Asian Journal of
Scientific Research

Research Article
Antibiotics Susceptibility Pattern and Virulence-associated Genes in Clinical and Environment Strains of *Pseudomonas aeruginosa* in Iraq

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Abstract

Background and Objective: *Pseudomonas aeruginosa* (*P. aeruginosa*) is considered one of the most dangerous opportunistic bacteria worldwide, live in hospitals and other environments such as sewage water and soil and cause different infections include pneumonia. The main aim of this study was to investigate antibiotics susceptibility pattern and genotypic detection of virulence genes in *P. aeruginosa* isolates from clinical and environment sources. **Materials and Methods:** A total of 120 *P. aeruginosa* isolates were collected from two sources, 60 isolates from sputum of patients with pneumonia and 60 isolates from sewage water. Antibiotics susceptibility testing was done according to Kirby-Bauer method. Polymerase chain reaction technique was performed to detect the prevalence of six virulence-associated genes. **Results:** All 120 isolates (100%) were resistance to amoxicillin. While, imipenem was the best efficient antibiotic against clinical and sewage water isolates. Fifty five clinical isolates (91.6%) and 41 sewage water isolates (68.3%) were multi-drug resistance (MDR). The *Pap A*, *Fim H*, *feoB*, *iutA*, *hly* and *kpsMTII* genes prevalence in clinical isolates with percentages 91.6, 100, 80, 95, 20 and 100%, respectively, while the same genes prevalence in sewage water isolates with percentages, 51.6, 68.3, 41.6, 35, 3.3, 5 and 41.6%, respectively. **Conclusion:** This study suggested that there was positive correlation between the high prevalence of virulence-associated genes and increase of antibiotics resistance in *P. aeruginosa* isolates.

Key words: *Pseudomonas aeruginosa*, antibiotics, virulence genes, pneumonia, sewage water

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INTRODUCTION

Pseudomonas aeruginosa is one of the most important pathogenic bacteria, it is Gram negative bacteria, obligate, aerobic, oxidase and coagulase positive, capsulated, motile, non-lactose fermenter, cause different nosocomial infections and distribution in hospitals and different environments such as sewage water[1,2]. All clinical isolates of *P. aeruginosa* are multi-drug resistance and resistant of different classes of antibiotics such as beta-lactams, fluoroquinolones and amino glycosides[3]. Recently, clinical isolates of *P. aeruginosa* has become a serious clinical problem worldwide due to an increase resistant to 3rd and 4th generation cephalosporins[4]. *Pseudomonas aeruginosa* is considered one of the most virulent bacteria able to live in different environments include, hospitals, sewage water, soil and it is able to infect human and wide range of animals[5,6]. Hypermucoviscosity, biofilm formation, lipopolysaccharides formation, capsule synthesis, adhesions and iron uptake are the most important virulence factors in most pathogens[7-9]. Many virulence factors such as capsular polysaccharides, adhesion, siderophores and fimbriae are play an important role in pathogenicity of *P. aeruginosa*[10]. Detection of the prevalence of virulence genes in pathogenic bacteria and other pathogens by polymerase chain reaction technique is a good method to identify virulence factors in all pathogens such as *Klebsiella pneumoniae* (*K. pneumoniae*) and *Escherichia coli* (*E. coli*)[11,12]. These virulence factors allowing pathogenic bacteria to cause different illness such as pneumonia, burns infections and bacteremia[13]. Any microorganism especially pathogenic bacteria can be a virulent when a single factor presented, sometimes the presence of various factors at the same time is required to decide the bacterial ability of causing infections[14]. Some previous and recent studies outside Iraq suggested that there was positive correlation between the high presence of virulence genes and high resistance of antibiotics in pathogenic bacteria isolated from clinical and environment sources[15-18]. In Iraq, there was no studies focus on this relationship, therefore, the main aim of the present study was to compare between 60 clinical and 60 sewage water isolates of *P. aeruginosa* according to antibiotics susceptibility pattern and the prevalence of virulence genes in these isolates.

MATERIALS AND METHODS

Samples and study design: This is a case control study performed in Department of Biology, Faculty of Science, University of Kufa, Iraq. A total of 120 isolates of *P. aeruginosa* were collected from two sources during the period from January-October, 2017; 60 isolates were collected from sputum of patients infected with pneumonia was considered as case study and 60 isolates were collected from sewage water was considered as a control study.

