromosomal loci (Singh *et al.,* 2006). Until 2009, 788 fully microbial genomes were sequenced (Lui *et al.,* 2009).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Equipments

Table 3.1. The equipment used in this study.

Equipment Name	Company _Origin
Autoclave	Daikyo_ Spain
Bench Top Centrifuge	Sigma_Germany
33130 Genetic analyzer systems	Applied Biosystems_ USA
Gel-electrophoresis apparatus	Ammersham_ USA
Hot Plate Magnetic Stirrers	Stuart _ England
Incubator	Prodit _ Italy
Ice-maker	Scotman
Microcentrifuge	Hettich _Germany
Microshaker	Camlab _England
NanoDrop Spectrophotometer	Thermo Scientific _USA
Orbital Shaker	Stuart _ England
PH-Meter	Hanna _Portugal
Refrigerated Bench Top Centrifuged 5810R	Eppendorff_ USA
Roller Mixer	Local
Sensitive Balance	Voyager _Switzerland
Thermal cycler	AppliedBiosystems_Singapore
UV. Transilluminater	HVD life Sciences _Austria
Water Bath	ShelLAB _USA
Water Distillater	Lab Tech _Korea

3.1.2. Chemicals

Table 3.2. The chemicals used in this study

Chemicals	Company _Origin
Agarose	Promega_ USA
Amyl alcohol (iso) LR C5H11OH	LaboratoryRasayan
Boric acid	Riedel-de Haen_ USA
Bromophenol Blue	BDH _ England
Chloroform	Scharlau _ Spain
Ethylene diamine tetraacetic acid (EDTA)	Sigma _Germany
Ethanol 99.9	Scharlau _Spain
Ethidium Bromide solution (10mg/ml)	Promega _USA
Glycerol	BDH _England
Cexadecyl Trimethyl Ammonium Bromide	Sigma _Germany
Hydrochloric Acid HCl	α -Alpha _Indian
Hi Di Formamide	Applied Biosystems_USA
Isopropanol (2-propanol)	Scharlau _ Spain
Sucrose	BDH _England
Sodium Chloride (NaCl)	Sigma _Germany
Sodium Hydroxide(NaoH)	Scharlau_Spain
Sodium Dodecyl Sulfate (SDS)	Sigma _Germany
Tris-base	Promega _USA

3.1.3. Culture media

The media which have been used in this study are listed in Table 3.3.

Table 3.3. Culture media used in this study

Culture Media	Company _Origin
Brain Heart Infusion Agar	Difco _USA
Brain Heart Infusion Broth	Difco _USA
Blood Agar	Oxoid_England
MacConkey Agar	LabM _UK
Mueller-Hinton Agar	Difco _USA
Nutrient Agar	Difco _USA
Cetrimide Agar	Difco _USA
Simmons Citrate Agar	Oxoid_England

3.1.4. Antibiotics

Sixteen antibiotic disks have been used in this study, supplied by (Bioanalyse/ Turkey) in table 3.4 illustrates these antibiotics, their codes, and potency (µg).

Table 3.4. The antibiotic disks used in this study

No.	Antibiotics	Code	Disc Potency
1.	Amikacin	AK	10 µg
2.	Tobramycin	TN	30 µg
3.	Gentamicin	CN	10 µg
4.	Ticarcillin/clavulanic acid	TNC	10 µg
5.	Ampcillin/Sulbactam	AMS	5 µg
6.	Ampcillin	Am	30 µg
7.	Piperacillin	PN	(20/10) µg
8.	Meropenem	MP	10 µg
9.	Imipenem	IMP	25 µg
10.	Cefepime	CFM	30 µg
11.	Ceftazidime	CFZ	10 µg
12.	Ceftriaxone	CRO	10 µg
13.	Cefuroxime	CTX	10 µg
14.	Ciprofloxacin	CFN	75 µg
15.	Aztreonam	An	10 µg
16.	Trimethoprim/Sulfamethoxazole	STX	10 µg

3.1.5. Primers

The primers listed in Table (3.5) have been obtained from Operon Incorporation (USA) Technologies in lyophilized forms. These primers were prepared to the required concentration by deionized distilled water according to the supplier recommendation.

Table 3.5. The primers used in this study

Method and primer	Oligonucleotide sequence (5–3) Forward and Reverse	Size of amplicons	Reference
Species specificPCR *16SrDNA*	F-GGGGGATCTTCGGACCTCA R-TCCTTAGAGTGCCCACCCG	956 bp	Spilker *et al.*, 2004
Virulence gene *Nan1*	F-AGGATGAATACTTATTTTGAT RTCACTAAATCCATCTCTGACCCGA	1316 bp	Stover *et al.*, 2000
Exo-S	F-CTTGAAGGGACTCGACAAGG R-TTCAGGTCCGCGTAGTGAAT	504 bp	Stover*et al.*, 2000
Las-B	F-GGAATGAACGAAGCGTTCTC R-GGTCCAGTAGTAGCGGTTGG	300 bp	Stover *et al.*, 2000
Tox-A	F-GACAACGCCCTCAGCATCACCAGC R-CGCTGGCCCATTCGCTCCAGCGCT	396 bp	Rawya*et al.*, 2008
Opr1	FATGAACAACGTTCTGAAATTCTCTT R-CTTGCGGCTGGCTTTTTCCAG	249 bp	De Vos *e al.*, 1993
ERIC PCR ERIC	F-ATGTAAGCTCCTGGGGATTCAC R-AAGTAAGTGACTGGGGTGAGCG		Ram *et al.*, 2011
Antibiotic resistance gene *VIM-1*	F-CAGATTGCCGATGGTGTTTGG R-AGGTGGGCCATTCAGCCAGA	523 bp	Dong *et al.*, 2008
IMP-1	F-GGAATAGAGTGGCTTAATTCTC R-GGAATAGAGTGGCTTAATTCTC	361 bp	JácomePrla*et al.*, 2012
SPM-1	F-CCTACAATCTAACGGCGACC R-TCGCCGTGTCCAGGTATAAC	831 bp	Dong *et al.*, 2008
Oxa-10	F-TATCGCGTGTCTTTCGAGTA R-TTAGCCACCAATGATGCCC	760 bp	Mirsalehian *et al*. 2010
Oxa-2	F-GCCAAAGGCACGATAGTTGT R-GCGTCCGAGTTGACTGCCGG	700 bp	Tanaz *et al.*,2010
VEB-1	F-CGACTTCCATTTCCCGATGC R-GGACTCTGCAACAAATACGC	643 bp	Mirsalehian *et al*. 2010
GIM	F-TCGACACACCTTGGTCTGAA R-AACTTCCAACTTTGCCATGC	477 bp	Ellington *et al.*, 2007
SIM	F-TACAAGGGATTCGGCATCG R-TAATGGCCTGTCCCATGTG	570 bp	Ellington *et al.*, 2007
OprD	F- ATGAAAGTGATGAAGTGGAGC R- CAGGATCGACAGCGGATAGT	500 bp	Pirnay *et al.*, 2002

3.1.6. Enzymes and other biological materials

Table 3.6. Enzymes and other biological materials used in this study

Enzymes and Other Biological materials	Concentration	Company (Origin)
Lysozyme	50mg/ml	USB (Germany)
Proteinase K	20mg/ml	Promega (USA)
Deoxynucleoside triphosphates mixture dNTPs; (dATP, dGTP, dTTP, dCTP)	0.2mM/µl pHm7.0	Promega (USA)
Undigested Lambda DNA (λDNA)	250µg/µl	promega (USA)
Molecular weight marker (100-1500bp.)	-----	Promega (USA)
Taq. DNA Polymerase	5unit/ µl	Sigma- Germany

3.1.7. Kits used for molecular methods

Table 3.7. kits used for molecular methods in this study

Kit name	Company(Origin)	CatalogueNo.	Purpose
Master Mix	GeneDirex-USA	MB203-0100	PCR amplification

3.1.8 Sample collection

A total of 225 clinical isolates of *P. aeruginosa* burn infection isolates were collected from different patients attending major hospitals in Duhok and Erbil / Iraq from both sexes, different ages and percentage of burn during the period from April 2015 to September 2015. On the basis of clinical judgment of infection, swabs of pus from infected burn wound were collected at the time of change of dressing.

3.2. Methods

3.2.1 Sterilization methods

All media, solutions and other autoclavable instruments were sterilized using autoclave at 121˚C (15 pound/in^2) for 15 min.

3.2.2 Culture media preparation

All culture media were prepared according to the manufactures instruction.

3.2.3. Culture media characterization of *P. aeruginosa*

All collected clinical isolates of suspected *P. aeruginosa* burn infection isolates were recultured on media including MacConkey agar, Cetrimide agar, Blood agar and Nutrient agar by streak plate method incubated at 37 ˚C for 24 hours (Cheesbrough, 2006).

3.2.4. Biochemical test and reagents

Oxidase test indicator: It was freshly prepared as 1by dissolving 0.05 gm of Tetra methyl-P-Phenlyene diamine dihydrochloride in 5 ml D.W. and stored in a dark container**.**

3.2.5. Preservation of bacterial strains

All isolates of *P. aeruginosa* burn infection isolates are maintained in 10ml brain heart infusion agar slants which prepared in screw-capped bottles and stored at 4˚C. All the confirmed *P. aeruginosa* burn infection isolates were stored for long storage period in 50 glycerol broth at -20 ˚C (Suhaim *et al.,* 2012). It was prepared by adding 500 µl of bacterial growth to 500 µl of 50 of sterilized glycerol in 1.5ml microcentrifuge tube.

After 24 hours, the viability and purity of the organism was checked by reculturing it in brain heart infusion broth as enrichment media then it was cultured on MacConkey agar and Cetrimide agar.

3.2.6. Antibiotic susceptibility test

The isolates were subjected to antibiotic sensitivity testing by the disc diffusion method on Mueller-Hinton agar according to the National Committee for Clinical Laboratory Standards and Manual of Antimicrobial Susceptibility Testing guidelines (Cheesbrough, 2006; CLSI, 2007). The inoculums were prepared by taking at least four morphologically similar colonies with a sterile loop into 10ml of brain heart infusion broth, incubated at 37 ˚C for 16 hr., a sterile cotton-wool swab was dipped in the broth and the excess of the broth was removed by turning the swab against the inside of the container. The inoculum was spread evenly over of the plate of Muller-Hinton agar in three directions, rotating the plates through the angle 60 ˚C to ensure even distribution and finally the swab passed around the edge of the agar surface, then the inoculum was left to dry for few minutes at room temperature. After that, antibiotic discs were placed on the inoculated plate using sterile forceps. Discs were readily placed at 30-36 mm distance to avoid overlapping of inhibition zone and these plates were incubated at 37 ˚C for 24 hour. The diameter of each inhibition zone was measured and interpreted as resistant or sensitive using the interpretative chart of the zone sizes.

3.2.7. Preparation of stock solutions and buffers

All stock solutions and buffers were prepared according to Maniatis *et al.*, (1982)

3.2.7.1. 1M Tris –HCl pH 8.0

This was prepared by dissolving 121.1gm of Tris-base in 800 ml of distilled water; pH was adjusted to 8.0 by adding few drops of 10 NHCl. The volume was made up to 1000 ml by adding distilled water.

3.2.7.2. 0.5 M EDTA pH 8.0

This was prepared by dissolving 186.1 gm of EDTA in 800 ml of distilled water; pH was adjusted to 8.0 by adding few drops of 10 M NaOH. The volume was made up to 1000 ml by distilled water, sterilized by autoclaving.

3.2.7.3. 10X Tris-Borate-EDTA buffer (10XTBE)

This was prepared by dissolving 108 gm of Tris-base and 55 gm of boric acid then 40ml of EDTA (0.5 M pH 8.0) was added, and dissolved in 800 ml of distilled water; pH was adjusted to 7.8 by adding few drops of 10N HCl. The volume was made up to 1000 ml by adding distilled water.

3.2.7.4. 6X Loading buffer

It was prepared by dissolving 0.25 gm of Bromophenol blue with 30 ml of glycerol; in 50ml of distilled water; pH was adjusted to 8.0 by adding few drops of 10N NaOH. The volume was made up to 100 ml by adding distilled water and kept at 4^{o}C.

3.2.7.5M Sodium chloride

This was prepared by dissolving 146.1 gm of sodium chloride in 400 ml of distilled water. The volume was made up to 500 ml by distilled water.

3.2.7.6. Sodium hydroxide (10M)

It was prepared by dissolving 40 gm of NaoH in 80 ml of distilled water; the volume was made up to 100 ml by distilled water.

3.2.7.7. 10 % SDS solution

It was prepared by dissolving 20 gm of SDS in 200 ml of distilled water with heating at 55 ˚C.

3.2.7.8. TE 25S solution

This buffer consists of 25 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0 and 0.3 M sucrose. This solution was prepared by dissolving 51.3 gm of sucrose, 12.5 ml of 1 M Tris-base and 25 ml of 0.5 M EDTA. The volume was made up to 500 ml by adding distilled water.

3.2.7.9.Tris-EDTA buffer (TE)

This buffer consists of 10mM Tris-HCl (pH 8.0), 1mM EDTA (pH8.0). This buffer was prepared by adding 5ml 1MTris-base and 1ml of 0.5M EDTA, the volume was made up to 500 ml with distilled water.

3.2.7.10. CTAB/NaCl

It was prepared by dissolving 20 gm of cetyl trimethlammonium bromide CTAB to 28 ml of heated 5 M NaCl the volume was made up to 100 ml by distilled water.

3.2.7.11. Chloroform isoamyl (24:1)

Comprised of 96 ml of Chloroform and 4 ml of Iso-amylalcohol and kept at 4 ˚C until to use.

3.2.7.12. Ethanol 70 %

Comprised of 70 ml of absolute Ethanol and 30 ml of distilled water.

3.2.7.13. Undigested lambda DNA

Undigested λ DNA with concentration of 25 ng/µl was prepared from the stock solution of λ DNA by diluting it to obtain required concentration by sterile deionized distill water.

3.2.8. Extraction of genomic DNA

One hundred isolates of *P. aeruginosa* are selected and subjected to molecular methods. Fifty isolates were selected randomly from each province (Erbil and Duhok). Genomic DNA extraction was carried out using traditional method and commercial kit (DNP ™ High yield DNA Purification). However, Prior to DNA extraction in each method, strains were cultured on MacConkey agar plate at 37 ˚C for 24 hrs and pure few single colonies from MacConkey agar plate was used for extraction. Extracted DNA was stored in a freezer at -20 ˚C, ready to be used later for PCR.

3.2.8.1. Extraction of genomic DNA by traditional method

Genomic DNA of fifty *P. aeruginosa* strains was prepared according to the method described by Kiesser, 1995. A single isolated *P. aeruginosa* colony was inoculated into 30 ml of brain heart infusion broth and after incubated at 37 ˚C for 24 hours with continuous shaking, the bacterial cells were harvested by centrifugation at

4000 rpm for 25 minutes. The pellet was resuspended in 3.5 ml TE25S buffer and mixed well and 100 µl of lysozyme (50 mg/ml) solution was added to this suspension, incubated at 37 ˚C for 1 hour. After the incubation, 50 µl of Proteinase K (20 mg/ml) and 200 µl of SDS (10 %) were added to the mixture and further incubated at 55 ˚C with shaking for 1 hour. Then 850 µl of NaCl (5 M) and 850 µl CTAB/NACL were added to the lysate and incubated at 55 ˚C in water bath for 10 minutes. The tubes were left at room temperature for 5 minutes. Then equal volume of chloroform/ isoamyl (24:1) was added and mixed gently by using mixer for 30 minutes and centrifuged at 4000 rpm for 30 minutes this step was repeated twice. The supernatant was transferred into a sterile tube and 0.6 volume of cooled isopropanol was added, and mixed gently by inverting them several times. Tubes were left at -20 ˚C for 30 minutes. The DNA mass was taken by hock class and washed with 1ml of 70 ethanol. The DNA mass was air dried for few minutes. The nucleic acid was dissolved in 500 µl TE buffer.

3.2.8.2. Extraction of genomic DNA using commercial kit

Genomic DNA was extracted from one hundred *P. aeruginosa* strains using High yield DNA Purification Kit according to the manufacture instructions (Bioneer-Korea).

3.2.9. Determination of the concentration and purity

The concentration and purity of genomic DNA for each sample were determined using NanoDrop Spectrophotometer by recording the concentration and purity of each sample. This work had been done in the Scientific Research Center-Veterinary Faculty-Duhok University.

