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MOLECULAR CHARACTERAZATİON OF VİRULENCE FACTORS AMONG ANTİBACTERİAL RESİSTANT *Pseudomonas Aerugınosa* ISOLATED FROM BURN INFECTİONS FROM DUHOK AND ERBİL HOSPİTALS/IRAQ.

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ABSTRACT

In the current study, 225 isolates of *P. aeruginosa*burn infection have been collected from major hospitals in Duhok and Erbil / Iraq, from April 2015 till 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 of these isolates were selected (fifty from each province including; Erbil and Duhok Province) and subjected to 16 different antibiotics using disc diffusion method showed high resistancy to most tested antibiotics, 90% of these isolates were multidrug resistant, Imipeneme was considered as the most effective antibiotic effect on these isolates with resistant rate 47%.The genome all of these isolates were successfully amplified producing a single band of the *16sr*DNA 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*with35(35%)respectively.

KEYWORDS:Burn infection, , *P. aeruginosa*, *PCR*, *16sr*DNA, Virulence genes

INTRODUCTİON

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, and those suffering from urinary tract infections, and patients suffering from burn wounds (Wisplinghoff and Seifert , 2016). 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* 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

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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). So that the aim of this study Screening of antimicrobial sensitivity profile of *P. aeruginosa*, Application of Specific-species PCR technique for the confirmation of *P. aeruginosa* isolates at molecular level.

MATERİALS AND METHODS

A total of 225 clinical isolates of *P. aeruginosa* from burn infection isolates were collected from different patients attending major hospitals in Duhok and Erbil / Iraq from both genes with different ages having various degree percentages of burn during the period from April 2015 till 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. All collected clinical isolates of suspected *P. aeruginosa* fromburn infection were cultured 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).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). Genomic DNA was extracted from one hundred *P. aeruginosa* strains using High yield DNA Purification Kit according to the manufacture instructions (Bioneer-Korea). Result of DNA extract was visualized by UV light after being electrophoresed on 1% agarose gel (Maniatis *et al.*, 1982). Table 1 shows the primers sequences and amplification bands sizes of *16srDNA, nan-1, exo-s, las-b, tox-a, opr-1*gene which used for Polymerase chain reaction by adding 12.5 µl of master mix(GeneDirex-USA), 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 2 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(Maniatis *et al.*, 1982).

Table (1): Molecular weight and sequencing of Primers used

Gene	Forward and Reverse primer	Size product	Refrences
16SrDNA	F 5′ GGGGGATCTTCGGACCTCA 3′ R 5′ TCCTTAGAGTGCCCACCCG 3′	956	Spilker *et al.*, 2004
Nan1	F 5′ AGGATGAATACTTATTTTGAT 3′ R 5′ TCACTAAATCCATCTCTGACCCGA 3′	1316	Stover *et al.*,2000
Exo-S	F 5′CTTGAAGGGACTCGACAAGG 3′ R 5′TTCAGGTCCGCGTAGTGAAT3′	504	Stover*et al.*,2000
Las-B	F 5′GGAATGAACGAAGCGTTCTC3′ R 5′GGTCCAGTAGTAGCGGTTGG3′	300	Stover *et al.*,2000
Tox-A	F 5′GACAACGCCCTCAGCATCACCAGC3′ R 5′CGCTGGCCCATTCGCTCCAGCGCT3′	396	Rawya*et al.*, 2008
Opr1	F 5′ATGAACAACGTTCTGAAATTCTCTT3′ R 5′CTTGCGGCTGGCTTTTTCCAG3′	249	De Vos *e al.*,1993

Table (2:) PCR amplification conditions of *16SrDNA,Nan1,Exo-S,Las-B,*Tox-Aand*Opr1*gene

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of *Pseudomonas* spp.