Sample collection and *P. aeruginosa* identification: Non-duplicate 60 sputum samples were collected in sterile containers (Himedia, India) from patients infected with pneumonia (diagnosis by specialized physician) admitted in Al-Najaf hospital in Al-Najaf City, Iraq (mouth should be rinsed out with distilled water, breath and cough deeply, coughed up from the lungs, not saliva or mucous, early in the morning upon rising) and homogenized with distilled water. Non-duplicate 60 samples of sewage water was collected in sterile containers (Himedia, India) from purified water station in Al-Najaf City, Iraq, during the period from January- October, 2017. Immediately, all collected samples were transferred to the Laboratory of Microbiology, Department of Biology, Faculty of Science, University of Kufa. All sputum and sewage water samples were streaked (by sterile swab, Bioanalyse, Turkey) onto blood agar (Oxoid, UK) surface, MacConkey agar (Oxoid, UK) surface and chocolate agar (Oxoid, UK) surface and incubated 24 h at 37°C. Any bacterial growth was identified by standard biochemical tests according to MacFaddin[19] such as Gram positive, oxidase positive, coagulase positive, motility positive and growth on mannitol salt agar.

Antibiotics susceptibility testing: Disc diffusion method was used for antibiotics susceptibility test according to method by Kirby-Bauer[20]. Ten different antibiotics were used provided from Bioanalyse Company, Turkey as follows: Amoxicillin (25 µg), amoxiclav (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone(30 µg), amikacin (30 µg), tobramycin (10 µg), gentamicin (15 µg), tetracycline (30 UI) and imipenem (10 µg). Clinical and Laboratory Standards Institute[21] was used as a guideline of antibiotic susceptibility and resistance according to bacterial zone diameter. According to the results of antibiotics sensitivity test, any bacterial isolate was resist to at least three different types of antibiotics classes was considered as a MDR, if was susceptible to only one type of antibiotic class was considered as a XDR and when resist to all types of antibiotics classes was considered as a PDR (CLSI[21]).

Phenotypic detection test of extended spectrum beta-lactamase: This test was done according to method by Aljanaby and Alhasnawi[11] as follow: Amoxiclav disc (30 µg) was

Table 1: All genes and primer sequences used in this study

Genes	Primer sequences	Product size (bp)	References
iutA	F: ATGAGCATATCTCCGGACG	587	Moulin-Schouleur *et al.*[22]
	R: CAGGTCGAAGAACATCTGG		
kpsMTIII	F: GCGCATTTGCTGATACTGTTG	272	
	R: CATCCAGACGATAAGCATGAGCA		
fimH	F:TGCAGAACGGATAAGCCGTG	508	
	R:GCAGTCACCTGCCCTCCGGTA		
papA	F:ATGGCAGTGGTGTTTTGGTG	720	Yun *et al.*[23]
	R:CGTCCCACCATACGTGCTCTC		
feoB	F: AATTGGCGTGCATGAAGATAACTG	470	
	R:AGCTGGCGACCTGATAGAACAATG		
hly	F:GACCCGGCACAAGCATAAGC	384	
	R:CCACCTGCAGCAACAAGAGG		

placed in the center of Mueller Hinton agar plate (Oxoid, UK). Ceftriaxone disc (30 µg), cefotaxime disc (30 µg) and ceftazidime disc (30 µg) were placed with distance of 15 mm from center to center of amoxiclav disc and incubated at 37°C overnight. If inhibition zone was increased towards the amoxiclav disc then considered as positive result.

DNA extraction, primer sequences and thermo cycling conditions: This method was done according to procedure by Aljanaby and Alhasnawi[11]. Briefly, all *P. aeruginosa* isolates were heated in boiling water at 100°C for 30 min and centrifugation at 8000 rpm for 10 min, the supernatant was considered as a DNA template. The primer sequences are mentioned in Table 1. The multiplex PCR program consisted of an initial denaturation step at 95°C for 4 min, followed by 35 cycles of DNA denaturation at 52°C for 60 sec, primer annealing at 55°C for 60 sec extension 72°C for 60 sec. After the last cycle, a final extension step at 72°C for 5 min. Five microliter aliquots of PCR product were analyzed by gel electrophoresis with 2% agarose[22,23].

Statistical analysis: GraphPad prism computer software version 8 was used in this study for comparison between samples according to Chi-squared test. The p<0.05 was considered statistically significant.