3.2.10. Gel electrophoresis

3.2.10.1. Agarose gel electrophoresis

Agarose gel was prepared with different concentrations according to the required application. For example 1 (w/v) of agarose gel in TBE was used for the detection of geneomic DNA by adding 1 gm of agarose to 100 ml of 1X TBE buffer and it is dissolved by heating at boiling temperature. Then the agarose was left to cool at 55 ˚C, before pouring in a casting plate to solidify. A required comb was placed near one edge of the gel, and the gel was left to cast. 1XTBE was poured into the gel tank and the gel plate was placed horizontally in an electrophoresis tank. The DNA samples were prepared by adding 1 µl of loading buffer and mixed with 5 µl DNA samples, and then the samples were added carefully to individual wells. Power was turned on at 45 V for 15 minute and 85 V for 1-2 hours to run DNA or at 5-8 v/cm. Agarose gels were stained with ethidium bromide by immersing them in distilled water containing the dye at a final concentration of 0.5 µg/ml for 15-30 minute. After de-staining shortly in distilled water, DNA bands were visualized by U.V. illumination at 366 nm wavelength on U.V. transilluminator. Then the gels were documented using Digital camera (Maniatis *et al.*, 1982).

3.2.11. PCR-amplification

3.2.11.1. Species-specific PCR-amplification

Internal fragment of *16srDNA* gene with molecular weight 956 bp. was amplified using one pair of *16srDNA* primer including (Forward and Reverse). Master-mix was prepared for 100 of *P. aeruginosa* isolates plus control in each test. The amplification reaction consist of 25 µl as final volume for each sample containing 12.5 µl of master mix, 1µl of each primer including forward and reverse (10 pmol/µl), 4 µl of genomic DNA (25-50 ng/ µl) and 6.5 µl of sterile deionized distil water. All prepared reaction tubes were placed in the thermal cycler to carry out the amplification. The

Amplification conditions were illustrated in Table 3.8. The presence of the PCR product was confirmed electrophoretically using 1.5 (w/v) of agarose in TBE Buffer. Molecular marker (1500-100bp) was used to determine the molecular weight of PCR product.

Table 3.8 Amplification conditions of Species-Specific PCR analysis using *16srDNA* primer.

Initial denaturation	Denaturation	Annealing	Extension	Final Extension
95˚**C** 2 min	94 ˚C 20 sec.	54 ˚C 20 sec.	72˚C 40 sec	72˚C 5 min
1 cycle		25cycles		1 cycle

3.2.11.2. Screening of virulence related genes using PCR amplification

Five primers (*nan-1, exo-s, las-b, tox-a, opr-1*) were used for the detection of virulence related genes including; Putative neuraminidase, Exoenzyme S, elastase A zinc metalloprotase, Exotoxin A and outer membrane lipoprotein among 100 selected *P. aeruginosa* strains.The amplification reactions for each gene were carried out in 25 µl volumes containing 12.5 µl of master mix, 1 µl of each primer including forward and reverse (10 pmol/ µl for *nan-1, exo-s* 12.5 pmol/ µl for *las-b* 30 pmol/ µl for *opr-1* and 0.5 pmol/ µl for tox-a) and 4 µl (25-50 ng) of genomic DNA. The volume was completed to 25 µl by adding 6.5 µl of sterile de-ionized distilled water. The Amplification conditions of each gene were illustrated in Table 3.9 After aplification, the presence of the PCR product was confirmed electrophoretically using 1.5 (w/v) of agarose in TBE Buffer. Molecular marker (1500-100bp) was used to determine the molecular weight of PCR product.

Table 3.9 Amplification conditions of PCR- based analysis for the detection of different virulence markers among *P. aeruginosa* isolates using *nan-1, exo-s, las-b, tox-a* and *opr-1* primers

Primer	Initialdenaturation	Denaturation	Annealing	Extensin	Final extension
Nan-1	94˚C 5 min. Cycle	94˚C 30 sec.	54C^{o} 1 min 36 cycles	72˚C1min.	72 ˚C 90 sec. 1cycle
Exo-s	95˚C 2 min. 1 cycle	94˚C 30 sec.	60 ˚C 1min. 35 cycles	72 C^{o}1min	72˚C 5 min. 1 cycle
Las-b	94˚C 3min 1 cycle	94˚C 30 sec.	60 ˚C 1min. 30 cycles	72˚C 90sec.	72˚C 5 min. 1 cycle
Tox-a	94˚C 2 min 1 Cycle	94˚C 2 min.	68˚C 1 min. 30 cycles	72˚C 1min.	72˚C 7min. 1cycle
Opr-1	95˚C 2 min 1 cycle	94˚C 40 sec.	57˚C50 sec. 25 cycles	72˚C 20sec.	72˚C 5 min. 1 cycle

3.2.11.3. Screening of antibiotic resistance related genes using PCR amplification

Eight primers were used for the detection of metallo-B-lactamases (MBL) and extended-spectrum B-lactamases (ESBL) Genes. (*vim-1, imp-1, spm-1, gim, sim, oxa10, oxa2,veb-1*), among 100 selected *P. aeruginosa* strains. The amplification reactions for each gene were carried out in 25 µl volumes containing 12.5 of master mix, 1µl of each primer including forward and reverse (10 pmol/µl except for vim-1 20 pmol/µl and for *oxa-10* 0.5 pmol/ µl) and 4 µl (25-50 ng) of genomic DNA. The volume was completed to 25 µl by adding 6.5 µl of sterile de-ionized distilled water. The Amplification conditions of each gene were illustrated in Table 3.10. After amplification, the presence of the PCR product was confirmed electrophoretically using 1.5 (w/v) of agarose in TBE Buffer. Molecular marker (1500-100bp) was used to determine the molecular weight of PCR product.

Table 3.10 The amplification conditions of PCR- based analysis for the detection of different antibiotic resistance markers among *P. aeruginosa* isolates using *imp-1,spm-1 ,oxa-2,veb-1,gim sim, oxa-10 and vim-1*primer

Primer	Initial denaturation	Denaturation	Annealing	Extensin	Final extension
ipm-1	95˚C 5 min. 1 cycle	95˚C 1 min.	50.6˚C1min 30 cycles	68 ˚C1 min.	68˚C 10 min. 1 cycle
Spm-1	95˚C 5 min. 1 cycle	95˚C1 min.	64 ˚C1 min. 35 cycles	68 ˚C 1min.	68˚C 10 min. 1 cycle
Oxa-2	94˚C 5 min. 1 cycle	95˚C 30 sec.	56 ˚C90sec. 35 cycles	72˚C 1 min.	72˚C 10 min. 1 cycle
Veb-1	94˚C 5 min 1Cycle	94˚C 45 sec.	64˚C 1 min. 36 cycles	72˚C 1 min.	72˚C 7min. 1cycle
Gim	94˚C 5 min 1 cycle	94˚C 1 min.	56˚C 1 min. 36 cycles	72˚C 1 min.	72˚C 5 min. 1 cycle
Sim	94˚C 5 min 1 cycle	94˚C 1 min.	64˚C 1 min. 36 cycles	72˚C 1 min.	72˚C 5 min 1 cycle
Oxa-10	95 ˚C 4 min. 1 cycle	95˚C 45 sec.	56˚C 1 min. 30 cycles	72˚C 1 min	72˚C 5 min 1 cycle
Vim-1	95˚C 5 min 1 cycle	95˚C 1 min.	62˚C 1 min. 30 cycles	68˚C 1 min.	68˚C 10 min 1 cycle

3.2.11.4. ERIC-PCR amplification

Universal primers based on Entrobacterial Repetitive Intergenic Consensus ERIC sequences were used to DNA amplification. The amplification reactions for each sample were carried out in 25 µl volumes containing 12.5 µl of master mix, 1.5 µl of each primer including forward and reverse (10 pmol/µl), and 4 µl (25-50 ng) of genomic DNA. The volume was completed to 25 µl by adding 5.5 µl of sterile de-ionized distilled water. The Amplification conditions ERIC were illustrated in Table 3.11 After amplification, the presence of the PCR product was confirmed electrophoretically using 1.5 (w/v) of agarose in TBE Buffer. Molecular marker (1500-100bp) was used to determine the molecular weight of PCR product. PCR patterns were visually evaluated, and a matrix was compiled, in which each taxon was reported in a separate column and the presence/absence of DNA fragments of the same length was

reported as 1/0 in rows. The matrix was analyzed with UPGMA method (Unweighted Pair Group Method with Arithmetic Mean) Cluster analysis.

Table 3.11 Amplification conditions of ERIC-PCR analysis using ERIC primer.

Primer	Initial denaturation	Denaturation	Annealing	Extensin	Final extension
ERIC	94˚C 7 min.	94˚C 30 sec.	38˚C 1 min	72˚C 5 min.	72˚C 15 min.
	1 cycles	34 cycles			1cycles

3.2.11.5. PCR Amplification of DNA sequences

The amplification reactions for 15 samples (*oprD* gene) was carried out in 50 µl volumes containing 25 µl master mix, 2 µl of primer including forward and reverse (0.6 pmol/µl) and 8 µl (25-50 ng) of genomic DNA. The volume was completed to 50 µl by adding 13 µl of sterile de-ionized distilled water. The Amplification conditions of primer was illustrated in Table 3.12. The presence of PCR products was confirmed by mixing 5 µl of each PCR product with 1 µl of loading buffer and passing mixture through 1(w/v) agarose in TBE Buffer. Molecular marker (1500-100bp) was used as a ladder to confirm the correct size of PCR product and running at 5-8 v/cm for 1 hr. followed by staining in Ethidum bromide 0.5 µg/ml for 20 min then visualization of the bands under UV transillumination.

Table 3.12 Amplification conditions of DNA sequences PCR analysis using *OprD* primer**.**

Primer	Initial denaturation	Denaturation	Annealing	Extensin	Final extension
oprD	95˚C 5 min.	94˚C 30 sec.	65 ˚C 45sec	72 ˚C45sec.	72˚C 10 min.
	1 cycles	35 cycles			1 cycles

3.2.11.6. Sequence determination and analysis

The sequences of the reaction products were determined using a capillary electrophoresis sequencer **(**ABI 3130 DNA sequencer) This work had been done in South Korea. Raw sequences were reviewed by visual inspection with Chromas 5 V software to form contig of each target gene using forward and reverse sequences. Single nucleotide sequences analysis was performed by multiple sequence alignments of the corresponding *OprD* gene for 15 *P. areuginosa* isolates with *Str.PA12* (accession number KJ482587.1) *as* reference strain using BioEdit (Version 8). MEGA4was used for phylogenetic analysis and construction of neighboring trees.

4. RESULTS

4.1. Microbiological investigations

4.1.1. Characterization and incidence of *P. aeruginosa*

The comes about of refined of all isolates on MacConkey and Cetrimide agar affirmed to be *P. aeruginosa* and guaranteed them from any defilement amid transporting of tests from distinctive clinics in Erbil and Duhok/ Iraq. These isolates were found lactose ferment creating negative pale yellow colonies on MacConkey agar and on Blood agar β-hemolytic colonies. Colonies are surrounded by bluish green coloration on Nutrient agar due to produced soluble pyocyanin and pyoverdine which is a water-soluble, yellow-green pigment, the colonies pigments on selective media (Cetermide agar) are more obvious (Prescott *et al.,* 1993). *P. aeruginosa* burn contamination confines were affirmed by biochemical tests counting oxidase test. Citrate utilization too identify capacity of development at a temperature as tall as 42 ˚C (Cunliffe *et al*., 1995).

Table 4.1 Conveyance of collected *P. aeruginosa* from two areas in Iraq and their recurrence among male and females patients.

Provinces	Patients No.	Male No**. (%)**	Female No. (%)
Erbil	125	72(57.6)	53 (42.4)
Duhok	100	64(64)	36 (36)
Total	225	136(60.4)	89 (39.6)

Table 4.2 appears the connection of mode of burn with age, sex and total burn surface area (TBSA) in 225 patients tainted by *P. aeruginosa*. Fire burn was the generally overwhelming cause of burn wounds in 125 (55.5 %) patients, where was the scalded burn was the second common cause of burn harm in 85 (37.7 %) of patients and remaining 15 patients (6.6 %) got chemical (acid) burn. Forty two out of 125

patients (33.6 %) who procured burn harm by fire, were matured up to 12 years, while the remaining 83 (66.4 %) patients were more seasoned than 12 years. Patients influenced by fire burn had a place to both genders in which male were 88 (70.4 %) and female were 37 (29.6 %).In all patients influenced by fire burn, TBSA up to 15 was found in 29 (23.2 %) and TBSA > 15was found in 96 (76.8 %) patients. Thirty three out of 85 patients (38.8 %) who obtained burn harm by scald were matured up to 12 years, while the remaining 52 (61.1 %) patients were more seasoned than 12 years. Patients influenced by scald burn had a place to both genders in which male were 43 (50.5 %) and female were 42 (49.4 %). In all patients influenced by scald burn, TBSA up to 15 was found in 13 (15.2 %) and TBSA > 15was found in 72 (84.7 %) patients. Four out of 15 patients (26.6 %) who obtained burn damage by acid were matured up to 12 years while the remaining 11(73.3 %) patients were more seasoned than 12 years. Patients influenced by acid burn had a place to both genders in which male were 12 (80 %) and female were 3 (20 %). In all patients influenced by acid burn, TBSA up to 15 % was found in 5 (33.3 %) and TBSA > 15 % was found in 10 (66.6 %) patients.

Table 4.2 Connection of mode of burn with age, sex, and add up to burn surface region (TBSA) in 225 patients tainted by *P. aeruginosa*

Cause	No. of	Age		Sex		TBSA	
of burn	patients %	<12 years	>12 years	Male	Female	<15%	>15 %
		79	146	143	82	47	178
Flame	125(55.5)	42(33.6)	83(66.4)	88(70.4)	37(29.6)	29(23.2)	96(76.8)
Scald	85(37.7)	33(38.8)	52(61.1)	43(50.5)	42(49.4)	13(15.2)	72(84.7)
Acid	15(6.6)	4(26.6)	11(73.3)	12(80)	3(20)	5(33.3)	10(66.6)

4.1.2. Antibiotic sensitivity test

The comes about of anti-microbial sensitivity test for *P. aeruginosa* burn isolates (50 randomly chosen confines) from distinctive clinics in Duhok and Erbil territories are displayed in Table (4.2). It is clear that none of the isolates was sensitive to all anti-microbials; Imipenem anti-microbial as one of Carbapenems operators was found to be the most powerful of all other antimicrobial specialists utilized with a resistance rate of 47 %.

Table 4.3 Distribution of tested antibiotic susceptibilities of *P. aeruginosa* isolates from Duhok and Erbil

	Antibiotics	Class	Duhok	Erbil	Total
			Resistance No. (%)	Resistance No. (%)	Resistance No. (%)
1	Amikacin	Aminoglycoside	46 (92)	45 (90)	91 (91)
2	Tobramycin	Aminoglycoside	48 (96)	46 (92)	94 (94)
3	Gentamicin	Aminoglycoside	46 (92)	47 (94)	93 (93)
4	Ticarcillin/clavulanic acid	Beta-Lactam	35 (70)	36 (72)	71 (71)
5	Ampcillin/Sulbactam	Beta-Lactam	40 (80)	43 (86)	83 (83)
6	Ampcillin	Penicillin	43 (86)	42(84)	85 (85)
7	Piperacillin	Penicillin	44(88)	41 (82)	85(85)
8	Meropenem	Carbapenem	47 (94)	48 (96)	95 (95)
9	Imipenem	Carbapenem	25 (50)	22(44)	47 (47)
10	Cefepime	Cephalosporin	49 (98)	44 (88)	93 (93)
11	Ceftazidime	Cephalosporin	45(90)	46 (92)	91 (91)
12	Ceftriaxone	Cephalosporin	45 (90)	45 (90)	90 (90)
13	Cefuroxime	Cephalosporin	46 (92)	44 (88)	90(90)
14	Ciprofloxacin	Fluoroquinolone	27 (54)	29 (58)	56 (56)
15	Aztreonam	Monobactam	43 (86)	47(94)	90 (90)
16	Trimethoprim/ Sulfamethoxazole	Folate pathway inhibitor	23 (46)	31 (62)	54 (54)

Meropenem may be considered as the second most compelling anti-microbial against these isolates with a resistant rate of 53 %, the tried *P. aeruginosa* burn infection separates were shown a high resistant to most tried anti-microbials counting; Tobramycin, Gentamicin , Amikacin and Aztreonam with resistant rate of 94 %, 93 %, 91 % and 90 % respectively,ampicillin and Piperacillin which customarily considered as a front-line treatment for *P. aeruginosa* infection burn in isolates appeared low impact on these confines with resistance rate of 85 %. Resistance design of tried separates to Ticarcillin/clavulanic acid and Ampcillin/Sulbactam was found in arrange of 71 % and 83 % separately. Trimethoprim/Sulfamethoxazole and Ciprofloxacin moreover appeared moo impact on tried isolates with resistant rates 54 % and 56 % individually to these anti-microbials, the isolates too appeared high resistant rates to

the forth generation of Cephalosporins counting Cefepime, Ceftazidime, Ceftriaxone and Cefuroxime with resistance rates of 93 %, 91%, 90 %and 90 %, separately.

4.2. Molecular investigations

4.2.1. Extraction of genomic DNA

The normal concentration of genomic DNA extricated by conventional strategy was 1928.11 ng/µl with purity of 1.6,while the normal concentration of genomic DNA extricated by commercial pack was 115.25 ng/µl with exceedingly purity of 1.8 as appeared in Table 4.4.