Primer	Initialdenaturation	Denaturation	Annealing	Extensin	Final extension
16SrDNA	95˚C2min 1 Cycle	94 ˚C20 sec.	54 ˚C20 sec. 25cycles	72˚C40sec	72˚C5min1cycle
Nan-1	94˚C 5 min.	94˚C 30 sec.	54C°1 min	72˚C1min.	72 ˚C90 sec.
	1 Cycle		36 cycles		1cycle
Exo-s	95˚C 2 min.	94˚C 30 sec.	60 ˚C 1min.	72 C°1min	72˚C5 min.
	1 cycle		35 cycles		1 cycle
Las-b	94˚C3min	94˚C30 sec.	60 ˚C 1min.	72˚C90sec.	72˚C 5 min.
	1 cycle		30 cycles		1 cycle
Tox-a	94˚C2 min	94˚C2 min.	68˚C1 min.	72˚C 1min.	72˚C7min.
	1 Cycle		30 cycles		1cycle
Opr-1	95˚C 2 min	94˚C40 sec.	57˚C50 sec.	72˚C20sec.	72˚C5 min.
	1 cycle		25 cycles		1 cycle

RESULTS

The purfreel colonies on MacConkey and Cetrimide agar affirmedto be *P. aeruginosa* and guaranteed them from anydefilementamid transporting oftests from distinctive clinics in Erbil and Duhok/ Iraq. These isolates were found non lactose ferment creating negative pale yellow colonies on MacConkey agar and on Blood agar shows β-hemolytic colonies.Colonies are surrounded by bluish green coloration on Nutrient agar due to producedsoluble pyocyanin and pyoverdine which is a water-soluble, yellow-green pigment, the colonies pigments on selective media (Cetermide agar) are more obvious *P. aeruginosa* burn contamination confines were affirmed by biochemical tests, oxidase test. Citrate utilization too identify capacity of development at a temperature astallas 42 ˚C. İn table 3. show illustrat number of the patients in defferent genes with percintage in both province

Table (3): samples collected of *P. aeruginosa* from two areas in Iraq and their recurrence among male and females patients.

Provinces	Patients No.	MaleNo. **(%)**	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. appears the connection of mode of burn with age, sex and total burn surface area (TBSA) in 225 patients tainted by *P. aeruginosa*. Flime burn was the generally over whelming 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 15was found in 29 (23.2%) and TBSA > 15wasfound 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 scaldburn, TBSA up to 15was 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 acidwere 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 acidburn, TBSA up to 15 % was found in 5 (33.3%) and TBSA > 15 % was found in 10 (66.6%) patients.

Table (4): Connection of mode of burn with age, sex, and add up to burn surface region (TBSA) in 225 patients tainted by *P. aeruginosa*

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Cause of burn	No. of patients%	Age		Sex		TBSA	
		<12years	>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)

Results of anti-microbial sensitivity test for *P. aeruginosa* burn isolates (50 chosen isolates) from distinctive clinics in Duhok and Erbilterritories displayed

in Table 5. It is clear that none of the isolateswas sensitive to allanti-microbials; Imipenem anti-microbial as one of Carbapenemsoperatorswas found to be the most powerful over all other antimicrobialspecialistsutilized with a resistance rate of 47%.

Table (5): 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 be considered as the second most compting antibiotic these isolates with a resistantrate of 53%, the tested *P. aeruginosa* isolates were shown a high resistance to most tested antibiotics; Tobramycin, Gentamicin , Amikacin and Aztreonam showed 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 to Ticarcillin/clavulanic acidand Ampcillin/Sulbactam was found 71% and 83% respectively.Trimethoprim/Sulfamethoxazole and Ciprofloxacin moreover appeared moo impact tested isolates with resistance rates 54 % and 56 % respctively, the isolates also appeared a high resistant rates to the forth generation of Cephalosporins such as Cefepime, Ceftazidime, Ceftriaxone and Cefuroxime with resistance rates of 93%, 91%, 90 %and 90%, separately. Concerning molecular test , all of the selected 100 *P.aeruginosa* isolates were successfuly produced amplified products with single band of the *16Sr-DNA* as the species specific locus with a molecular weight of almost 956 bp as appeared in Figure 1, this results affirmed at the molecular

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level that all these strains were in reality*P. Aeruginosa*

