RESEARCH ARTICLE

Detection of Some Virulence Factors and Antibiotics Susceptibility of *Psudomonas aeruginosa* Clinical Isolates

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ABSTRACT

The current study conducted by collecting 150 specimens from different sources include urine, wounds,burns,sputum, to identify *Pseudomonas aeruginosa* bacteria,the specimens were collected from medical laboratories for pathogenic analysis in Baquba city -Iraq during the period from 1/9/2019 – 1/7/2020 to detection virulence factors,antibiotic resistance of bacteria,the biofilm formation ,and the effect of sub minimum inhibitory concentration (Sub-MIC) of Ciprofloxacin antibiotics in biofilm production. The showed 35 (14.28 %) *P. aeruginosa* isolates were identified from the total clinical specimens by the standard identification methods,the percentage of distribution of isolates between the specimens source as following urine (10.6%) wounds (16.36%), burns (35%) and sputum samples (15%). The result of investigation of virulence factors of *P aeruginosa* isolates reveal that lipase 30 (85.71%), haemolysin and gelatinase production were 35 (100%), and extended spectrum-lactamase enyme (ESBLS) Production were 30 (85.71%). Biofilm formation by 35 *P. aeruginosa* showed that 33 (94.28%) of total (35) isolates form biofilms, as following: 15 (42.85%) isolates form strong biofilm, 8 (22.85%) form moderate bifilm,10 (28.57%) produce weak biofilm,and 2 (5.71 %) not production,antibiotic resistance of isolates showed the highest percentage of resistance against ticarcillin (95%), cephalothin (90%) and ceftazidime (63%) whereas the lowest resistance for colistin, ofloxacin, imipenem were 31%, 25%, 16% respectively, the influence of sub-minimum inhibitory concentrations (Sub-MIC) (8 µg/mL) of ciprofloxacin on Biofilm formation showed the reduction of biofilm formation degree from strong in 15 isolates (100%) to weak formation in 8 isolates (53.34%).

Key words: Biofilm Sub–MIC, Antibiotic, *P. aeruginosa*, Virulence factors.

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INTRODUCTION

P. aeruginosa is gram-negative, bacilli shape bacteria belong to pseudomonadiaceae family, encapsulated, facultative aerobic, widespread presence in water . air,soil,an opportunistic, nosocomial bacteria responsible for serious infections specifically in weak immunity persons as in human immunodeficiency virus (HIV) patients.[1]

P. aeruginosa invades several tissues and organs of individuals include airway (lungs), urinary tract (kidenys), burns, wounds, when reach the blood, causing septicemia.[2] It causes pneumonia, endocarditis, burns and wound infections, otitis, and keratitis[3,4] (pathogen) due to mortality rates as high as 50%. The bacterium is also present in hospitals environment, on medical system, tools and considered problem due to their ability to attach to medical surfaces such as catheters.[5] For example the relation between ventilator and pneumonia and other sepsis infections, the high ability of *P. aeruginosa* to cause serious infections because the bacterium has many virulence factors such as lipopolysaccharide as endotoxins, flagella for motility and transmission of bacteria, pili important for attachment of pathogen to the host cells,[6] posses several toxins and enzymes such as exotoxin resulting in the inhibition of protein, other exoenzymes as a cytotoxin that is required for colonization, invasion and bacterial dissemination during infection named exoenzyme S., *ExoU* has a toxic effect on macrophages and as a cytotoxin with phospholipase activity that affects epithelial cells and causes lung infection.[7-10] In addition to the other virulence factors such as lipase, proteases, hemolysin, elastase, proteases, pyocyanin, lectins, rhamnolipids.[11-13]

The important virulence factor biofilm formation for bacteria, usually one main cause of resistance to antibiotics.[14] Biofilm formation isolates found more in hosts suffered from immunity diseases and in individuals lungs disease, the middle ear infection, as well as patients with contact lenses, catheters, and other implants. Biofilms can supply all living pathogen with high antibiotic resistance and give barrier from antibiotics, host immune responses, and facilitate transport

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microorganisms gens, coding the expression of drug resistance of bacteria.[15] Quorum sensing was used to regulation virulence factors and biofilm production by Q Sensing.[16]

The bacterium's main mechanisms used to resist antibiotics can be divided into intrinsic by minimizing the cytoplasmic membrane's permeability, and efflux pumps, anthor method of antimicrobial drugs performed by mutation processes named acquired resistant.[17] Multi-drug resistance bacterial cell in biofilm can cause prolonged and recurrent infections in cystic fibrosis persons.[18]

The increase in the percentage of multidrug resistance *of P. aeruginosa* causes different diseases. The current study carried to investigate antibiotic resistance of bacteria, virulence factors and the biofilm formation between clinical isolates, the effect ciprofloxacin stress on the formation of biofilm.