Table 4.4. Average concentration and purity of genomic DNA extracted by traditional method and commercially Kit.

Provinces	Patients No.	Male No**. (%)**	Female No. (%)
Erbil	125	72(57.6)	53 (42.4)
Duhok	100	64(64)	36 (36)
Total	225	136(60.4)	89 (39.6)

The result of conventional strategy was characterized by generation of high quantity with generally high quality is appeared in Figure 4.1. In any case, this strategy has a few restrictions counting; time expending and endeavors particularly when it is utilized for a large scale compared to the commercial kit (Wang and Storm, 2006).

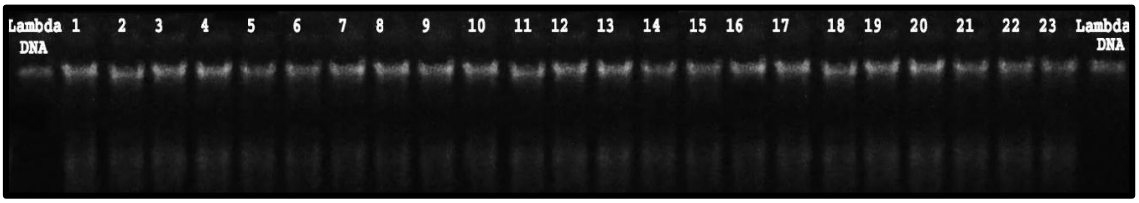

Figure 4.1. Genomic DNA extricated from twenty three isolates of *P. aeruginosa* utilizing conventional strategy. Electrophoresis was performed on 1agarose gel and run with 5v/cm. The to begin with and final path speak to uncut λ DNA marker (25ng) as standard molecular marker.

Commerciall kit strategy was found as a exceptionally proficient strategy for fast DNA surrender are reasonable for PCR amplification, with high purity and a huge number of tests can be extricated. Be that as it may, this strategy might be unacceptable on the off chance that DNA tests are to be subjected to other sorts of molecular examinations such as cloning and in numerous tests since of moo abdicate quantity. In this way, it might lead to constrain the utilize of this commercial kitin future.

4.2.2. PCR Analysis

4.2.2.1. *P. aeruginosa* species-specific PCR amplification

In rehashed tests, all of the 100 *P.aeruginosa* isolates were effectively amplified and they created a single band of the *16Sr-DNA* as the species specific locus in all strains with a molecular weight of almost 956 bp as appeared in Figure 4.2, the fruitful amplication of *16srDNA* amplicon in all chosen tests was encourage affirmed at the molecular level that all these strains were in reality *P. aeruginosa.*

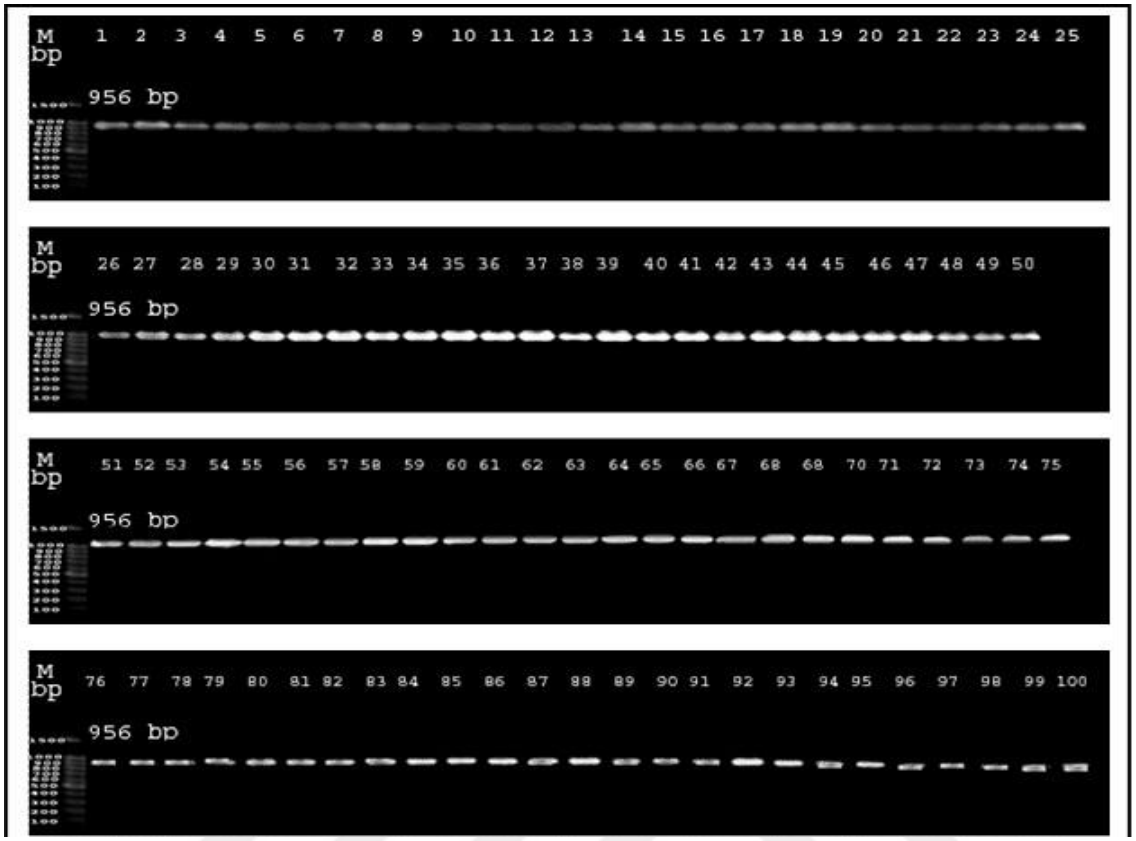

Figure.4.2. Species- Particular PCR amplification for (1-100) *P. aeruginosa* strains delivered with *16srDNA* amplicon with molecular molecular weight 956 bp. Electrophoresis was performed on 1agarose gel and run with 5v/cm, for 1.30 hour. Path M contained DNA molecular weight marker (1500-100bp).

4.2.2.2. Prevalence and distribution of virulence genes

In this consider all 100 *P. aeruginosa* isolates were subjected to PCR strategies to decide the prevalence rates of virulence related genes counting (*opr-1, tox-A, exo-S, las-B,* and *nan-1*) marker and their dispersion. The comes about of these tests are portrayed independently agreeing to person Governorate (Erbil and Duhok).The comes about of the tried isolates from Erbil area are appeared in Fig. 4.3, 4.4, 4.5, 4.6 and 4.7. It has been found that the prevalence of *oprI* marker was generally high among these isolates accounting for 90 % while *toxA* gene accounted for 88 %; while *exoS* gene shown among 86 % of these confines. The prevalence of *lasB* and *nan-1* was the most reduced among these confines with rates 84 % and 38 % respectively.

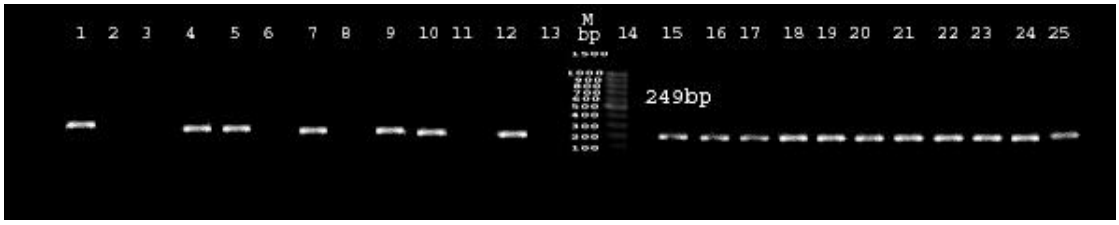

Figure 4.3. PCR amplification of *P. aeruginosa* isolates collected from Erbil area created *oprI* amplicon with molecular weight 249bp. Electrophoresis was performed on 1% agarose gel and run with 5v/cm, for 1.30 hours. Path M contained DNA molecular weight marker (1500-100bp).

Figure 4.4. PCR amplification of *P. aeruginosa* isolates collected from Erbil area created *toxA* amplicon with molecular weight 396 bp. Electrophoresis was performed on 1% agarose gel and run with 5v/cm, for 1.30 hours. Path M contained DNA molecular weight marker (1500-100bp).

Figure 4.5. . PCR amplification of *P. aeruginosa* isolates collected from Erbil area created *exoS* amplicon with molecular weight 504 bp. Electrophoresis was performed on 1.5 % agarose gel and run with 5v/cm, for 2 hours Path M contained DNA molecular weight marker (1500-100bp).

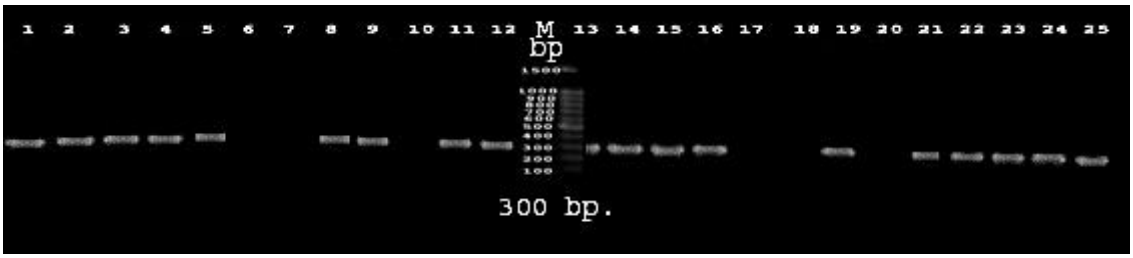

Figure 4.6. PCR amplification of *P. aeruginosa* isolates collected from Erbil area delivered appeared *lasB* amplicon with molecular weight 300 bp. Electrophoresis was performed on 1 % agarose gel and run with 5v/cm, for 1.30 hours.Way M contained DNA molecular weight marker (1500-100bp)

Figure 4.7. PCR amplification of *P.aeruginosa* isolates isolated from Erbil territory delivered appeared *nan-1* amplicon with molecular weight 1316 bp. Electrophoresis was performed on 1 % agarose gel and run with 5 v/cm, for 1.30 hours. Path M contained DNA molecular weight marker (1500-100bp)

Fig. 4.8, 4.9, 4.10, 4.11 and 4.12 speak to the prevalence of the five virulence genes related genes among *P.aeruginosa* separates in Duhok territory, it has been found that these isolates shown some way or another comparative design as those in Erbil territory, *oprI* marker accounted for 90 % though, 84 % of these isolates shown *tox A* gene, 86 % of these separates shown *exoS* gene, the presence of *lasB* gene among these isolates accounted for 80 % and *nan1* was the most reduced virulence genes these confines with a rate of 32 %.

Figure 4.8. PCR amplification of *P. aeruginosa* isolates collected from Duhok area created *oprI* amplicon with molecular weight 249 bp. Electrophoresis was performed on 1 % agarose gel and run with 5 v/cm, for 1.30 hours. Path M contained DNA molecular weight marker (1500-100bp).

Figure.4.9. PCR amplification of *P.aeruginosa* isolates collected from Duhok area created *tox A* amplicon with molecular weight 396 bp. Electrophoresis was performed on 1 % agarose gel and run with 5 v/cm, for 1.30 hours. Path M contained DNA molecular weight marker (1500-100bp)

Figure 4.10. PCR amplification of *P.aeruginosa* isolates collected from Duhok area created *exo S* amplicon with molecular weight 504 bp. Electrophoresis was performed on 1 % agarose gel and run with 5 v/cm, for 1.30 hours. Path M contained DNA molecular weight marker (1500-100bp).

Figure 4.11. PCR amplification of *P.aeruginosa* isolates collected from Duhok erea created *las B* amplicon with molecular weight 300 bp. Electrophoresis was performed on 1 % agarose gel and run with 5 v/cm, for 1.30 hours. Path M contained DNA molecular weight marker (1500-100bp).

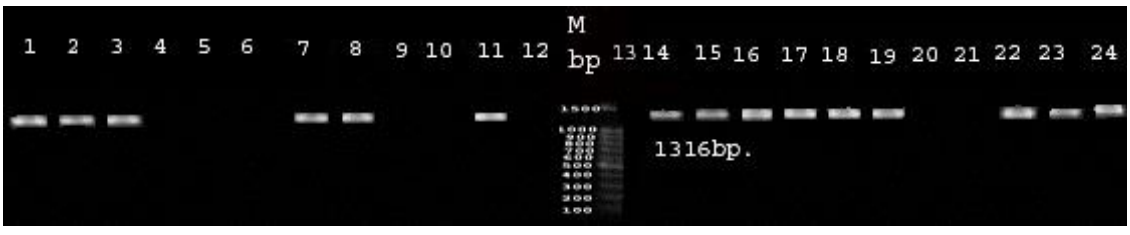

Figure 4.12. PCR amplification of *P.aeruginosa* isolates collected from Duhok erea created *nan-1* amplicon with molecular weight 1316 bp. Electrophoresis was performed on 1 % agarose gel and run with 5 v/cm, for 1.30 hours. Path M contained DNA molecular weight marker (1500-100bp).

The in general comes about of virulence related genes prevalence among *P.aeruginosa* isolates collected from the two territories in Iraq, as it were 10 % of these isolates lacked the nearness of any of the tried virulence markers.The comes about of separates that harbored these markers are summarized in Table 4.5. From these comes about, it got to be clear that *opr I* was the most overwhelming marker among all other virulence related genes accounting for 90 % taken after by *toxA* and *exoS* accounting for 86 % and 86 %,individually, while the prevalence of *lasB* gene was found with a rate of 82 % and *nan1* in rate of 35 %. The comes about of this ponder Table 4.5 also uncovered that are were contrasts in the prevalence rates of these genes among two areas.

Table 4.5 Prevalence of virulence markers among *P. aeruginosa* isolates collected from Erbil and Duhok provinces.

Province	Virulence markers				
	oprI(%)	*toxA(%)*	*exoS(%)*	*lasB(%)*	*Nan1*(%)
Erbil	45(90)	44(88)	43(86)	42(84)	19(38)
Duhok	45(90)	42(84)	43(86)	40(80)	16(32)
Total	90(90)	86(86)	86(86)	82(82)	35(35)

4.2.2.3. Prevalence and distribution of antibiotic resistance genes

4.2.2.3.1 Prevalence and distribution of metalo β-lactamase(MβL) genes

In this ponder all 100 *P. aeruginosa* separates were subjected to PCR procedures to decide the prevalence rates of metalo β-lactamase (MβL) related genes counting (*vim-1, imp1,spm-1,sim and gim*) marker and their dissemination. The comes about of these tests are depicted independently agreeing to person territory (Erbil and Duhok). The comes about of the tried isolates from Erbil area are appeared in Fig. 4.13, 4.14, 4.15, 4.16 and 4.17. It has been found that the prevalence of *vim-1* marker was moderately high among these separates accounting for 24 (48 %), while *imp1*

gene accounted for 23 (46 %), *spm-1* gene shown among 20 (40 %) of these confines. The prevalence of *sim* and *gim* was the most reduced among these separates with rates 13 (26 %) and 12 (24 %) respectively.

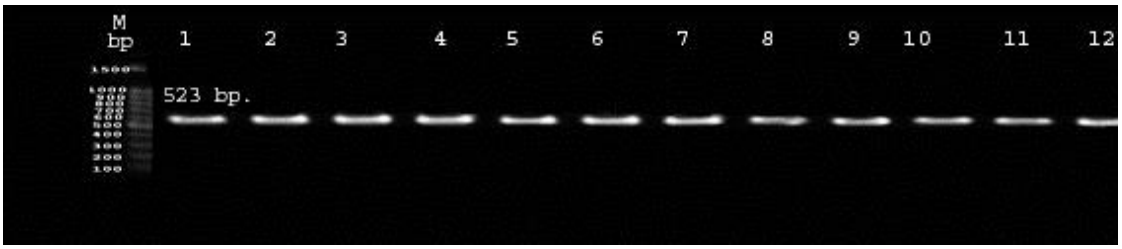

Figure 4.13. PCR amplification of *P. aeruginosa* separates collected from Erbil erea delivered *vim1* amplicon with molecular weight 523 bp. Electrophoresis was performed on 1 % agarose gel and run with 5 v/cm,for 1.30 hours.Lane M contained DNA molecular weight marker (1500 100bp).