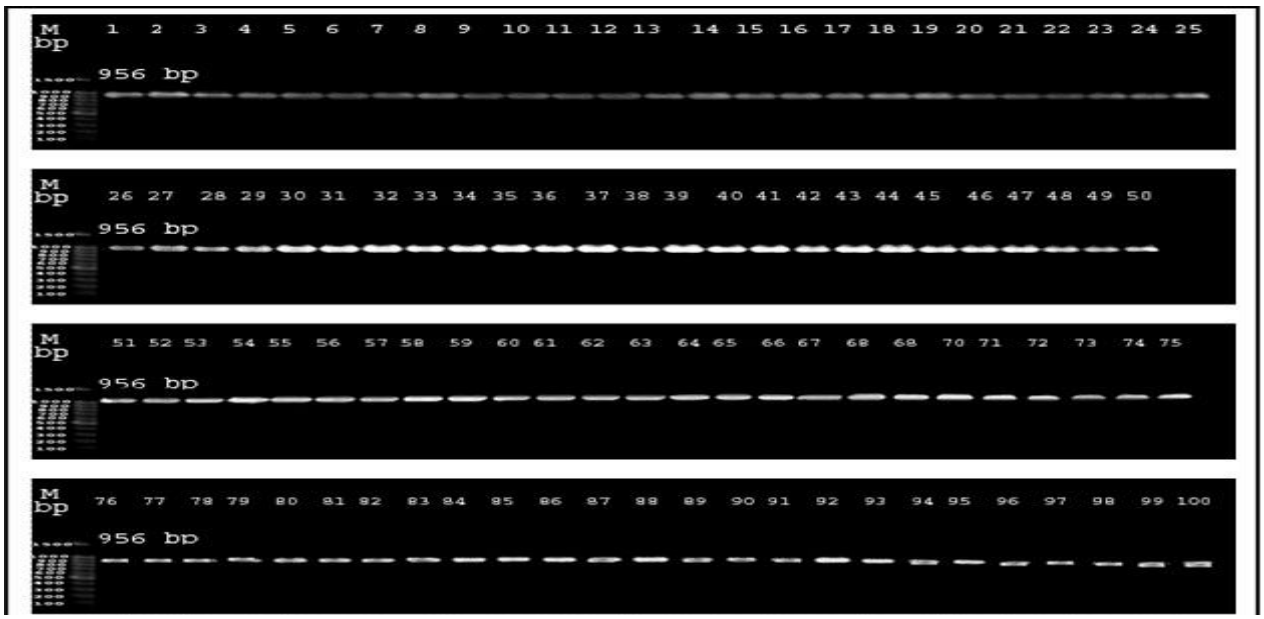

Fig. (1): PCR products bands of *16srDNA* of *P. aeruginosa* on 1.5% agarose gel and run with 5v/cm, for 1.30 hour. line M contained DNA marker (1500-100bp).

In this consider all 100 *P. Aeruginosa* isolates were subjected to PCR strategies to decide the prevalence rates of virulence related genes linke (*opr-1, tox-A,exo-S,las-B,* and *nan-1*) marker and their dispersion show the results in table 6and figure 2,3,4,5,6

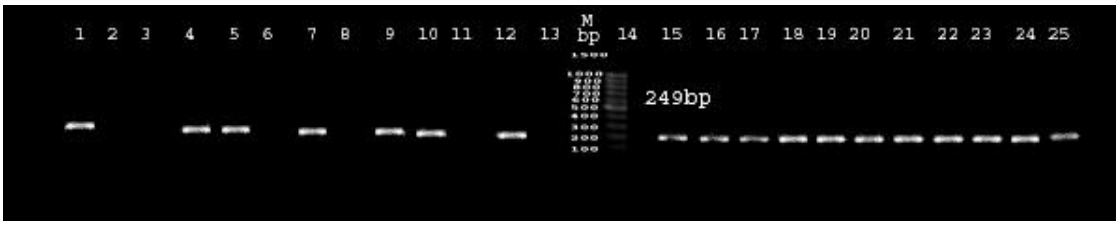

Fig. (2): PCR amplification of *oprI* gene with molecular weight 249bpon 1% agarose gel and run with 5v/cm, for 1.30 hours.line M contained DNA molecular weight marker (1500-100bp).