MATERIALS AND METHOD

Collection of Clinical Specimens

One hundred fifty specimens were collected from different sources include urine (150), wounds (55), burns (20), sputum (20), to identify *P. aeraginosa* bacteria, sterile cottons swabs, and container were used for collection from medical laboratories for pathogenic analysis in Baquba city -Iraq during the period from 1/9/2019–1/7/2020.

Isolation and Identification of Bacteria

All specimens were cultured on the MacConkey agar, incubated the plates aerobically for 24 hours at 37°C, expected *P. aeruginosa* isolate was subcultured on the cetrimide agar for confirmatory tests include blue-green pigment pyocyanin and growth at 42°C, then the pure colony of bacteria were selected to performed specific biochemical tests include oxidase, catalase, oxidase, IMVIC tests,[19] confirmation of the identification of isolates perform by used VITEK2 compact system through special diagnostic kits of device-specific for *P aeruginosa*.[20]

The current study also includes detecting some virulence factors in bacterial isolates, including MBLS enzyme, gelatinase, protease, hemolysin, and lipase.[21]

Antibiotic Susceptibility

The Kirby-Bauer disk diffusion technique[22] were used for detection of the antibiotic sensitivity of 35 *P. aeruginosa* isolates identified from clinical specimens. A total of 10 antibiotics disc were used in the current study. They include piperacillin /tozabactum, ceftazidime, imipenem, gentamicin, amikacin, ofloxacin, colistin, ciprofloxacin, ticarcillin, cephalothin, take part of pure colony and placed in 0.5 McFarland solution to obtain suspension culture with 5×1.5^8 CFU/mL concentration, by sterile cotton swabs spread 0.1 mL on the Muller–Hinton agar plate, leave for 15 minutes, five antibiotic discs placed on each plate with 15 mm space between each disc, incubation overnight at 30°C temperature degree, after incubation area inhibition zone /mm were recorded and interpreted the results of the susceptibility of isolates to antibiotics by comparing with Clinical and Laboratory Standards Institute (CLSI).[23] MIC of ciprofloxacin a were determined for the 15 pseudomonas isolates of strong biofilm formation by preparing serial dilutions on the Muller Hinton agar in final concentration, 4,8,16,32, 64,128,256,512, and 1024 according to,[24] and investigate the effect of sub-MIC of ciprofloxacin to the biofilm formation.

Detection of Biofilm Formation

Microtiter plate method used to detection of this important virulence factor by 35 *bacterial* isolates according to[25] bacterial suspension with 1.5×10^8 prepared by inoculating 4-5 colonies on Muller Hinton medium, incubating the inoculums overnight at a suitable temperature for growth, and adjusted by comparing with standard McFarland (0.5), transferred 200 uL of bacterial suspension into polystyrene plates containing 96 flat-bottom wells, broth without culture were used as control, sealed the plates and incubate over night, washed the well three times by sterile saline after the removal of culture suspension, fixed the stuck cells by 200 uL of methanol for 10–15 minutes,The methanol removed, dry plates in room temperature, added 200 uL of crystal violet 0.1% leave 10–15 minutes,washed the plates 3 times by distilled water, the wells were left to dry at room temperature, recorded the absorbency by enzyme-linked immunoassay (ELISA) reader at wavelength 630 nm.[26] The result of the degree of bioflim production by bacterial isolates obtained by comparing the mean of optical densities of isolates (ODi) and the optical density of control (ODc) results and isolates classified into four types according to a degree of biofilm formation as following: non-produce, weak, moderate, and strong.