RESULTS

Antibiotics susceptibility: All clinical isolates 100% (60/60) were resistance to amoxicillin, that were resistant to other antibiotics with different percentages as follow: Amoxiclav 91.6% (55/60), cefotaxime 88.3% (53/60), ceftazidime 81.6% (49/60), ceftriaxone 78.3% (47/60), amikacin 75% (45/60), tobramycin 63.3% (38/60), gentamicin 58.3% (35/60), tetracycline 45.6% (28/60) and imipenem 11.6% (7/60). Also, all sewage water isolates 100% (60/60) were resistance to amoxicillin, while, was resistant to

Table 2: Numbers and percentages of *P. aeruginosa* strains isolated from clinical source and sewage water that were resistant to antibiotics

	Clinical isolates	Sewage water isolates
Antibiotics	60 (100%)	60 (100%)
Amoxicillin (25 µg)	60 (100)	60 (100)
Amoxiclav (30 µg)	55 (91.6)	41 (68.3)
Cefotaxime (30 µg)	53 (88.3)	41 (68.3)
Ceftazidime (30 µg)	49 (81.6)	40 (66.6)
Ceftriaxone (30 µg)	47 (78.3)	39 (65.0)
Amikacin (30 µg)	45 (75.0)	29 (48.3)
Tobramycin (10 µg)	38 (63.3)	29 (48.3)
Gentamicin (15 µg)	35 (58.3)	21 (35.0)
Tetracycline (30 UI)	28 (45.6)	21 (35.0)
Imipenem (10 µg)	7 (11.6)	2 (3.3.0)

Table 3: Numbers and percentages of MDR, XDR and PDR of *P. aeruginosa* strains isolated from clinical source and sewage water

	Clinical isolates	Sewage water isolates	
Resistances	60 (100%)	60 (100%)	p-value*
MDR	55 (91.6)	41 (68.3)	0.0061
XDR	9 (15.0)	5 (8.3)	0.5237
PDR	1 (1.3)	1 (1.3)	1.0000

Compare between clinical isolates and sewage water isolates, MDR: Multi-drug resistance, XDR: Extensive-drug resistance, PDR: Pan-drug resistance

amoxiclav and cefotaxime with percentage 68.3% (41/60), ceftazidime 66.6% (40/60), ceftriaxone 65% (39/60), amikacin and tobramycin 48.3% (29/60), gentamicin and tetracycline 35% (21/60) and imipenem3.3% (2/60) (Table 2). Out of 60 clinical isolates, there were 55 isolates (91.6%) were MDR, 9 isolates (15%) were XDR and 1 isolate (1.3%) was PDR. Out of 60 sewage water isolates, there were 41 isolates (68.3%) were MDR, 5 isolates (8.3%) were XDR and 1 isolate (1.3%) was PDR. The results proved that there was significant differences (p-value = 0.0061) in the prevalence of MDR of clinical isolates as compared with sewage water isolates (Table 3). While, there was no significant differences (p-value = 0.3282) between clinical isolates (18/60, 30%) and sewage water isolates (11/60, 18.4%) in the prevalence of ESBL-producing isolates (Table 4).
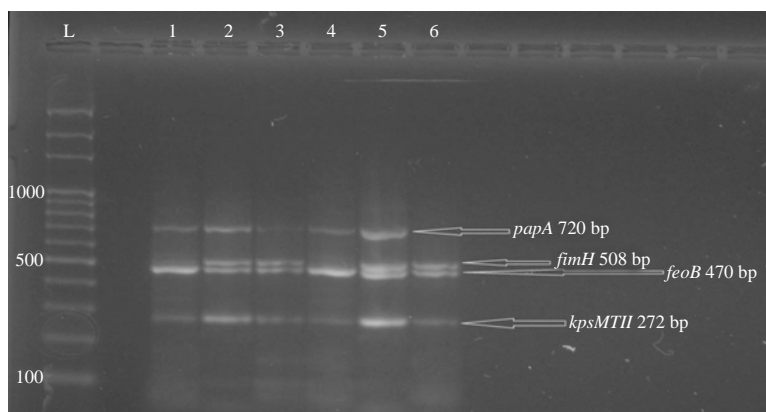
Fig. 1: Gel-electrophoresis of PCR amplification of *P. aeruginosa* DNA using *papA*, *fimH*, *feoB* and *kpsMTII* genes specific primers show positive results at (720, 508, 470 and 272) bp, respectively
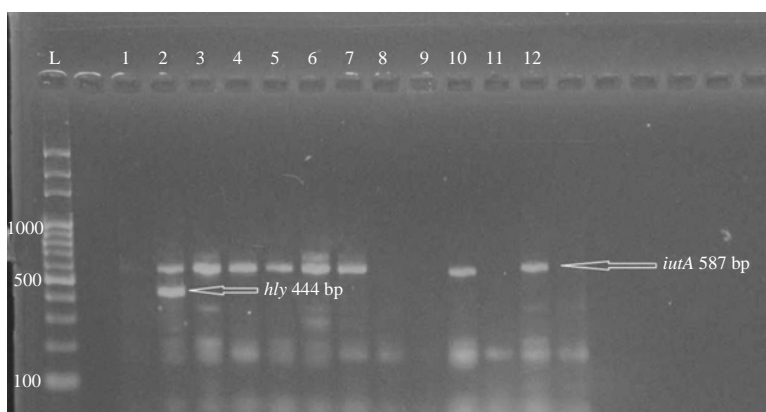