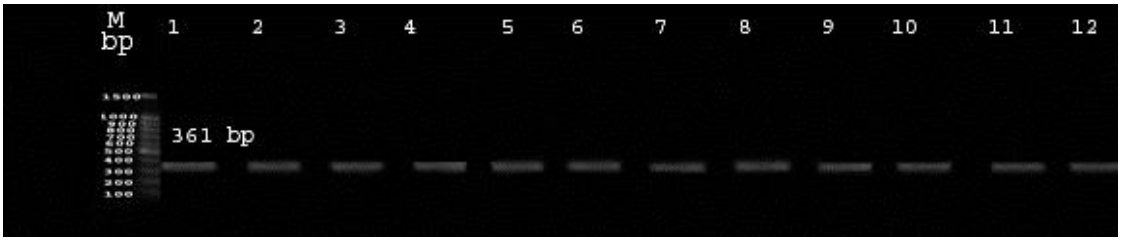

Figure 4.14. PCR amplification of *P. aeruginosa* confines confined from Erbil area created *imp1* amplicon with molecular weight 361 bp. Electrophoresis was performed on 1% agarose gel and run with 5 v/cm, for 1.30 hours. Path M contained DNA molecular weight marker (1500 100bp).

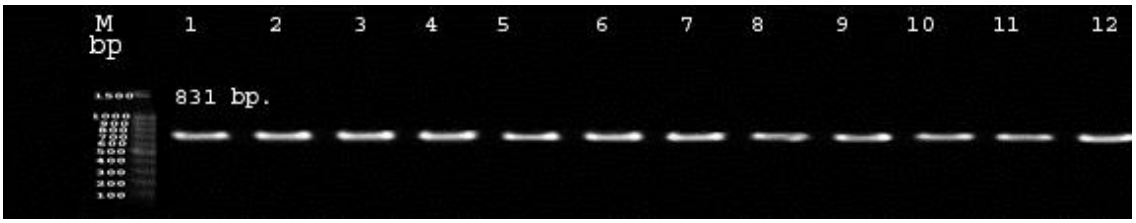

Figure 4.15. PCR amplification of *P. aeruginosa* isolates collected from Erbil area delivered *spm* amplicon with molecular weight 831 bp. Electrophoresis was performed on 1 % agarose gel and run with 5 v/cm,for 1.30 hours.Lane M contained DNA molecular weight marker (1500 100bp).

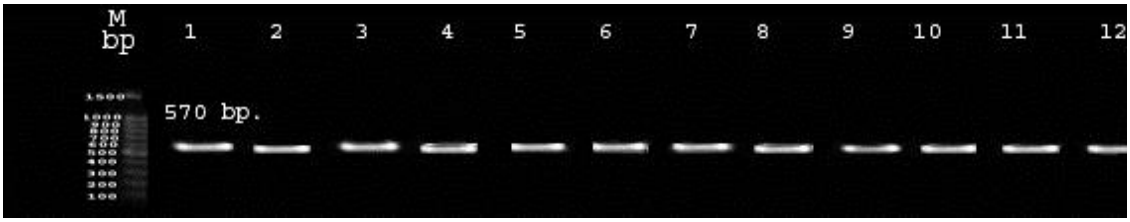

Figure 4.16. PCR amplification of *P. aeruginosa* isolates collected from Erbil erea delivered *Sim* amplicon with molecular weight 570 bp. Electrophoresis was performed on 1 % agarose gel and run with 5 v/cm,for 1.30 hours.Lane M contained DNA molecular weight marker (1500 100bp).

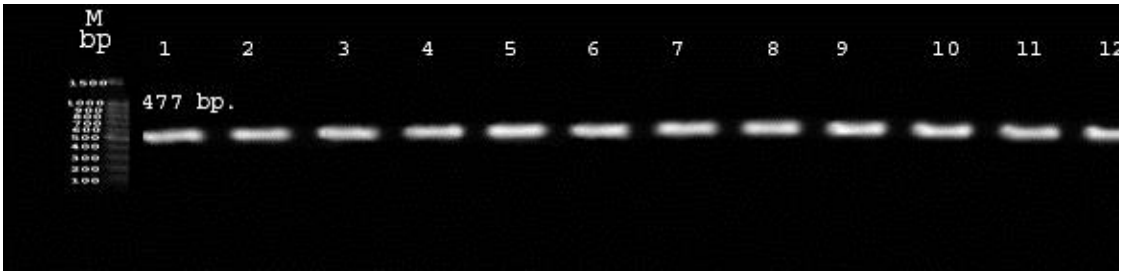

Figure 4.17. PCR amplification of *P. aeruginosa* isolates collected from Erbil erea created *gim* amplicon with molecular weight 477 bp. Electrophoresis was performed on 1 % agarose gel and run with 5 v/cm,for 1.30 hours.Lane M contained DNA molecular weight marker (1500 100bp).

Fig. 4.18, 4.19, 4.20, 4.21 and 4.22 speak to the prevalence of the five MβL related gene among *P.aeruginosa* confines in Duhok area. It has been found that these isolates shown by one means or another comparable design as those in Erbil area *vim1* marker accounted for 22 (44 %)whereas, 22 (44 %) of these confines shown *imp1* gene, 15 (30 %) shown *spm1* gene, while the nearness of *sim* gene among these separates accounted for 14 (28 %) and *gim* was the least virulence genes among these isolates with a rate of 12 (24 %).

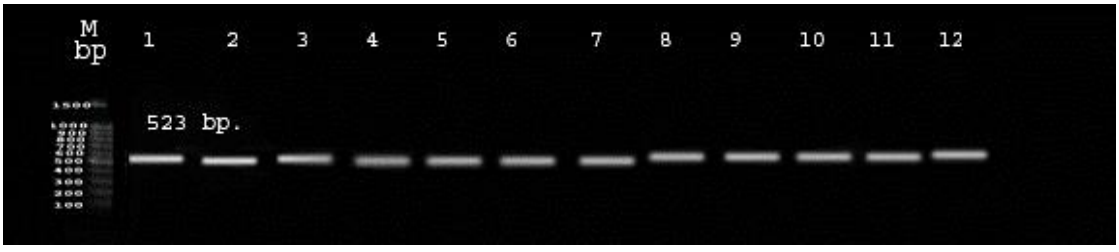

Figure 4.18. PCR amplification of *P. aeruginosa* isolates collected from Duhok area created *vim1* amplicon with molecular weight 523 bp. Electrophoresis was performed on 1% agarose gel and run with 5v/cm,for 1.30 hours.Lane M contained DNA molecular weight marker (1500 100bp).

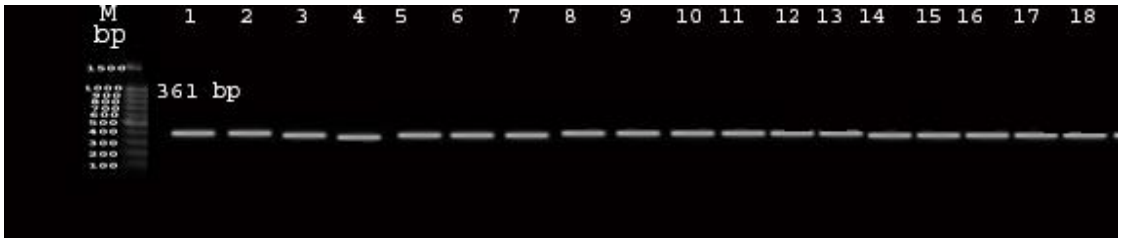

Figure 4.19. PCR amplification of *P.aeruginosa* isolates collected from Duhok area delivered *imp 1* amplicon with molecular weight 361 bp. Electrophoresis was performed on 1 % agarose gel and run with 5 v/cm,for 1.30 hours.Lane M contained DNA molecular weight marker (1500 100bp)

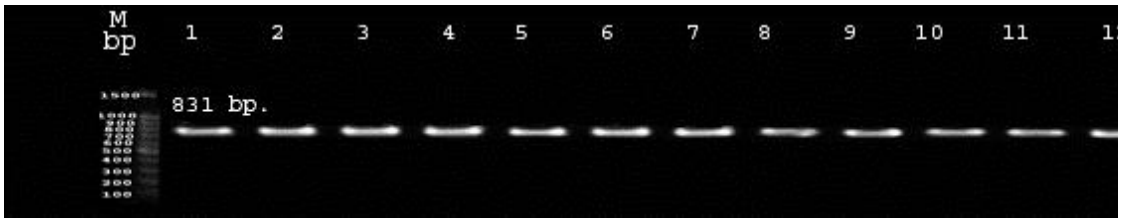

Figure 4.20. PCR amplification of *P. aeruginosa* isolates collected from Duhok area delivered *spm 1* amplicon with molecular weight 831 bp.Electrophoresis was performed on 1 % agarose gel and run with 5 v/cm,for 1.30 hours.Lane M contained DNA molecular weight marker (1500 100bp).

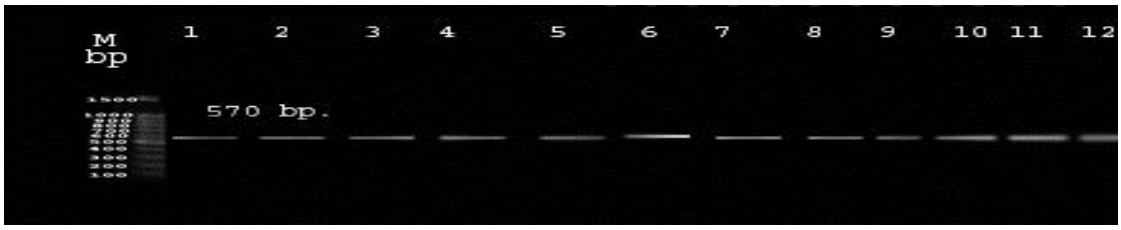

Figure 4.21. PCR amplification of *P. aeruginosa* isolates collected from Duhok area delivered *sim* amplicon with molecular weight 570 bp. Electrophoresis was performed on 1 % agarose gel and run with 5 v/cm,for 1.30 hours.Lane M contained DNA molecular weight marker (1500 100bp).

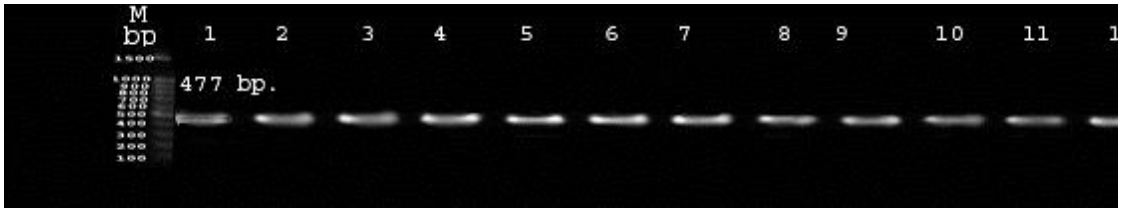

Figure 4.22. PCR amplification of *P. aeruginosa* isolates collected from Duhok area created *gim* amplicon with molecular weight 477 bp.Electrophoresis was performed on 1% agarose gel and run with 5v/cm,for 1.30 hours.Lane M contained DNA molecular weight marker (1500 100bp).

The generally.comes about of MβL related genes prevalence among *P. aeruginosa* separates collected from the two territories in Iraq, it is found out of 100 *P. aeruginosa* confines, 47 strain (47 %) were imipenem resistance and 46 strains of them (97.8 %) were carrying genes responceple for MβL generation (46 % of the add up to number of *P. aeruginosa*). The comes about of isolates that harbored these markers are summarized in Table 4.6. From these comes about, it got to be clear that *vim1* was the most overwhelming marker among all other MβL related genes accounting for 46 % taken after by *imp1* and *spm1* bookkeeping for 45 % and 35 %, separately, while the prevalence of *sim* gene was found with rate 27 % and *gim* in rate of 24 %.

Table 4.6 prevalence of MβL markers among *P. aeruginosa* isolates collected from Erbil and Duhok provinces.

Province	MβL markers				
	Vim1(%)	*Imp1 (%)*	*Spm1 (%)*	*Sim (%)*	Gim (%)
Erbil	24(48)	23(46)	20(40)	13(26)	12(24)
Duhok	22(44)	22(44)	15(30)	14(28)	12(24)
Total	46(46)	45(45)	35(35)	27(27)	24(24)

4.2.2.3.2 Prevalence and distribution of extended spectrum β-lactamase (ESβL) genes

In this think about all 100 *P. aeruginosa* isolates were subjected to PCR methods to decide the prevalence rates of (ESβL) related genes counting (*oxa10, veb1*, and*oxa2*) marker and their conveyance. The comes about of these tests are portrayed independently concurring to person territory (Erbil and Duhok).The comes about of the tried confines from Erbil territory are appeared in ig. 4.23, 4.24 and 4.25 it has been found that the prevalence of *oxa10* marker was generally high among these confines accounting for 45 (90 %) while *veb1* gene accounted for 33 (66 %), though *oxa2* gene shown among 17 (34 %) of these confines.

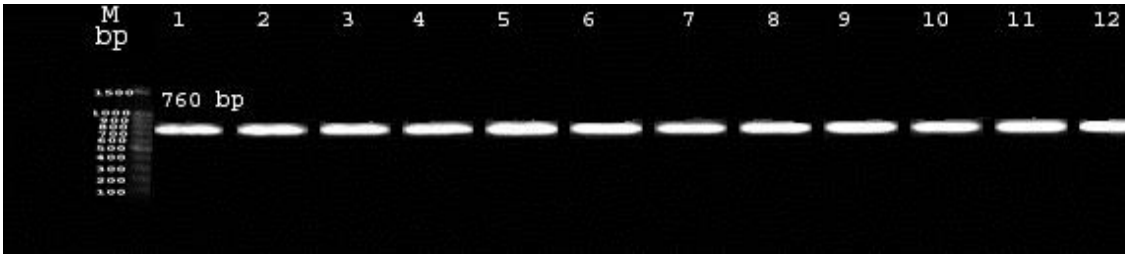

Figure 4.23. PCR amplification of *P. aeruginosa* isolates collected from Erbal area delivered *oxa 10* amplicon with molecular weight 760 bp. electrophoresis was performed on 1 % agarose gel and run with 5 v/cm,for 1.30 hours.Lane M contained DNA molecular weight marker (1500 100bp).

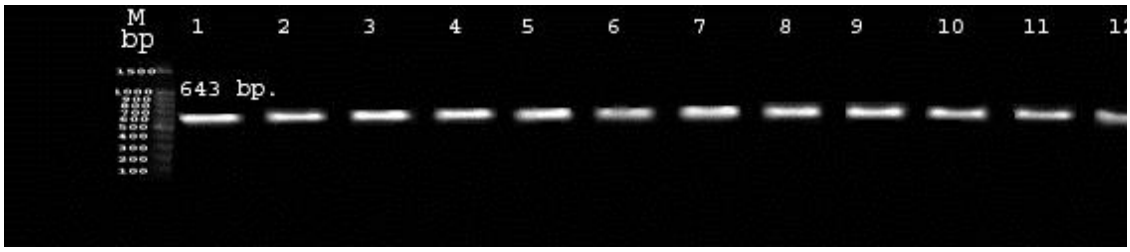

Figure 4.24. PCR amplification of *P. aeruginosa* isolates collected from Erbal area delivered *veb 1* amplicon with molecular weight 643 bp. electrophoresis was performed on 1 % agarose gel and run with 5 v/cm, for 1.30 hours.Lane M contained DNA molecular weight marker (1500 100bp).

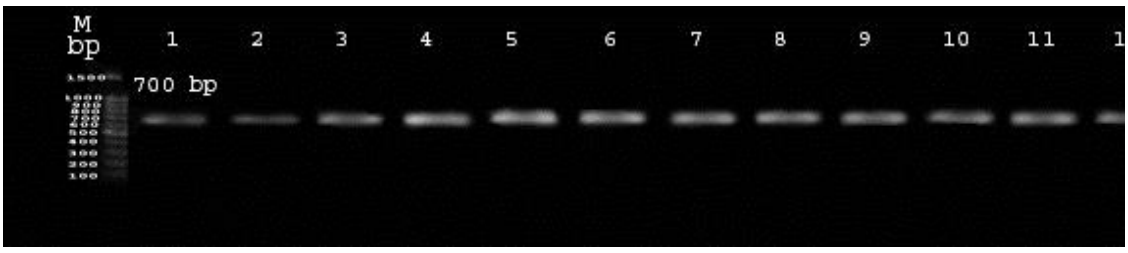

Figure 4.25. PCR amplification of *P. aeruginosa* isolates collected from Erbal area delivered *oxa 2* amplicon with molecular weight 700 bp. electrophoresis was performed on 1 % agarose gel and run with 5 v/cm, for 1.30 hours.Lane M contained DNA molecular weight marker (1500 100bp).

Figures (4.26, 4.27 and 4.28) represent the prevalence of the three ESβL related genes *P. aeruginosa* separates in Duhok area it has been found that these separates shown by one means or another comparative design as those in Erbil area. *Oxa 10*

marker accounted for 46 (92 %), though 30 (60 %) of these isolates displayed *veb 1* gene and 19 (38 %) of these isolates shown *oxa 2* gene.