RESULTS AND DISCUSSION

Bacterial Identification

Thirty-five (14.28%) *P. aeruginosa* isolates were identified from the total clinical specimens by the standard identification methods include colony characteristic on MacConky agar after overnight incubation at 37°C, and confirmatory test by subculture on cetrimid pseudomonas agr at 42°C for the growth of isolates on the media and production of pyocyanin (blue–green) specific and differentiate *P. aeruginosa* from other species,the results of biochemical tests were performed give negative reaction for gram stain (-ve), catalase, indol, methyl red,voges proskauer,and positive for citrate utilization, oxidase, gelatin hydrolysis, motility,the diagnosis of all isolates confirmed by VITEK2 compact system. The percentage of distribution of isolates between the source of the specimen as the following urine (10.6 %) the result agrees with the study of[27] wounds (16.36%), burns (35%) the percentage of infection consistency with results of[28] performed in Baghdad hospitals to the isolation of bacteria. *P. aeruginosa* obtained from sputum samples (15%), another study Aaron *et al* found (83%) *P. aeruginosa* isolates in studies for Bacterial cultures from sputum (Table 1).[29]

Virulence Factors Detection

The result of investigation of virulence factors of *P. aeruginosa* isolates reveal 30 (85.71%) were positive for lipase production

this near the result of Alim A., *et al.*,[30] the results showed that 35 (100%) of isolates give positive test for gelatinase production near the result of as Alihiand Hassan 2015,[31] Hemolysin production were investigated by inoculate the isolate on the blood agar medium the result showed that all 35 (100 %) produced beta hemolysin Hamed 2017 recorded the same result[32] extended-spectrum-lactamase enyme (ESBLS) production by bacteria isolates according to double discsynergy test according to[33] reveal that 30 (85.71%) isolates can produce the enzyme this in consistence with Hayford O, *et al*.,[34] (Table 2).

Microtiter plate method used for the detection of quantitative assay of biofilm formation by 35 *P. aeruginosa* the study showed that 33 (94.28%) of the total 35 isolates form biofilms, this indicates the high ability of bacteria to production, the degree of biofilm formation illustrate in Table 3 showed that 15 (42.85%) isolates form a strong biofilm, 8 (22.85%) form moderate biofilm,10 (28.57 %) produce weak biofilm, and 2 (5.71%) not production. The results showed more ratios of formation than the study of Pittaya Maita1, and Khaemaporn Boonbumrung were they recorded that *P. aeruginosa* from clinical isolates 79.4% biofilm production and 20.6% non-biofilm production[35] control gene expression in bacterial cell by quorum sensing mechanism that allows bacteria to form biofilm under unsuitable conditions,[36] quorum sensing effect to the pathogenicity of biofilm production *P. aeruginosa* by regulating the biofilm production and others virulence factors.[37]

Antibiotics Susceptibility of Isolates

Figure 1 The results of bacterial resistance isolates ten antibiotics according to the Kirby-Bauer disk diffusion technique, 22 showed that highest percentage of resistance against ticarcillin (95%) cephalothin (90%) the result was close to hameed 2017,[32] gentamicin (48%) the result similar to[38] ceftazidime (63%) agree with Kaur 2018,[39] amikacin (40%), ciprofloxacin (38%), this result confidence with fattimaetal 2017.[40] piperacillin/tozabactam, colistin, ofloxacin 58, 25, and 25% respectively, and the lowest resistance for imipenem (16%). The elevation of resistance among bacteria to antibiotics, because high quantity of antibiotics ude in treatment of infection and cause widespread of MDR microorganisms.[41]

P. aeruginosa similar to other gram-negative bacteria, has specific enzymes this enzyme is able to hydrolysis of β-lactam antibiotics.[42] ESBLs which increase the ratios of resistance to aztreonam, penicillins, and cephalosporins.[43,44]

Sub-MIC Effect of Ciprofloxacin on Biofilm Formation

The rang of MIC of ciprofloxacin for the 15 strong biofilm *P. aeruginosa* formation (16–520 ug/mL). The result of current study for the influence of sub–MIC (8 ug/mL)effect of ciprofloxacin on Biofilm formation of 15 showed the reduction of biofilm formation degree from strong in 15 isolates (100%) to weak formation in 8 isolates (53.34 %). The strong formation isolates distributed among clinical source in UTI 8 (46.66%), 33 (20%), for each of wound and burns,and sputum 2 (13.33%)