Fig. 2: Gel-electrophoresis of PCR amplification of *P. aeruginosa* DNA using *iutA* and *hly* genes specific primers show positive results at (587 and 444) bp, respectively

Table 4: Numbers and percentages of ESBL-producing *P. aeruginosa* strains isolated from clinical source and sewage water

	Clinical isolates	Sewage water isolates	
ESBL	60 (100%)	60 (100%)	p-value*
Positive ESBL	18 (30)	11 (18.4)	0.3282
Negative ESBL	42 (70)	49 (81.6)	

Compare between sputum and sewage water isolates, ESBL: Extended spectrum beta-lactamase

Table 5: Numbers and percentages of prevalence of virulence genes in *P. aeruginosa* strains isolated from clinical source and sewage water

Virulence genes	Clinical isolates 60 (100%)	Sewage water isolates 60 (100%)	p-value*
PapA	55 (91.6)	31 (51.6)	<0.0001
FimH	60 (100.0)	41 (68.3)	<0.0001
hly	12 (20.0)	2 (3.3)	0.0175
feoB	48 (80.0)	25 (41.6)	<0.0001
iutA	57 (95.0)	21 (35.0)	<0.0001
kpsMTII	60 (100.0)	25 (41.6)	<0.0001

Compare between clinical isolates and sewage water isolates

Genotypic detection of virulence genes: The results proved that there were significant increase ($p < 0.05$) in the prevalence of virulence genes in clinical isolates as compared with sewage water isolates (Table 5). The *PapA*, *FimH*, *feoB*, *iutA*, *hly* and *kpsMTII* genes were prevalence in clinical isolates with percentages 91.6, 100, 80, 95, 20 and 100%, respectively, while the same genes were prevalence in sewage water isolates with percentages, 51.6, 68.3, 41.6, 35, 3.3 and 41.6%, respectively (Fig. 1 and 2).

DISCUSSION

This study showed that the clinical and sewage water isolates of *P. aeruginosa* were MDR with percentages 91.6 and 68.3%, respectively with significant increase ($p = 0.0061$) in the prevalence of clinical isolates as compared with

sewage water isolates. Multi-drug resistance pathogens such as *P. aeruginosa*, *K. pneumoniae* and *S. aureus* become one of the most important medical problems worldwide[11]. *Pseudomonas aeruginosa* is one of the most important multi-drug resistance bacteria found in hospitals environments, water, soil and cause different kinds of infections include pneumonia and liver abscess[24]. Mostly encountered MDR bacterial strains such as 3rd and 4th generation cephalosporins resistance *P. aeruginosa* is common and prevalent in a patients infected with pneumonia and water environments[25,26]. Water and air borne pathogens such as *P. aeruginosa* and *E. coli* could cause different kinds of illness such as pneumonia and heavy diarrhea[27,28]. Multi-drug resistance *P. aeruginosa* is inherently resistant to different drug classes and is able to acquired resistance to all effective antibiotics[29] and elaborates inactivating enzymes that make β-lactams ineffective such as extended spectrum β-lactamases[30]. The antibiotics susceptibility pattern of sewage water isolates of *P. aeruginosa* is mostly overlooked and rarely studied. One of the most important of antibiotics resistance strategies in *P. aeruginosa* is efflux pumps, this resistance mechanism has been increasingly recognized as a major component of resistance in *P. aeruginosa*[31]. Eighteen clinical isolates (30%) and 11 sewage water isolates (18.4%) were ESBLs-producing strains according to phenotypic test in this study. Extended spectrum β-lactamases (ESBLs)-producing *P. aeruginosa* have been associated with different infections such as pneumonia, liver abscess and burns infections and became a serious health problem in worldwide lead to treatment failure in hospitals[32]. Extended spectrum β-lactamases are plasmid mediated enzymes that hydrolyze broad spectrum β-lactams antimicrobials. These ESBLs are transmitted by plasmids between bacteria to bacteria in different environments and hospitals lead to emergence a new ESBLs-producing bacteria[11]. If infections were accrued caused by ESBLs-producing bacteria, can be predicated by the clinical characteristics of patients, can lead to a batter selection of antimicrobials and help to improve the outcome of infections[33]. The efflux system in *P. aeruginosa* is mostly encoded by chromosomal genes and capable of ejecting of antibacterial agents and fall into five families, multi-drug and toxic compound extrusion, ATP binding cassette, major facilitator superfamily, small multi drug resistance and the resistance nodulation division[34]. Because the efflux system is encoded by chromosomal genes and these genes are able to transfer from bacteria to other in hospitals environment and between person to person, therefore, in this study, clinical isolates of *P. aeruginosa* were high MDR isolates as compare with sewage water isolates.