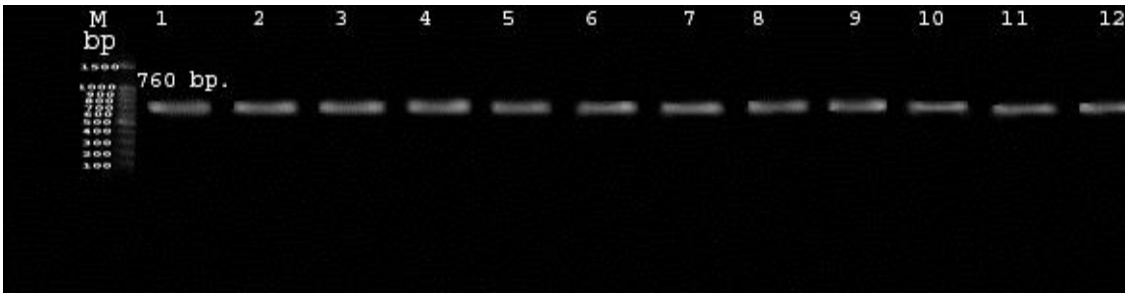

Figure 4.26. PCR amplification of *P. aeruginosa* isolates collected from Duhok area created *oxa 10* amplicon with molecular weight 760 bp. electrophoresis was performed on 1 % agarose gel and run with 5 v/cm,for 1.30 hours.Lane M contained DNA molecular weight marker (1500 100bp).

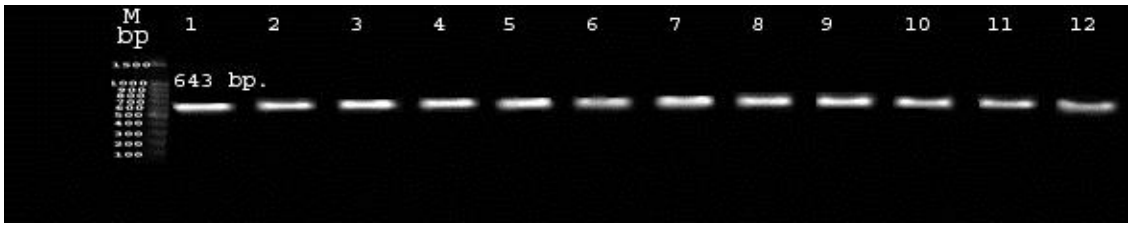

Figure 4.27. PCR amplification of *P.aeruginosa* isolates collected from Duhok area created *veb 1* amplicon with molecular weight 643 bp. electrophoresis was performed on 1 % agarose gel and run with 5 v/cm, for 1.30 hours.Lane M contained DNA molecular weight marker (1500 100bp).

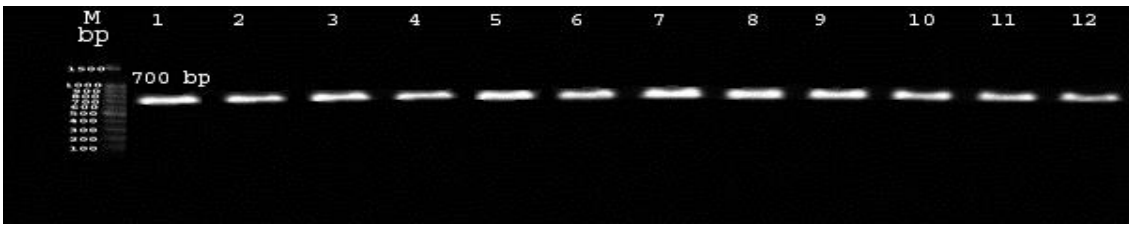

Figure 4.28. PCR amplification of *P. aeruginosa* isolates collected from Duhok area created *oxa2* amplicon with molecular weight 700 bp. electrophoresis was performed on 1% agarose gel and run with 5 v/cm, for 1.30 hours.Lane M contained DNA molecular weight marker (1500 100bp).

The in general comes about of ESβL related genes prevalence among *P. aeruginosa* isolates collected from the two areas in Iraq shwoed out of 100 *P.*

aeruginosa isolates more than 90 % resistace for ceftazidime, cefotaxime cefepime and aztreonam exist. The comes about uncovered that more than (93 %) of the separates were multidrug resistant and 91 (91%) of the confines were ESBL positive, 91 (91 %), 66 (66 %)and 36 (36 %) strains among 91 ESBL-producing strains amplified *oxa10, veb-1* and *oxa2* individually. The comes about of isolates that harbored these markers are summarized in Table (4.7).

Table 4.7 Prevalence of ESβL markers among *P. aeruginosa* isolates collected from Erbil and Duhok provinces.

Province	ESβL markers		
	Oxa10 (%)	*Veb1 (%)*	*Oxa2 (%)*
Erbil	45(90)	33(66)	17(34)
Duhok	46(92)	30(60)	19(38)
Total	91(91)	66(66)	36(36)

4.2.2.4. ERIC-PCR DNA fingerprint analysis

ERIC-PCR fingerprints of amplified DNA parts gotten by agarose gel electrophoresis were recorded. The positions of the bands on each path and each gel were normalized utilizing the molecular weight marker (1500-100bp). The molecular weights of amplicons have been decided by photo-Capt computer program. The zero-one manual strategy was utilized to number the bands and at that point the information were entered on the the following site: http://insilico.ehu.es/dice_upgma/, and the dendrograms were drawn. Identified and characterized 100 isolates of *P. aeruginosa* were subjected to genomic DNA differing diversity investigation utilizing ERIC-PCR fingerprinting strategy with ERIC-type primer. All these confines were effectively creating different amplicons extending from 5-21 bands with distinctive molecular weights extending from 1400<100bp.The most frequent band was 730 bp. which was intensified by 62 strains, and the slightest frequent were 1420, 1380, 1300, and 1200 bp. watched in as it were two strains. The comes about of dendogram examination

appeared that ERIC–PCR profiles separated of the 100 confines into two fundamental clusters, group A accounting 92 %, while group B speaking to as it were 8 each one can be classified into two subgroups as appeared in Figure (4-30) . Inside group A; forty six (46 %) isolates were had a place to subgroup A1, 46 isolates of the considered strains were in subgroup A2 (46 %),4 (4 %) strains were alloted inside subgrop B1 and as it were 4 strains were had a place to subgroup B2 (4 %), inside each subgroups complex patterns of fingerprints have been gotten for all strains.

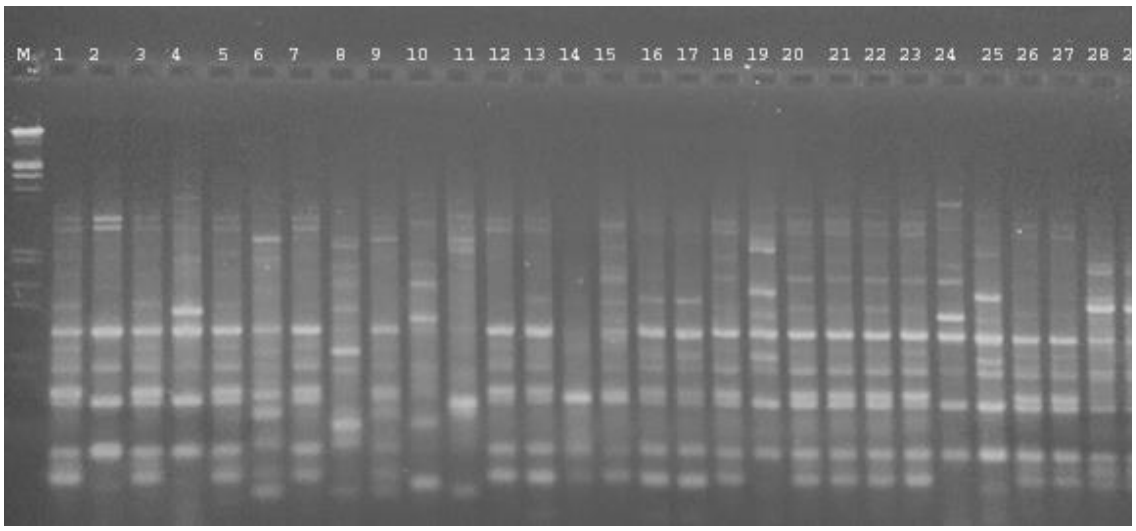

Figure 4.29. DNA-banding profiles of visual *P. aeruginosa* strains by gel electrophoresis taking after ERIC-PCR. Electrophoresis was performed on 1.5% agarose gel and run with 5v/cm, for 3 hours.Lane M contained DNA molecular weight marker (1500 100bp).

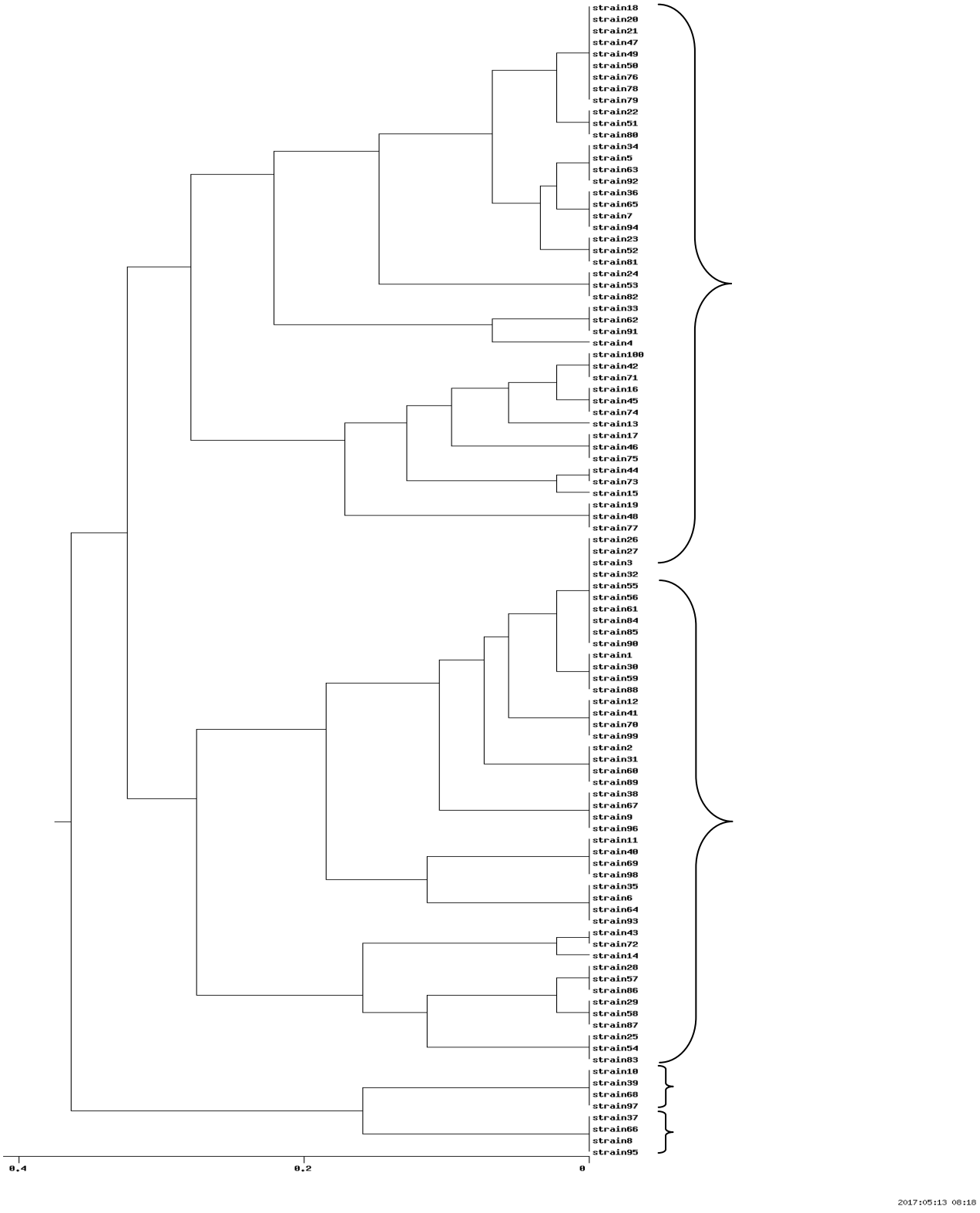

Fig.4.30. Dendrogram *Pseudomonas aeruginosa* isolates from burn inefctions constructed with the use UPGMA

4.2.2.5 Single nucleotide sequences analysis of *oprD* genes

In this study, fifteen isolates of clinical strains *P. aeruginosa* were selected and subjected to single nucleotide polymorphisms (SNPs) analysis using *OPrD* gene which encodes *OprD* porine. The partial sequence (503 bp.) of *OPrD gene* for clinical isolates of *P. aeruginosa* were aligned and compared with *P. aeruginosa* Str.PA12 (accession number KJ482587.1) which was used as a reference strain. From the analysis of sequenced region, nineteen point mutations were observed at 19 polymorphic sites accounting 3.77 % of the whole sequenced fragment of *OprD* gene. All mutations were point substitutions (SNPs) type, out of 19 point mutations, six of them were (6/19) transversions type accounting 31.57 %, while thirteen 68.42 % (13/19) were transitions. Only one SNPs at 328/503 site due to transversion mutation resulted amino-acid change (sense mutation), all other mutations were silent substitutions. No SNPs were singletons (observed in only one *OprD* type) among all mutations. From the sequencing, alignments and comparing fragment of *OprD* gene with reference strain, four allelotypes have been identified, the first allelotype involved six clinical strains (*P. aureoginosa* 1,3,4,8, 13 and 14) resembled 100 to the reference strain and lack any mutations at this region, *P. aureoginosa* strain 2 has unique profile undergoing only seven mutations as shown in Table (4.8), while six sequenced strains aligned in one allelotype *(P. aureoginosa* 6, 7,9, 10, 11 and 15) have 17 point mutations and, *P. aureoginosa* strain 5 has 18 point mutations, mutation has been identified among nine *P. aeruginosa* strains.

Table 4.8. Single-nucleotidepolymorphism(s) position with amino-acid replacements

SNP	No.
Total	19 (3.77%)
Point substitutions (Transitions)	13/19 (68.42%)
Pointsubstitutions (Transversions)	6/19 (31.57 %)
Amino acid replacement	1/19
Silent substitutions	18/19

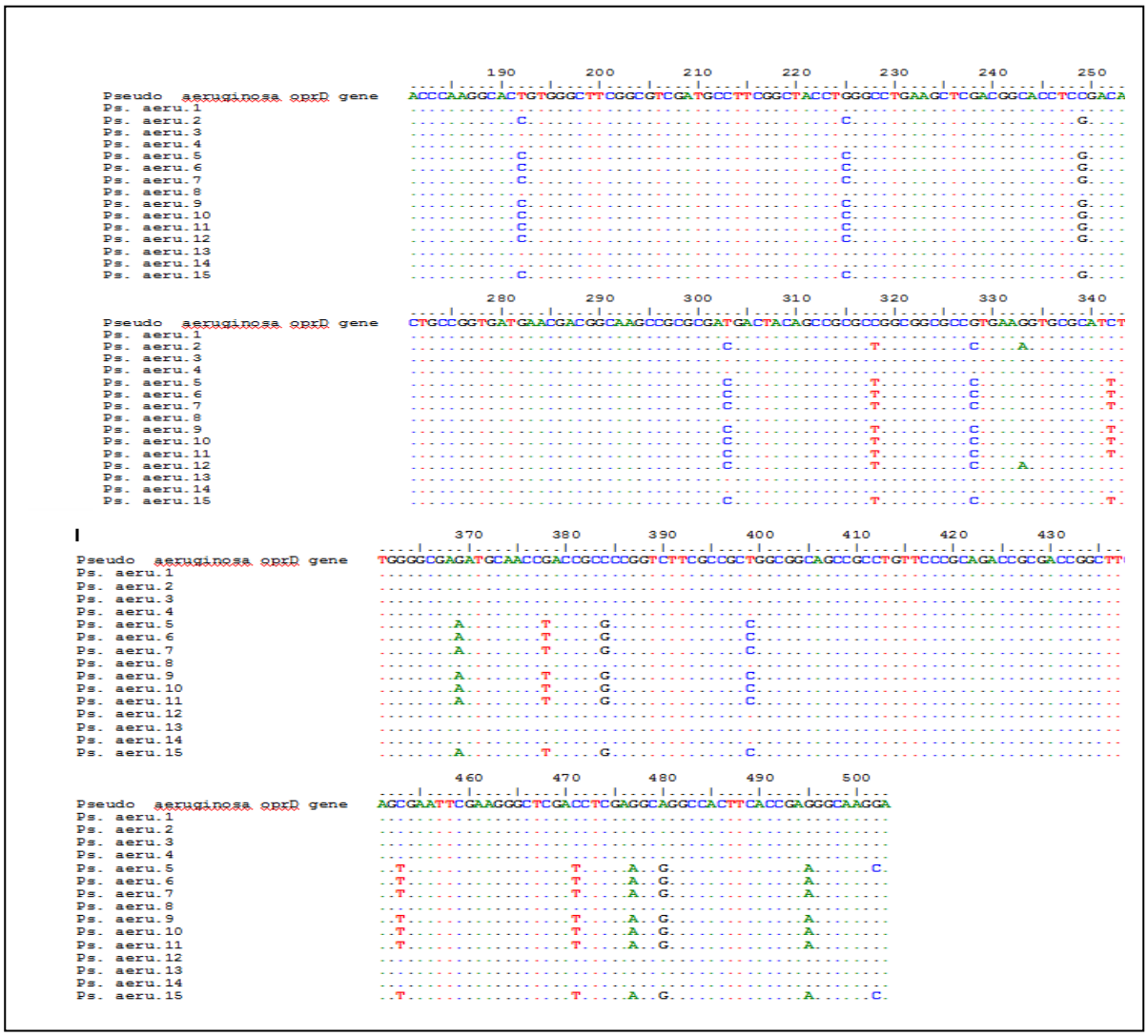

Figure 4.31 Multiple sequence alignments of the DNA sequences of *OPrD* gene for 15 selected isolates of *Ps. aureoginosa* with *P. aeruginosa Str.PA12* as a reference strain

Amino acid variants and silent SNPs were compared between selected *Ps. aeruginosa* strains with *P. aeruginosa* Str.PA12 references strain. It was revealed that nine strains (*Ps.*2, *Ps.*5, *Ps.*6, *Ps.*7, *Ps*9, *Ps.*10, *Ps.*11, *Ps.*12 and *Ps.*15) shared the same amino acid replacements G328C (Val 110 Lucien) as shown in Figure 4-32.