Fig. (3): PCR amplification of *toxA* gene with molecular weight 396 bp. on 1% agarose gel and run with 5v/cm, for 1.30 hours.line M contained DNA molecular weight marker (1500-100bp).

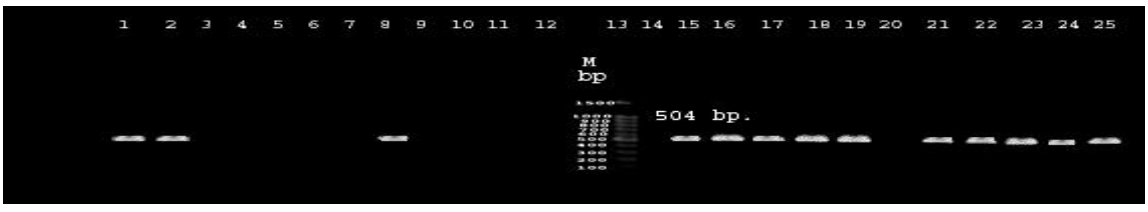

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Fig. (4): PCR amplification of *exoS* gene with molecular weight 504 bp. on 1.5% agarose gel and run with 5v/cm, for 2 hours line M contained DNA molecular weight marker (1500-100bp).

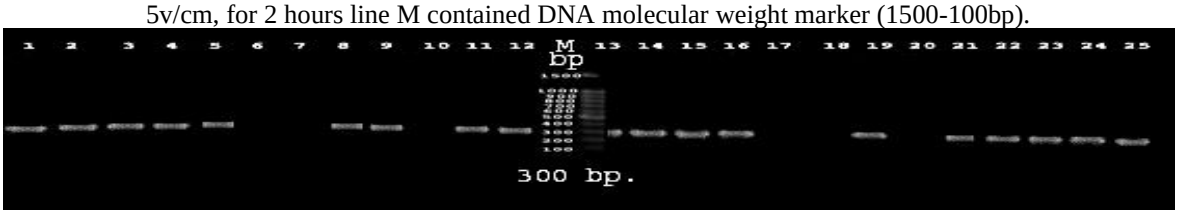

Fig. (5): PCR amplification of *lasB* gene with molecular weight 300 bp. on 1% agarose gel and run with 5v/cm, for 1.30 hours.line M contained DNA molecular weight marker (1500-100bp)

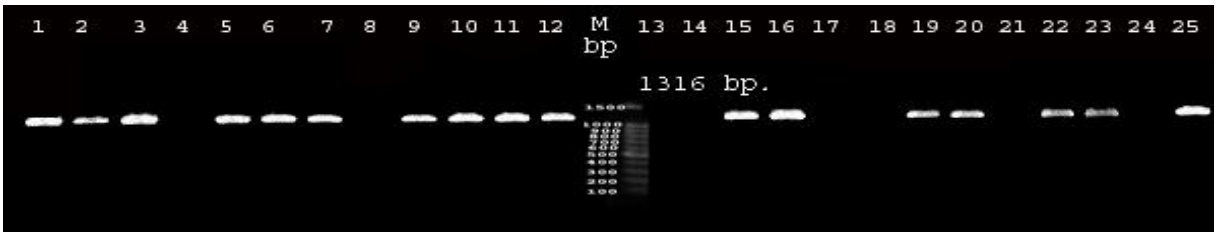

Fig. (6): PCR amplification of *nan-1*gene with molecular weight 1316 bp.on 1% agarose gel and run with 5v/cm, for 1.30 hours.line M contained DNA molecular weight marker (1500-100bp)

Table (6): 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)