Infections caused by *P. aeruginosa* are well known to be persistent and resilient in hospitals and environments due to the tendency of the species to form biofilms[35]. Biofilms and outer membrane lipoproteins are play an important role in protecting Gram negative bacteria from different antimicrobials effect[36]. In this study, all 60 clinical isolates (100%) were harbored *fimH* gene, 55 isolates (91.6%) were harbored *PapA* gene and 12 isolates (20%) were harbored *hly* gene. While sewage water isolated were harbored the same genes in low prevalent, 41 isolates (68.3%), 31 isolates (51.6%) and 2 isolates (3.3%), respectively. Many studies suggested that outer membrane lipoproteins and biofilms act as biodegradable effect on β-lactamase antibiotics. Beta-lactamases enzymes are secreted and maintain their activity inside of biofilm matrix and decompose β-lactam antibiotics before these antibiotics reach the bacterial cells[7-37]. In the present study, all 60 clinical isolates (100%) were harbored *kpsMTII* gene that responsible for capsule synthesis while only 25 isolates (41.6%) were positive for this gene. Almost *P. aeruginosa* produce large amounts of extra-capsular polysaccharides and mucopolysaccharide mass to produce strain with more virulent[38]. The *kpsMTII* is important gene have an essential role in capsule synthesis and pathogenicity of *P. aeruginosa*[39,40]. *Pseudomonas aeruginosa* is able of producing and secreting siderophores for iron uptake, siderophores are weakly soluble and found in most environments such as sewage water. In this study, *feoB* gene was found in 80 and 41.6% of clinical isolates and sewage water isolates, respectively. While, *iutA* gene was predominated in 95 and 35% of clinical isolates and sewage water isolates, respectively. This results are in agreement with previous studies proved that the most mucoidgram-negative bacteria isolated from clinical isolates were harbored virulence gene responsible for siderophores synthesis[7-41]. Siderophore are compounds produced by bacterial cells to iron uptake from iron-binding proteins of the host and they have strong-affinity extracellular ferric chelators, which, they have an important role in pathogenesis and virulence of bacteria, virulence of these compounds are remain unclear[42]. Most Gram negative bacteria such as *Pseudomonas aeruginosa* are able to produce many types of toxins, one of the most important toxins is hemolysins. These toxins are proteins and lipids that cause lysis of red blood cells by destroying their cell membrane[43]. In the present study, 12 clinical isolates (20%) and 2 sewage water isolates (3.3%) were harbored *hly* gene. Hemolysins are able to enhance the production of outer membrane vesicles and release of the cytolysin[44]. In Gram negative bacteria, outer membrane turnover and outer membrane vesicles production provide a general mechanism

of protection against clinical and environment source of envelope stress like antimicrobials and agents[45,46].

CONCLUSION AND FUTURE RECOMMENDATIONS

Clinical strains of *P. aeruginosa* isolated from sputum of patients infected with pneumonia were harbored virulence genes and were high antibiotics resistance more than those isolated from sewage water. Therefore, clinical strains of *P. aeruginosa* are more virulent than those isolated from environment isolates. It is recommended to analyze the antibiotic susceptibility pattern and virulence-associated genes of other pathogens such as *K. pneumonia* and *S. aureus* and conduct the comparison to get more pronounced data on virulence genes.

SIGNIFICANT STATEMENT

This study discovered that the environment isolates of *P. aeruginosa* has new virulence-associated genes don't found before. So, this study could be beneficial in determining the detail mechanism of virulence gene and formulation of antibiotics to treat the diseases related to it. This study will help researchers to discover more details about virulence genes in clinical and environments isolates of *P. aeruginosa* and also to modify the antibiotics according to the virulence genes discovered to treat the disease. Thus, best theory on it may be arrived at.

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