Figure 4.32. Multiple sequence alignments of the amino acids sequences of *oPrD* gene of 15 selected isolates of *P. aeruginosa* with *P. aureoginosa Str.PA12* as a reference strain Shows sense mutation at G328C (Val110Lucien).

Dendogram analysis of studied strains revealed that the clinical strains were classified into two main clusters show in Fig. 4.33 the first cluster divided into two subgroups; (*P. aureoginosa* strains1,3,4,8,13 and 14) were assigned with references strain *P. aeruginosa* Str.PA12); (*P. aureoginosa* strains2 and 12); the other cluster also divided into two subgroups (*P. aureoginosa* strains 5and 15) and *(P. aureoginosa* 6,7, 9,10 and *Ps aeru* 11). Nearly 53.3 % of studies isolates were assigned with reference strain *P. aeruginosa* Str.PA12, whereas 46.6 % of strains were assigned with other cluster. The distribution of *OprD* variants was analyzed by UPGMA software analysis.

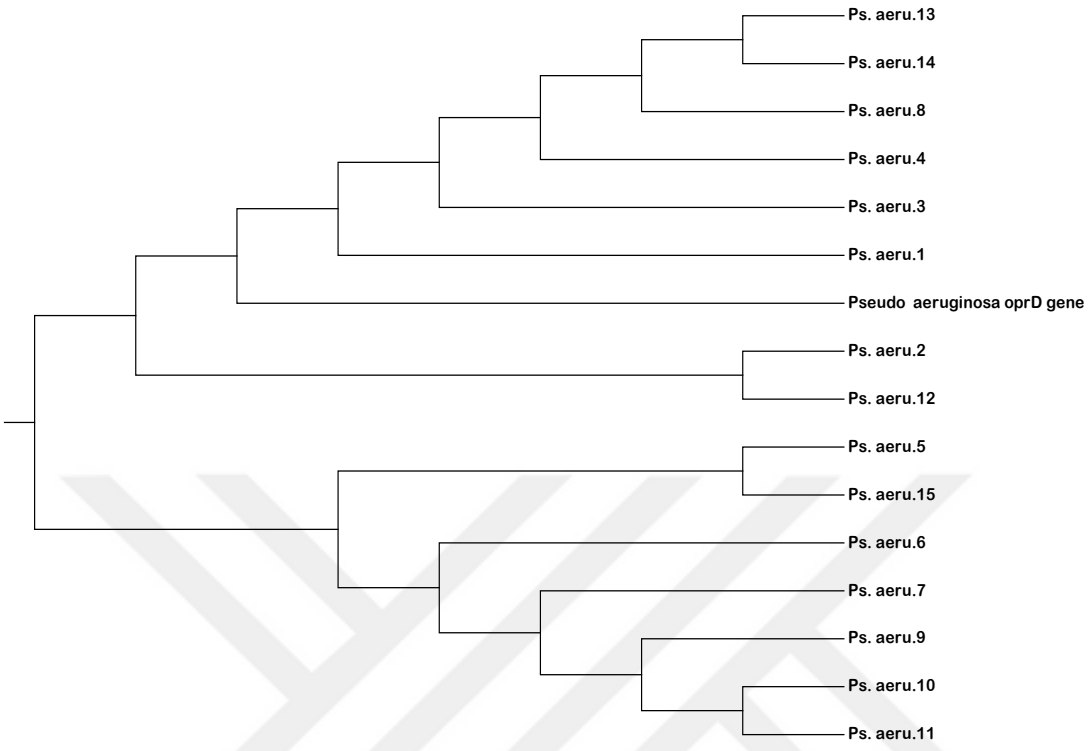

Figure 4.33. Dendrogram analysis *P. aeruginosa* isolates and the distribution of *oprD* variants constructed with the use of (UPGMA).

5. DISCUSSION AND CONCLUSIONS

5.1 Microbiological investigations

5.1.1 Characterization and incidence of *P. aeruginosa*

The comes about of *P. aeruginosa* isolates frequency are appeared in Table 4.1 out of 225 collected *P.aeruginosa* isolates from two areas (Erbil, and Duhok) in Iraq uncovered that 143 were separated from males tallying 63.5 % and 82 strains confined from females tallying 36.4 %.The comes about of table 4-2 were in understanding with the comes about of numerous other studies about, for illustration, it was assessed that fire burn was the generally overwhelming cause of burn wounds in 25 (80.6 %) patients and the second scald and at then the acid burn. The more seasoned male than 12 year and the TBSA> 15 was the most noteworthy rate among the harmed in all case of burn (Naqvi *et al*., 2005). Total burn surface area is found to be the most critical hazard figure for nosocomial contamination (Oralancul *et al.*, 2002). *P. aeruginosa* remains the driving pathogen causing burn wound disease (Lari and Bahrami, 1998). It survives well in the clinic environment. Once it is set up, it can endure for months inside a unit, posturing as Multi drug resistant nosocomial disease hazard for patients being treated there. Hands of staff individuals can ended up momentarily sullied and exchange contamination among patients (Douglas, 2001; Edwards, 2003).

5.1.2 Antibiotic sensitivity test

In a few considers the affectability of Imipenem against *P. aeruginosa* was moderately more, i.e. 86 % 78 %, 88 % and 91.6 %, separately (Neely and Holder, 1999; Xu and Sun, 1998). The resistance of *P. aeruginosa* was much higher (48 %) against this drug in a ponder conducted by Singh *et al.*, in Korea in 2001 (Song *et al.*, 2001). By one means or another comparative rates have been detailed and proposed that this rate ought to be considered since these operators are favored in empiric treatment

for serious bacterial infections caused by β-lactam resistant bacteria (Paterson, 2006). The development of Carbapenem resistance due to the generation of Carpabenemase chemical in Gram-negative organisms is an expanding universal open well being issue . Discovery of carbapenems resistance separate in a healing center environment postures not as it were a restorative issue, but moreover a genuine concern for infection control administration (Paterson, 2006; Hodiwala *et al*., 2013). Carbepenems are valuable in treatment of a few cases of multi-drug resistance strains of *P. aeruginosa* (Douglas, 2001). Comparative thinks about counting more seasoned antimicrobial specialists are restricted, but a few of these operators stay valuable for the treatment of chosen patients (Nicolle, 2005). Another thinks about appear comparable comes about, 100 % separates of *P. aeruginosa* were resistance to Amikacin, 95 % to Gentamicin 94 % to Aztreonam and 91 % to Tobramycin (Prasanna and Thomas, 1999; Lari, 1998; Naqvi *et al*., 2005). Ampcillin and Piperacillin comes about appear a comparative design in think about conducted in Pakistan where more than 87 % strains of *P. aeruginosa* were resistance to Ampcillin and Piperacillin (Naqvi *et al*., 2005).The broad utilize of fourth era cephalosporins as the driving drive behind the rise of extended-spectrum β lactamase (ESBL) creating organisms has been appeaed in numerous thinks about (Paterson, 2006). It has been found that the genes that encode ESBLs are habitually found on the same plasmids as genes that encode resistance to aminoglycosides and trimethoprim- sulfamethoxazole (Yasufuku *et al*., 2011).This implies that ESBL producing are commonly multidrug resistance, which postures a specific challenge for the treatment of nosocomial diseases. Unseemly empiric antimicrobial treatment for nosocomial- or community- obtained diseases has been detailed to contribute essentially for more prominent mortality rates in the seriously care unit (ICU). Other than, insufficient antimicrobial treatment of disease was the most critical free determinant of healing center mortality (Paterson, 2006).

5.2. Molecular investigations

5.2.1. Extraction of genomic DNA

The determination of the suitable strategy for DNA extraction Fig. 4.1 depends on the sum of the test accessible, number of tests to be extricated and the accessibility of specific equipment (Santella, 2006).

5.2.2 PCR analysis

5.2.2.1. *P. aeruginosa* species-specific PCR amplification

Numerous considers have detailed that the *16srDNA* amplification appeared in Fig. 4.2 is a prerequest for any assist molecular examination (Theodore *et al*., 2004). The gotten result was in understanding with a consider conducted in Baghdad Territory utilizing the same primer (*16srDNA* gene) for the identifecation of *P. aeruginosa* which delivered the same molecular weight (Altaai *et al.*, 2014). In any case, this molecular weight was found distinctive from that gotten by Gabriela and Anthony 2009 by utilizing the same primer sequence. This variety may be credited to the truth that genes often contain multiple coding smaller than expected- and microsatellite rehashes that are exceedingly energetic components of genomes. In this manner, recombination occasions inside these pair rehashes lead to changes in rehash numbers, which in turn changes the sequences. This variety may give the useful differences and permits fast adjustment to the environment and/or elusion of the host immune system (Levdansky *et al*., 2007). This strategy may give quick and vigorous comes about in a brief time for the affirmation ofspecific-species recognizable proof of *P. aeruginosa* and may also clear the way for advance and more points of interest molecular applications.

5.2.2.2. Prevalence and distribution of virulence genes

Comes about in Fig. 4.3 to 4.12 appeared are in assention with a number of related distributed thinks about; for example, Khattab and associates 2015 found that 100 % of *P.aeruginosa* isolates have *oprI* genes and most reduced prevlance with *Nan1* gene. In another consider conducted in Poland by Wolska and Szweda 2009 appeared that the predominance of virulance genes among *P.aeruginosa* disconnected from burin infection *lasB, toxA,exoS,* at rates 96.8 %, 88.7 %, and 75.8 %, separately. Fazeli and Momtaz 2012 also decided the virulance factors of *P.aeruginosa* confined from burin disease and found variable prevalence rates for *exoS* and *toxA* as taking after; 67.6 %, 35.2.% separately.The contrasts in prevalence of *P.aeruginosa* virulance genes due to geographic locale was too detailed by Rasol, 2013 in Duhok territory and by Karimian *et al.,* 2012 in Iran who recommended that the climate of each locale, traditions, nourishment, levels of open wellbeing and hospital's cleanliness may be considered as variables that credited to the nearness of varieties in the prevalence rates of virulence genes of *P. aeruginosa* strains among diverse districts.

5.2.2.3. Prevalence and distribution of antibiotic resistance genes

5.2.2.3.1 Prevalence and distribution of metalo β-lactamase (MβL) genes

In this ponder, five sorts of MβLgenes have been detailed among *P. aeruginosa* (*vim1, imp1, spm1, sim* and *gim*) Fig. 4.13 to 4.22 and Table 4.6 *spm1,sim* and *gim* have barely been identified past their locale of to begin with location, i.e. the proliferation of *spm1* sorts is limited to Brazil, while *gim* and *sim* sorts are rare and constrained to Germany and Seoul/Korea individually, this is the to begin with report of (*sim* and *gim*) gene in Iraq compared with numerous other ponders realted to MβL genes among *P. aeruginosa* for case (Ellington *et al.,* 2007; Khorvash *et al.,* 2014; Zafer *et al.,* 2014) were not identified in that ponder, this contention in the rate of strains carrying MβL-genes may be due to varieties in the prevalence with distinctive healing centers and geographic regions, since there are no standard rules for location of MβLs,

diverse thinks about have detailed the utilize of diverse strategies, PCR investigation is the gold standard strategy for the location of MβL generation, but it is not doable in schedule microbiology research facility (Gibb *et al*., 2002 ; Pandya *et al*., 2011). *vim1* and *imp1* genes show a around the world spread and proceed to be recognized at expanding rates around the world (Livermore *et al*., 2012). These rates in this think about are in assention with a number of related distributed ponders, for illustration, in one think about in Egypt, it was found that out of 100 confines of *P. aeruginosa,* 25 separates (25 %) were resistance to imipenem and as it were 15 (60 %) of them were carrying MβL genes (Khosravi *et al.,* 2013). Fang *et al.,* (2008) moreover found that 36 % of *P. aeruginosa* strains needed these MβL related genes. Moreover, in another think about conducted in Iran by Sheik *et al.,* (2014) appear that the prevalence of MβL genes among *P. aeruginosa* separated from burn diseases *vim1* and *imp1* at rates 31.3 and 14.6, individually. Another consider conducted in Malaysia appeared the predominance of MβL genes among *P.aeruginosa vim1* at rates (56 %), though (44 %) confines carrying the *imp 1* gene (Khosravi *et al*., 2013). In one consider in Egypt by Essa and Afifi (2007) appear 40 isolates resistance to imipenem and 20 isolates imipenem sensitive of *P. aeruginosa* and found all imipeenm resistance isolates were carrying *vim1* gene and no one of them had *imp1* and too none of the sensitve imipenem isolates had any MβL genes. Azim *et al.,* (2010) moreover decided the MβL genes among *P. aeruginosa* confined from burn and found variable predominance rate for *imp1* as 51.5%. Numerous considers like (Wirth *et al.,* 2009; Gaspareto *et al.,* 2007; Sader *et al,* 2005) appear the prevalence of MβL genes among *P. aeruginosa spm1* at rate 19.3 %, 35.71 % and 55,60 % individually too in this think about the prevalence of MβL genes among *P. aeruginosa* confined from burn infections *sim* and *gim* genes at the rates 20 % and 18 % separately,

5.2.2.3.2 Prevalence and distribution of extended spectrum β-lactamase (ESβL) genes

Comes about in the Fig. 4.23 to 4.28 and Table 4.7 are understanding with a number of related distributed thinks about for illustration in one study about in Iran appear the prevalence of ESβL genes among *P. aeruginosa* collected from burn infections in tow area, in the to begin with area 92 (87.61 %) isolates were positive for *Oxa10* while as it were 5 (4.76 %) positive for *oxa-2* from 105 (30 %) isolates were positive for ESBLs from 350 confines of *P. aeruginosa* where was the second area, 46 (38.33 %) of *P. aeruginosa* isolates were positive for ESBLs of which 29 (63 %) were positive for *Oxa10* and as it were 1 (2.1 %) isolates were positive for *Oxa-2 (*Alipour *et al.*, 2010). Another consider in Korea moreover appear high prevalence of ESβL genes among *P. aeruginosa Oxa-10* (13.1 %), where is as it were (2.3 %) *Oxa-2* from 64 confines and no isolates were harbored for *veb1* (Lee *et al*., 2005). Mrsalehian *et al.*, (2010) also appear high prevalence of multidrug resistance (87.05 %) and generation of 50 (74.62 %), *Oxa10* and 21 (31.34 %) *veb1* from 67 separates ESBL-producing. Another ponder in Bulgaria appear a add up to of 101 of the examined *P. aeruginosa* isolates , the prevalence of *veb1* and *Oxa10* at rate 33.1 %, and 41.3 %, individually (Strateva *et al.*, 2007). The high prevalence of *Oxa-10,* and *VEB-1* among *P. aeruginosa* collected in the current ponder demonstrated high-level resistance to penicillins, CAZ and CTX, as proposed by other considers (Mrsalehian *et al.*, 2010).