DİSCSSİON

The results of *P. aeruginosa* isolates frequency shown in Table 4 out of 225 collected *P.aeruginosa* isolates from two cities (Erbil, and Duhok) in Iraq that 143 were from males (63.5 %) and 82 were from females (36.4 %). It was assessed that fire burn was the generally over whelming cause of burn wounds in 25 (80.6 %) patients and the second scald followed by the acid burn. The more seasoned male than 12 year and the TBSA> 15was the most noteworthy rate among the harmed in all case of burn (Naqvi *etal*., 2005). Total burn surfaceareais found to be the mostcriticalhazard 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 insidea unit, posturing as Multi drug resistant nosocomial disease hazard for patients beingtreated there. Hands of staff individuals can ended up moment arily sullied and exchange contamination among patients (Edwards, 2003).In a few considers the affectability of Imipenem against *P. aeruginosa* was moderate lymore, i.e. 86 % 78%, 88 % and 91.6%, separately (Neely and Holder, 1999). 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 synthesis of Carpabenemase enzymes in Gram-negative organisms is anexpanding universal open well being issue. Discovery of carbapenems resistance isolates in a healing center environment postures not as it were a restorativeissue, but moreover a genuine concern for infection controladministration (Hodiwala *etal*., 2013). Carbepenems are valuable in treatment of a few cases of multi-drug

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resistancestrains of *P. aeruginosa* (Douglas, 2001). Comparative thinks about more powerful antimicrobial agents are restricted, but a few of these drugs stay valuable for the treatment of certain patients (Nicolle, 2005). Another thinks about appearcomparablecomes about, 100 % isolates of *P. aeruginosa* were resistanceto Amikacin, 95 % to Gentamicin 94 % to Aztreonam and 91 % to Tobramycin (Naqvi *etal*., 2005).Ampcillin and Piperacillin recorded comparative design conducted in Pakistan where more than 87 % strains of *P. aeruginosa* were resistance to Ampcillin and Piperacillin (Naqvi *etal*., 2005).The broad utilize of fourthera cephalosporins as the driving rise of extended-spectrum β lactamase (ESBL) creating organisms in numerous issue (Paterson, 2006). It has been found that the genes that encode ESBLs arehabituallyfound on the same plasmids as genes that encode resistance to aminoglycosides and trimethoprim- sulfamethoxazole (Yasufuku *etal*., 2011).This implies that ESBL producing are commonly multidrug resistance, whichpostures a specific challenge for the treatment of nosocomial diseases.Unseemlyempiric antimicrobial treatment for nosocomial- or community-obtained diseases has been detailed to contributeessentiallyfor more prominentmortality rates in theseriouslycare unit (ICU).Other than,insufficientantimicrobial treatment ofdiseasewas the most critical free determinant of healing center mortality (Paterson, 2006).Numerous considers have detailed that the *16sr*DNAamplification appeared in Fig. 1 is a prerequest for any assist molecular examination (Theodore *etal*., 2004). The gotten result was in understanding with a consider conducted in Baghdad Territory utilizing the same primer(*16sr*DNAgene) for the identifecation of *P.aeruginosa* which delivered the same molecular weight,comes about in Fig. 2 to 6 appeared are in assention with a number of related distributed stuelies; 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 study conducted in Poland by Wolska and Szweda 2009 appeared that the predominance of virulance genes among *P.aeruginosa* isplates not from burin infection are *lasB, toxA,exoS,* at rates 96.8%, 88.7%, and 75.8%,separately. Fazeli and Momtaz 2012 also reported virulance factors of *P.aeruginosa* from burin disease at rates for *exoS* and *toxA* astaking after; 67.6 %, 35.2.% respectively .The contrasts in prevalence of *P.aeruginosa* virulance genes due to geographic locale was also 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 well being and hospital's cleanliness may be considered as variables that credited to the nearnessofvarieties in the prevalence rates of virulence genes of *P. aeruginosa* strains among diverse districts.

CONCLUSİON

It can be concluded that molecular methods can be. fast. and effective discriminating instruments in terms of identifying microorganisms which multi-drug resistance solates of *P. aeruginosa* posture agenuine chanceas they are spread broadly and score higher percentages with time.

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