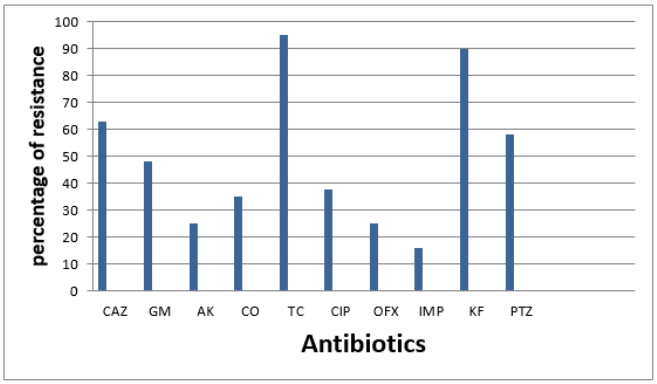

Figure 1: The percentage ratio of antibiotic resistance of (35*)* *P. aeruginosa* isolates
Amikacin (AK = 25%), gentamicin(GM=48%), ceftazidime (CAZ = 63 %), imipenem (IPM =16%), ciprofloxacin, (CIP=38%) piperacillin/ taozabactam (PTZ = 58%), colistin (CO=35%), Cephalothin (KF=90%), Ticarcillin (TC=95%), Ofloxacin (OFX=25%).

Table 1: Distribution of 35 bacterial isolates according to a source.

Sources of specimens	*Specimens number*	*Isolate number*	*%*
UTI	150	16	10.6
Wounds	55	9	16.36
Burns	20	7	35
Sputum	20	3	15
Total	245	35	14.28

Table 2: Positive virulence factors of (35) *P. aeruginosa* understudy

Virulence factor	*Number of (+ve) isolates*	*%*
Lipase	30	85.71
Gelatinase	35	100
Biofilm	33	94.28
ESBL senzyme	30	85.71
Haemolysin	35	100

Table 3: Distribution of biofilm degree among 35 *P. aeruginosa* isolate

Isolates number and source	*Biofilm formation isolates*							
	Strong		*Moderate*		*Weak*		*No biofilm formation*	
	No	*%*	*No*	*%*	*No*	*%*	*NO*	*%*
UTI (16)	7	20	4	11.42	4	14.28	1	2.85
Wounds(9)	3	8.57	3	8.57	2	5.71	1	2.85
Burns (7)	3	8.57	1	2.85	3	8.57	-	-
Sputum(3)	2	5.71	-	-	1	2.85	-	-
Total (35)	15	42.85	8	22.85	10	28.57	2	5.71

Table 4: Effect of sub-MIC ciprofloxacin on biofilm formation among 10 isolates

	Before CIP treatment		*After subMIC ciprofloxacin treatment*					
Source isolate	*Strong (15)*		*Strong*		*Moderate*		*Weak*	
	No	*%*	*No*	*%*	*NO*	*%*	*No*	*%*
UTI	7	46.66	2	13.33	1	6.66	4	26.66
Wounds	3	20	-	-	1	6.66	2	13.33
Burns	3	20	1	6.66	1	6.66	1	6.66
Sputum	2	13.33	-	-	1	6.66	1	6.66
Total	15	100	3	20	4	26.66	8	53.34

converted into weak degree were 4 (26.66%), 2 (13.33%), 1 (6.66), 1 (6.66%) respectively,the result consistence with study of Gillis and Iglewski, 2004 they found that Azithromycin inhibit biofilm production,[45] and agreement with the finding of Høiby *et al*.,[46] in his study for the detection of MIC ciprofloxacin effect he showed that the effective treatment of bacteria in cystic fibrosis individuals. The reduction effect agree with the.[47] He found alternation of ability of clinical isolates of *P. aeruginosa* by use Sub MIC of piperacillin/ tazobactam by decreasing biofilm formation, Kaplan, 2011 studies disagree and he found that some antibiotics in low concentrations not inhibit but induce biofilm formation in bacterial species Table 4.[48]

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