5.2.2.4. ERIC-PCR genotyping

Phylogenetic investigation is a capable instrument to screen the molecular.the study of disease transmission of microbes and their conveyance in the geographical locale, with the progress of molecular biology distinctive classification strategies based on PCR strategy had been recognized with extraordinary dependable for separation of microorganism by creating DNA fingerprints that are particular indeed for person strains (Prabhu *et al.*, 2010). ERIC-PCR procedure consider as one of method that demonstrate to be basic, quick, touchy with dependable for investigation of hereditary

differing genes and the study of disease transmission of microorganisms (Leung *et al.*, 2004). ERIC PCR examination could be a great microbial source tracking (MST) approach (Diab and Al-Turk, 2011). The clonal transmission of bacteria inhealing center situations utilizing ERIC PRC fingerprinting designs have been illustrated by other considers (Ramazanzadeh *et al.,* 2013; Edelstein *et al.,* 2003). It has been utilized by a few thinks about and connected for genotyping of diverse bacterial separates (Macedo *et al.,* 2011; Shuan *et al.,* 2011; Guimaraes *et al.,* 2011;Nath *et al.,* 2010). Depending on the result of phylogenetic investigation based on ERIC PCR strategy these isolates were doled out into two main clusters which jutting four genotypes as appeared in Figure (4.30), This strategy may be simple, quick with capable strategy for genotyping, estimation the genetic relationship of *P.aeruginosa* strains and could be utilized for assurance of bacterial transmission follow in the community and hospitals. ERIC-PCR uses specific primers related to ERIC (entero-bacterial repetitive intergenic consensus) sequences, the repetitive sequences that found either in gram negative or gram positive bacteria. ERIC elements have been found in non-coding and inter-genic locales (Sharples and Lloyd, 1990). These components are 126 bp long, exceedingly preserved and incorporate a central core altered repeat. The position of ERIC components in enterobacterial genomes shifts between diverse species and has been utilized as a genetic marker to characterize separates inside a bacterial species (Son *et al.,* 2002). So ERIC-PCR can be utilized to increment the proficiency in ponder of genetic dissemination of *P. aeruginosa* comparison of ERIC -PCR profiles uncovered a moo level of likeness among the strains analyzed. ERIC components have been profoundly moderated inside the eubacteria kingdom for at slightest hundreds of millions of a long time. ERIC-PCR permits clear qualifications between diverse bacterial species and strains which contain these tedious components (De Bruijn, 1992). This method have been utilized for genotyping of distinctive species and diverse strains of bacteria. ERIC-PCR has too been utilized for *P. aeruginosa* isolates from patients with cystic fibrosis and keratitis in a few nations (Pinna *et al*., 2008).

5.2.2.5 Single nucleotide sequences analysis of *oprD* genes

Later improvements in molecular strategies have revolutionized the location and characterization of microorganisms in a wide range of therapeutic demonstrative fields (Regmi *et al*., 2015). PCR coupled with sequencing has ended up a effective apparatus for epidemiological examination of unused and rising irresistible infections (Speers, 2009). Sequences analysis of known anti-mycobacterial drug-resistant genes is regularly utilized to anticipate resistance to anti-microbials. Be that as it may, a few polymorphisms in such genes may serve a phylogenetic reason or maybe than resistance to drugs (Regmi *et al*., 2015).The *oprD* gene codes for a specialized pore protein, *oprD* which permits particular penetration of essential amino acids and their auxiliary analogs like the carbapenem anti-microbials imipenem and meropenem (Valones *et al*., 2009).

In this study, SNPs of *oPrD* gene has been applied in order for molecular characterization of this gene and genotyping of *P. aeruginosa* isolates which enrolled in public hospitals in our region.The multiple alignments of sequencing fragment of *oprD* gene revealed most mutations are none sense mutations and only one mutation is sense mutation. However, in other previous studies, variability with multiple amino acid changes with a micro-scale mosaic structure were detected due to multiple recombination events (Pirnay *et al.*, 2002). For such a variation to be considered a SNP, it must occur in at least 1 % of the population studied, SNPs are believed to play an important role for the susceptibility to disease and the response to drugs.

RECOMENDATIONS

- The Introduction of the molecular subtle elements of *P. aeruginosa* burn infections is fundamental to create effective methodologies for the anticipation contaminations and urological complications related with *P. aeruginosa*
- Since PCR innovation is increasingly accessible indeed in clinic research facilities, the utilize of 16srDNAgene as a molecular marker is exceedingly suggested especially in taking care of huge number of tests and may give quick and strong comes about in a brief time for affirmation of specific genus-species of *P. aeruginosa*
- More molecular studies are required for the detection of most *P. aeruginosa* strains creating *MβL* and *ESβLs* enzymes that are dependable for resistant to antimicrobial agents.
- More concern on microbiological studies for the assurance of sources of diseases by pathogenic specialists counting *P. aeruginosa* through collecting tests from clinic environment and wellbeing care staff in expansion to the clinical samples.

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APPENDIX INDEX

APPENDIX 1

FARKLI MOLEKÜLER TİPLENDİRME METOTLARI KULLANILARAK *PSEUDOMONAS AERUGİNOSA'NIN* EPİDEMİYOLOJİK OLARAK ARAŞTIRILMASI

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GENİŞLETİLMİŞ ÖZET

1. ÖZET

Bu çalışmada kullanılan 225 adet *P. aeruginosa* izolatı Nisan 2015-Eylül 2015 tarihleri arasında Duhok ve Erbil / Irak'taki büyük hastaneden toplandı. Bunların 136'sı (% 60,4) erkeklerden, 89'u (%39.6) kadınlardan izole edildi. Rasgele seçilen (Erbil ve Duhok bölgesi dahil her ilden 50) 100 adet *P. aeruginosa* izolatı antibiyotik duyarlılığı testine tabi tutuldu. Ticari kit kullanılarak çıkarılan genomik DNA konsantrasyonunun ortalaması 1.8 saflık ile 115.25 ng/µl, geleneksel yöntemle çıkarılan genomik DNA'nın ortalama konsantrasyonunun saflık değeri 1.6 saflık ile 1930.22 ng/µl bulundu. Tüm bu izolatların

P. aeruginosa olduğu moleküler seviyede teyit edilmesi için tüm suşlarda 16srDNA loküsünün yaklaşık 956 bp'lik bir moleküler ağırlığa sahip olan tek bir bandını üreten genom başarıyla amplifiye edildi. Beş virülanla ilişkili genin (Opr-1, tox-A, ekzo-S, las-B ve nan-1) taranması sonucu bu izolatların onda birinin (10) herhangi bir test edilmiş virülans belirteçlerinden yoksun olduğunu ortaya koydu. Patojenite varlığı göstergesi için bir belirteç olarak oprI, diğer tüm virülans belirteçleri arasında izolatların 90'ında (90) en baskın belirteçti ve bunu takiben toks-A ve ekzo-S her ikisi de 86'sında (86) idi. las-B geninin yaygınlığı 82'inde (82) ve nan-1 35'inde (35) ile bulundu. Beş metalo β-laktamaz (MβL) geninin (Vim-1, imp1, spm-1, sim ve gim) saptanmasının sonuçları patojenite varlığının göstergesi olan Vim1, diğer tüm

antibiyotik direnç belirteçleri arasında 46 (46) iken en baskın belirteç, bunu takiben imp1and spm-1 miktarları sırasıyla 45 (45) ve 35 (35) iken, sim geni miktarı 27 (27) ve gim 24 (24) bulundu. Genişletilmiş Spektrum β-laktamaz (ESβL) genlerini (oxa10, veb1 ve oxa2) içeren, patojenite belirteci olan oksa10 diğer tüm antibiyotik direnç göstergelerinin 91'ini (91), takip eden veb1 ve oksa2 sırasıyla 66(66) ve 36(36) olduğu belirlendi. *P. aeruginosa* izolatları (100) aynı zamanda iki ana kümeye atamak için ERIC-PCR parmak izi analizine tabi tutuldu. İzolatların 92'si A Grubu ki; 24 izolat (46) alt grupA1'e aitti ve incelenen suşların 46'sı (46) alt grup A2 idi, grup B sadece %8'i temsil ederken 4'er (4) adet iki alt grupta oldukları belirlendi. Bu çalışmada da, P. aueroginosa klinik suşlarından onbeş izolat seçildi ve OprD porin kodlayan OPrD geni kullanılarak tek nükleotid polimorfizmlerine (TNPs) tabi tutuldu ve kullanılan P. aureoginosa Str.PA12 (erişim numarası KJ482587.1) ile karşılaştırıldı. Bir referans suş olarak. OPrD geninin tüm sıralı fragmanının 3.77'sini muhafaza eden 19 polimorfik bölgede on dokuz nokta mutasyon ikamesinin (TNP) olduğu gözlemlendi. Bunlardan altısı (6/19) transversiyon tipi muhasebe 31.57 iken, on üçü 68.42 (13/19) geçiş idi. Transversiyon mutasyonu nedeniyle 328/503 bölgesinde sadece bir tane SNP sadece amino asit değişikliği (sens mutasyonu), diğer tüm mutasyonlar sessiz yer değiştirmelerle sonuçlandı. Dört allelotip tespit edildi.

Dokuz suşun aynı amino asit değiştirmelerini G328C'yi (Val 110 Lucien) paylaştığı ortaya çıkarıldı. Çalışılan suşların dendogram analizi, klinik soyların iki ana küme halinde sınıflandırıldığını ortaya koydu; ilk küme iki alt gruba ayrıldı; (*P. aureoginosa* suşları1, 3, 4, 8, 13 ve 14); (*P. aureoginosa* suşları 2 ve 12); diğer küme aynı zamanda iki alt gruba (*P. aureoginosa* suşlar 5 ve 15) ve (*P. aureoginosa* 6,7, 9,10 ve *P. aureoginosa* 11) ayrılmıştır. Çalışma izolatlarının yaklaşık 53.3'ü referans suş P. aureoginosa Str.PA12 ile ayrılırken, suşların 46.6'sı diğer küme ile görevlendirildi.

Anahtar kelimeler: ERIC, PCR, *OprD, P. aeruginosa*, Tek nükleotid dizileri,Yanık enfeksiyonu, *16srDNA*

2. LİTERATÜR BİLDİRİŞLERİ

Pseudomonas aeruginosa, organizmaları enfeksiyona sokan ve hastane enfeksiyonlarına neden olan fırsatçı bir patojendir. Kistik fibrozis (KF) hastalarının kronik akciğer enfeksiyonlarının başlıca nedenlerinden biridir ve birçok ortamda hastane kaynaklı enfeksiyonların önemli bir nedenidir. *P. aeruginosa*, çok çeşitli ortamlarda (su, toprak ve hayvanlarda) hayatta kalmak için çok uygundur ve ortak günlük çevrede yaygındır.

P. aeruginosa enfeksiyonları, kanser hastalarında, üriner sistem enfeksiyonlarına yakalanan hastalarda ve yanık yaraları geçiren hastalarda ortaya çıkabilir. *P. aeruginosa'nın* neden olduğu enfeksiyonlar, patojenin antimikrobiyal ajanların sergilediği belirgin direnç nedeniyle tedavisi genellikle zordur. *P. aeruginosa*, yanık yarasının başlıca kolonizörü olarak bulunur çünkü nemli yanık yara yüzeyinde gelişir ve çapraz bulaşma yoluyla yanık hastalarına genellikle erişir. Hastaları yakmak için önemli bir nozokomiyal enfeksiyon tehdidi olarak devam etmektedir. Birden fazla antimikrobik ilaçlara karşı direnç ortaya çıkması sıklıkla *P. aeruginosa* enfeksiyonunun tedavisini zorlaştırmaktadır. Bu ciddi enfeksiyona neden olabilir ve bu nedenle bu hastalarda mortalite oranı yüksek olur. Moleküler araçlar tanıtıldı ve hastalıkları geleneksel yöntemlere göre daha hassas ve özgün bir şekilde doğru tanılamada çok yardımcı olduğu bulundu. Bu araçların kullanımı, özellikle polimeraz zincir reaksiyonuna (PCR) dayanan teknikler, bulaşıcı hastalık etkenlerinin karakterizasyonu, tespiti, teşhisi ve taksonomisi üzerinde büyük bir etkiye sahiptir. Bu bağlamda, PCR teknolojilerine dayanan birçok farklı DNA işaretleyicisi olmuştur son çeyrek yüzyılda gelişti. *P. aeruginosa* türlerinin filogenetik gruplarının tanımlanması için PCR tabanlı teknik kullanılmıştır. DNA Dizisi, nükleotid bazlarının sırasını belirliyor, modern DNA dizileme teknolojisiyle elde edilen sıralama hızının hızlı olması, insan genom projesinde insan genomunun diziliminde etkili olmuştur.

3. MATERYAL VE YÖNTEM

Duhok ve Erbil / Irak'taki büyük hastanelere gelen farklı hastalardan, Nisan 2015 ile Eylül 2015 arasındaki dönemde cinsiyetler, farklı yaş ve yanık yüzdeleri açısından toplam 225 klinik izolat *P. aeruginosa* yanık enfeksiyonu izolatı toplandı. enfeksiyöz yanık yarasından gelen pus sürüntüleri, pansuman değiştirme anında toplandı. İzolatlar, Ulusal Klinik Laboratuvar Standartları Komisyonu ve Antimikrobiyal Duyarlılık Testi kılavuz ilkelerine göre Mueller-Hinton agar üzerinde disk difüzyon yöntemi ile antibiyotik duyarlılık testine tabi tutuldu. *P. aeruginosa'nın* yüz izolatı seçilmiş ve moleküler yöntemlere tabi tutulmuştur. Her ilden elli izolat rastgele seçilmiştir (Erbil ve Duhok). Genomik DNA ekstraksiyonu, geleneksel yöntem ve ticari kit (DNP TM Yüksek verim DNA Saflaştırma) kullanılarak gerçekleştirildi. Her bir numune için genomik DNA'nın konsantrasyonu ve saflığı, her numunenin konsantrasyonunu ve saflığını kaydetmek suretiyle NanoDrop Spektrofotometre kullanılarak belirlendi. Agaroz jel, istenen uygulamaya göre farklı konsantrasyonlarda hazırlandı. Agaroz jelleri etidyum bromür ile boyandı. DNA bantları U.V. tarafından görselleştirildi. U.V.'de 366 nm dalga boyunda aydınlatma transillüminator.

Molekül ağırlığı 956 bp olan 16srDNA geninin dahili parçası. (ileri ve geri) dahil olmak üzere bir çift 16srDNA primer kullanılarak amplifiye edildi. Master-mix, her testte 100 *P. aeruginosa* izolatı artı kontrol için hazırlandı. Virülanla ilgili genlerin tespiti için aşağıdakileri içeren beş primer (nan-1, exo-s, las-b, tox-a, opr-1) kullanılmıştır; Seçilen 100 *P. aeruginosa* soyundan ekanotoksin A ve dış zar lipoproteini varsayımsal nöraminidaz, ekzoenzim S, elastaz, bir çinko metalloprotaz, ayrıca sekiz primer, metalo-B-laktamazların (MBL) ve genişlemiş spektrumlu B-laktamazların tespiti için kullanılmıştır ESBL) Genler. (vim-1, imp-1, spm-1, gim, sim, oksa10, oksa2, veb-1), seçilen 100 *P. aeruginosa* suşu arasından seçildi.

Entrobacterial Repetitive Intergenic Consensus ERIC dizilerine dayanan evrensel primerler, DNA amplifikasyonunda kullanılmıştır. PCR kalıpları görsel olarak değerlendirildi ve her bir taksonun ayrı bir sütunda rapor edildiği ve aynı uzunluktaki DNA fragmanlarının varlığı / yokluğunun sıralar halinde 1/0 olarak rapor edildiği bir matris derlendi.

4. BULGULAR TARTIŞMA ve SONUÇ

MacCokey ve Cetrimide agarında tüm izolatların kültürlenmesinin sonuçları, *P. aeruginosa* olarak doğrulandı Erbil ve Duhok / Irak'taki farklı hastanelerdeki numunelerin taşınması esnasında herhangi bir bulaşmadan korunmasını sağlamıştır. Bu izolatlar, MacConkey agarında ve Kan agar β-hemolitik koloniler üzerinde negatif açık sarı koloniler üreten laktoz fermentasyonunda bulundu. *P. aeruginosa* ile enfekte 225 hastada. Alev yanması, 125 (55.5) hastada yanık yaralanmalarının başlıca nedeni idi; burada yanık yaralanmasının 85 (37.7) hastada ikinci yaygın neden olduğu ve 15 hastanın (6.6) yanık olduğu kimyasal asit) yanar. Alevle yanık hasarı alan 125 hastanın 42'si (33.6) idi. Duhok ve Erbil illerinde farklı hastanelerdeki *P. aeruginosa* yanık enfeksiyonu izolatlarının (rasgele seçilen 50 izolat) antibiyotik duyarlılık testi sonuçları. İzolatların hiçbirinin tüm antibiyotiklere duyarlı olmadığı açıktır; Carbapenems ajanlarından biri olarak imipenem antibiyotiği, % 47 direnç oranı ile kullanılan diğer tüm antimikrobiyal ajanlar arasında en güçlü olduğu bulunmuştur. Geleneksel yöntemle ekstrakte edilen genomik DNA'nın ortalama konsantrasyonu 1928.11 ng/µl, saflık değeri 1.6 iken, ticari kit ile elde edilen genomik DNA'nın ortalama konsantrasyonu 115.25 ng/µl, saflık değeri 1.8 idi. Tekrarlanan deneylerde, 100 *P. aeruginosa* izolatının tümü başarıyla amplifiye edildi ve molekül ağırlığı yaklaşık 956 bp olan tüm suşlarda tür spesifik lokus olarak 16Sr-DNA'nın tek bir bandını ürettiler. Tüm 100 *P. aeruginosa* izolatı virülans ile ilgili genlerin prevalans hızlarını belirlemek için PCR tekniklerine tabi tutuldu; (opr-1, tox-A, ekzo-S, las-B ve nan-1) markeri ve dağılımı. OprI marker prevalansının, bu izolatların % 90'ını oluşturduğu, nispeten yüksek olduğu ve toxA geninin88'ini oluşturduğu bulunmuştur; exoS geni bu izolatların % 86'sı arasında görülür. LasB ve nan-1 prevalansı sırasıyla % 84 ve % 38 oranlarına sahip bu izolatların en düşüktü. Bu çalışmada tüm 100 *P. aeruginosa* izolatı, (vim-1, imp1, spm-1, sim ve gim) markeri ve bunların dağılımı dahil metalo β-laktamaz (MβL) ile ilişkili genlerin prevalans hızlarını belirlemek için PCR tekniklerine tabi tutulmuştur. Bu izolatların vim-1 markör prevalansının 24'ü (% 48), imp1 geni ise 23'ü (% 46), bunların 20'sinde (% 40) spm-1 geni oluşturduğunu tespit etmiştir izole eder. Sim ve gim yaygınlığı sırasıyla 13 (% 26) ve 12 (% 24) oranlarla bu izolatlar arasında en düşük olmuştur. 100

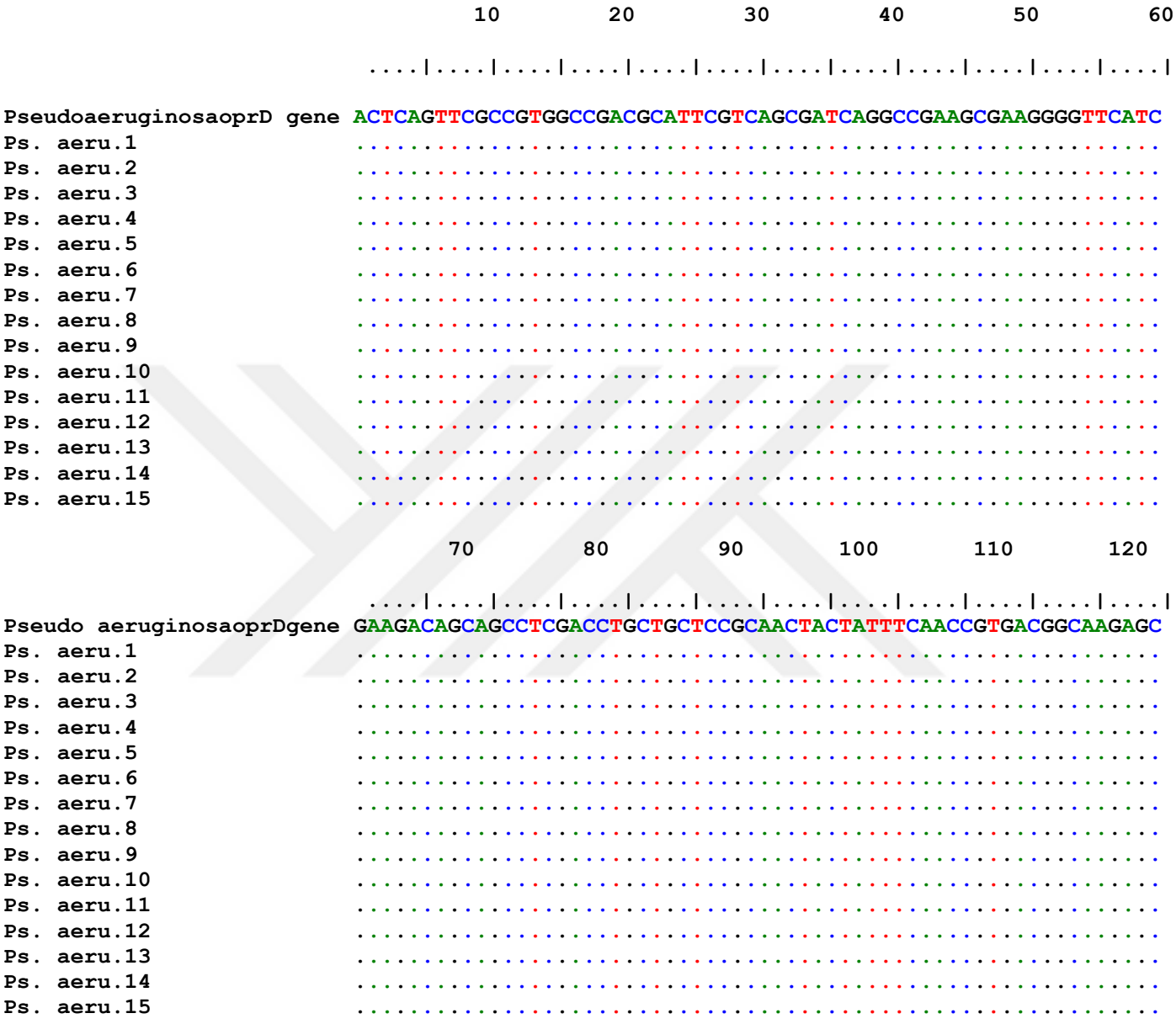
P. aeruginosa izolatı, (oxa10, veb1 ve oxa2) markeri ve bunların dağılımı dahil olmak üzere (ESβL) ilgili genlerin yaygınlık oranlarını belirlemek için PCR tekniklerine tabi tutuldu. Oxa10 markörünün prevalansının bu izolatlar arasında nispeten yüksek olduğu (45%) veb1 geni 33 (% 66), oxa2 geni ise 17 (% 34) izole edildi. Dendogram analizinin sonuçları, 100 izolatın iki ana kümeye ayrıldığını, A Grubu muhasebesinde92, B grubunun sadece8'ini temsil eden grup, Şekil 4-4'de gösterildiği gibi iki alt gruba ayrılabildiğini gösterdi.). Grup A; Çalışılan suşların 46 izolatı, A2 alt grubunda (% 46), 4 (% 4) subgropB1 suşunda, 4 suş sadece B2 alt grubuna (%4) aitti. Her alt grupta, tüm suşlar için karmaşık parmak izi kalıpları elde edilmiştir.

Bu çalışmada, *P. aueroginosa* klinik suşlarından on beş izolat seçildi ve *OprD* porine'i kodlayan OPrD geni kullanılarak tek nükleotid polimorfizmlerine (SNPs) tabi tutuldu. 19 polimorfik bölgede 19 noktalı mutasyon gözlendi ve bunların hepsi sıralı OPrD geninin fragmanının % 3.77'sini, 19 noktalı mutasyondan altısı, 6'sı (6/19) transversiyon tipi muhasebe % 31.57, üçüncüsü ise % 68.42'dir (13/19) geçişlerdi. Transversion mutasyonu nedeniyle 328/503 bölgesinde sadece bir SNP, amino asit değişikliği (sens mutasyon) ile sonuçlandı, diğer tüm mutasyonlar sessiz ikamelerdi. Dizilim, hizalamalar ve OPrD geninin referans suşla karşılaştırılması bölümünden beş allelotip tespit edildi; ilk alelotip altı klinik suş içeriyordu (P *aueroginosa* 1, 3, 4, 8, 13 ve 14), referans suşa100 benzer ve bu bölgede herhangi bir mutasyona sahip değildir, *P. aueroginosa* 2, Tablo (4.8) 'de gösterildiği gibi yalnızca yedi mutasyon geçiren benzersiz bir profile sahiptir ve altı sıralı suşlar bir allelotipe (*P. aueroginosa* 6,7,9,10,11 ve 15) 17 nokta mutasyonuna ve Ps'ye *P. aueroginosa* 5 suşu 18 nokta mutasyona sahiptir, dokuz *P. aueroginosa* suşları arasında mutasyon saptanmıştır.

Bu çalışmada, *P. aueroginosa* klinik suşlarından on beş izolat seçildi ve OprD porine'i kodlayan *OPrD* geni kullanılarak tek nükleotid polimorfizmlerine (SNPs) tabi tutuldu. 19 polimorfik bölgede 19 noktalı mutasyon gözlendi ve bunların hepsi sıralı OPrD geninin fragmanının % 3.77'sini, 19 noktalı mutasyondan altısı, 6'sı (6/19) transversiyon tipi muhasebe % 31.57, üçüncüsü ise % 68.42'dir (13/19) geçişlerdi. Transversiyon mutasyonu nedeniyle 328/503 bölgesinde sadece bir SNP, amino asit değişikliği (sens mutasyon) ile sonuçlandı, diğer tüm mutasyonlar sessiz ikamelerdi. Dizilim, hizalamalar ve OPrD geninin referans suşla karşılaştırılması bölümünden dört allelotip tespit edildi;

ilk alelotip altı klinik suş içeriyordu (*P. aueroginosa* 1,3,4,8,13 ve 14), referans suşa %100 benzer ve bu bölgede herhangi bir mutasyona sahip değildir, Ps. aeru.2, Tablo (4.8)'de gösterildiği gibi yalnızca yedi mutasyon geçiren benzersiz bir profile sahiptir ve altı sıralı suşlar bir allelotipe (*P. aueroginosa* 6,7,9,10,11 ve 15) 17 nokta mutasyonuna ve Ps'ye sahiptir. *P. aueroginosa* 5 suşu 18 nokta mutasyona sahiptir, dokuz *P. aueroginosa* suşları arasında mutasyon saptanmıştır..

APPENDIX 2


10 20 30 40 50 60
PseudoaeruginosaoprD gene ACTCAGTTCGCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATC
Ps. aeru.1
Ps. aeru.2
Ps. aeru.3
Ps. aeru.4
Ps. aeru.5
Ps. aeru.6
Ps. aeru.7
Ps. aeru.8
Ps. aeru.9
Ps. aeru.10
Ps. aeru.11
Ps. aeru.12
Ps. aeru.13
Ps. aeru.14
Ps. aeru.15
70 80 90 100 110 120
Pseudo aeruginosaoprDgene GAAGACAGCAGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGC
Ps. aeru.1
Ps. aeru.2
Ps. aeru.3
Ps. aeru.4
Ps. aeru.5
Ps. aeru.6
Ps. aeru.7
Ps. aeru.8
Ps. aeru.9
Ps. aeru.10
Ps. aeru.11
Ps. aeru.12
Ps. aeru.13
Ps. aeru.14
Ps. aeru.15

```
                                  130       140       150       160       170       180
                                  ....|....|....|....|....|....|....|....|....|....|....|
Pseudo aeruginosaoprDgene GGCAGCGGGGACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTC
Ps. aeru.1                ..............................................................
Ps. aeru.2                ..............................................................
Ps. aeru.3                ..............................................................
Ps. aeru.4                ..............................................................
Ps. aeru.5                ..............................................................
Ps. aeru.6                ..............................................................
Ps. aeru.7                ..............................................................
Ps. aeru.8                ..............................................................
Ps. aeru.9                ..............................................................
Ps. aeru.10               ..............................................................
Ps. aeru.11               ..............................................................
Ps. aeru.12               ..............................................................
Ps. aeru.13               ..............................................................
Ps. aeru.14               ..............................................................
Ps. aeru.15               ..............................................................

                                  190       200       210       220       230       240
                                  ....|....|....|....|....|....|....|....|....|....|....|
Pseudo aeruginosaoprDgene ACCCAAGGCACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGAC
Ps. aeru.1                ..............................................................
Ps. aeru.2                ..........C.....................................C.............
Ps. aeru.3                ..............................................................
Ps. aeru.4                ..............................................................
Ps. aeru.5                ..........C.....................................C.............
Ps. aeru.6                ..........C.....................................C.............
Ps. aeru.7                ..........C.....................................C.............
Ps. aeru.8                ..............................................................
Ps. aeru.9                ..........C.....................................C.............
Ps. aeru.10               ..........C.....................................C.............
Ps. aeru.11               ..........C.....................................C.............
Ps. aeru.12               ..........C.....................................C.............
Ps. aeru.13               ..............................................................
Ps. aeru.14               ..............................................................
Ps. aeru.15               ..........C.....................................C.............

                                  250       260       270       280       290       300
                                  ....|....|....|....|....|....|....|....|....|....|....|
Pseudo aeruginosaoprDgene GGCACCTCCGACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGC
Ps. aeru.1                ..............................................................
Ps. aeru.2                ........G.....................................................
Ps. aeru.3                ..............................................................
Ps. aeru.4                ..............................................................
Ps. aeru.5                ........G.....................................................
Ps. aeru.6                ........G.....................................................
Ps. aeru.7                ........G.....................................................
Ps. aeru.8                ..............................................................
Ps. aeru.9                ........G.....................................................
Ps. aeru.10               ........G.....................................................
Ps. aeru.11               ........G.....................................................
```

```
Ps. aeru.12                .....G.....................................................
Ps. aeru.13                ...........................................................
Ps. aeru.14                ...........................................................
Ps. aeru.15                .....G.....................................................

                                  310       320       330       340       350       360

                           ....|....|....|....|....|....|....|....|....|....|....|....|
Pseudo aeruginosaoprDgene  GATGACTACAGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAG
Ps. aeru.1                 ...........................................................
Ps. aeru.2                 ..C.........T.....C...A.....................................
Ps. aeru.3                 ...........................................................
Ps. aeru.4                 ...........................................................
Ps. aeru.5                 ..C.........T.....C.........T...............................
Ps. aeru.6                 ..C.........T.....C.........T...............................
Ps. aeru.7                 ..C.........T.....C.........T...............................
Ps. aeru.8                 ...........................................................
Ps. aeru.9                 ..C.........T.....C.........T...............................
Ps. aeru.10                ..C.........T.....C.........T...............................
Ps. aeru.11                ..C.........T.....C.........T...............................
Ps. aeru.12                ..C.........T.....C...A.....................................
Ps. aeru.13                ...........................................................
Ps. aeru.14                ...........................................................
Ps. aeru.15                ..C.........T.....C.........T...............................

                                  370       380       390       400       410       420

                           ....|....|....|....|....|....|....|....|....|....|....|....|
Pseudo aeruginosaoprDgene  TGGGGCGAGATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCG
Ps. aeru.1                 ...........................................................
Ps. aeru.2                 ...........................................................
Ps. aeru.3                 ...........................................................
Ps. aeru.4                 ...........................................................
Ps. aeru.5                 .....A.....T.....G.........C...............................
Ps. aeru.6                 .....A.....T.....G.........C...............................
Ps. aeru.7                 .....A.....T.....G.........C...............................
Ps. aeru.8                 ...........................................................
Ps. aeru.9                 .....A.....T.....G.........C...............................
Ps. aeru.10                .....A.....T.....G.........C...............................
Ps. aeru.11                .....A.....T.....G.........C...............................
Ps. aeru.12                ...........................................................
Ps. aeru.13                ...........................................................
Ps. aeru.14                ...........................................................
Ps. aeru.15                .....A.....T.....G.........C...............................
```

```
                                      430       440       450       460       470       480
                                ....|....|....|....|....|....|....|....|....|....|....|....|
PseudoaeruginosaoprDgeneCAGACCGCGACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCA
Ps. aeru.1              ...........................................................
Ps. aeru.2              ...........................................................
Ps. aeru.3              ...........................................................
Ps. aeru.4              ...........................................................
Ps. aeru.5              ..................A..........T..............T.....A..G
Ps. aeru.6              ..................A..........T..............T.....A..G
Ps. aeru.7              ..................A..........T..............T.....A..G
Ps. aeru.8              ...........................................................
Ps. aeru.9              ..................A..........T..............T.....A..G
Ps. aeru.10             ..................A..........T..............T.....A..G
Ps. aeru.11             ..................A..........T..............T.....A..G
```

```
                                490       500
                                ....|....|....|....|...
Pseudo  aeruginosa oprD gene    GGCCACTTCACCGAGGGCAAGGA
Ps. aeru.1                      .......................
Ps. aeru.2                      .......................
Ps. aeru.3                      .......................
Ps. aeru.4                      .......................
Ps. aeru.5                      .C............A......
Ps. aeru.6                      ............A........
Ps. aeru.7                      ............A........
Ps. aeru.8                      .......................
Ps. aeru.9                      ............A........
Ps. aeru.10                     ............A........
Ps. aeru.11                     ............A........
Ps. aeru.12                     .......................
Ps. aeru.13                     .......................
Ps. aeru.14                     .......................
Ps. aeru.15                     ............A......C.
```

CIRRICULUM VITE

Marwan Khalil QADER was born on 11 March 1983 in Duhok province in Iraq. After completing primary school in Mousl Province, he attended secondary and high school in Mosul. He graduated from Microbiology Department in Mosul University in 2005 and graduated M Sc. in Duhok University in 2010, Lecturer in Biology Department, college of Science, Duhok University since 2013 and puplishing many paper in defferent intrnational journal and sharing in many intrnational confrance. The main languages Arabic, Kurdish and English. He started his Ph.D. of Science degree at Van Yuzuncu Yil University in Van -Turkey on January 2014.

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