DOUBLE DISK SYNERGY TEST AND BIOFILM FORMATION IN CLINICAL ISOLATES OF PSEUDOMONAS AERUGINOSA

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Submitted: 10/2/2019; Accepted: 29/7/2019; Published: 21/9/2019

ABSTRACT

Background

Pseudomonas aeruginosa is an opportunistic nosocomial pathogen responsible for several infections. For such infections, limited antibiotics are suggested and combination therapy and subsequent synergetic effects may be useful.

Objectives

To determine antimicrobial susceptibility and biofilm formation of clinical isolates of *P. aeruginosa*, and to test synergy between commonly used antimicrobials.

Patients and Methods

Pseudomonas aeruginosa isolates were collected from several hospitals and community health laboratories. The isolate's identities were confirmed, disk diffusion antimicrobial sensitivity test was performed and double-disk synergy test was carried out to detect synergism between seven antimicrobial combinations. The ability to form biofilm was tested using microtiter plate assay.

Results

One hundred clinical isolates of *Pseudomonas aeruginosa* were tested. Twenty-two isolates were from community laboratories, and 78 were from hospital laboratories. Thirty-four isolates were from urine, 32 from burn wound tissue, 13 from blood and 21 from other specimens. Polymyxin B was the most effective agent (92%) followed by meropenem (65%), while 75% of the isolates were resistant to ticarcillin-clavulanate and 59% to netilmicin. Forty synergism observations were detected between ticarcillin-clavulanate & netilmicin combination and 12 between ticarcillin-clavulanate & meropenem.

Conclusion

Ticarcillin-clavulanate was least effective while polymyxin B was more effective against clinical isolates of *P. aeruginosa*. Double-disk synergy revealed synergism with ticarcillin-clavulanate & netilmicin combination, disk synergy results can aid in deciding combination therapy. Biofilm formation was common in *P. aeruginosa* but was not found to affect disk synergy.

Keywords: *Pseudomonas aeruginosa*, *Antimicrobial Combination*, *Disk Synergy*, *Biofilm.*

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https://doi.org/10.17656/jsmc.10207

INTRODUCTION

Pseudomonas aeruginosa, is a gram-negative opportunistic nosocomial pathogen, which is widely distributed in nature. As a pathogen, *P. aeruginosa* may cause serious infections in immunosuppressed patients, the infections are often difficult to treat, due to intrinsic and acquired resistance to many antimicrobials [1, 2].

Pseudomonas aeruginosa have several cell-associated and extracellular virulence factors; among those, flagella, type IV pili, and lipopolysaccharide [3, 4]. The organism produces exotoxin A, exoenzyme S, several proteases, and elastases, that have role in cell and tissue damage [4-7]. Biofilm formation serves as a survival factor for *P. aeruginosa* and provides more resistance to antimicrobials [8, 9]. *P. aeruginosa* nosocomial infections include burn wound infection and subsequent sepsis, bacteremia, hospital-associated and ventilator-associated pneumonia [10,11,12]. Other infections include urinary tract infection, mostly after catheterization [13], surgical site infection [14], and lung infection in patients with cystic fibrosis [15].

Because of multi-drug resistance, limited classes of antimicrobial agents, including aminoglycosides, carbapenems, some cephalosporins, fluoroquinolones, anti-pseudomonasl penicillin and polymyxins are suggested for *P. aeruginosa* infections [16]. Inappropriate chemotherapy and *e*mpirical therapy leads to selection of multidrug-resistant strains [17,18]. *P. aeruginosa* shows several resistance mechanisms, either intrinsic or acquired through chromosomal and plasmid resistance genes [19]. The organism also shows adaptive resistance due to exposure to sub-inhibitory antibiotic concentrations, growth in a biofilm, while swarming on epithelial surfaces is associated with increased resistance [4]. Unlike intrinsic and acquired resistance, adaptive resistance is not stable and reverts at the liminal of the inducing status [20].

Antimicrobial combinations are sometimes used to obtain a synergistic effect [21]. Possible benefits of combination therapy in *P. aeruginosa* infections include achieving *in vivo* antibiotic synergy, prevention of resistance emergence, and to improved adequacy of empiric therapy [22]. Unfortunately, the disadvantages include drug toxicity, creation of multidrug-resistant bacteria, and higher costs [23].

Double-disk synergy method is used to detect *in vitro* synergism and it's a simple and reliable method for various species [24, 25]. This method shows only qualitative information about the antimicrobial agent combinations, but with this method, it may be difficult to distinguish indifferent from synergistic interaction [26]. Time-kill curves method can be used to study quantitative drug interactions and to determine synergy, however it's limited by its cumbersome and laborious technique, and only a few drug combination concentrations can be effectively tested. Checkerboard method can be used to create multiple dilutions of the two antimicrobial agents; this can be performed either with microtiter trays or with broth macrodilution. However, with checkerboard, indifferent interaction can be differentiated from synergistic interaction but the method also have many limitations [21, 26].

This study was carried out to determine antimicrobial susceptibility and biofilm formation of clinical isolates of *P. aeruginosa*, and to test synergy between commonly used antimicrobials.

PATIENTS AND METHODS

Presumed *Pseudomonas aeruginosa* isolates were collected from several hospital and community health laboratories. The isolates were cultivated on MacConkey agar and cetrimide agar, incubated at 37ºC for 18-24 hours. *P. aeruginosa* isolates were confirmed according to the growth characteristics, including, lactose non-fermenter colonies on MacConkey agar, growth on the selective medium cetrimide agar, soluble pigment formation by some isolate, Gram smear features, and positive oxidase test. VITEK 2® System (Biomerieux, France) with gram-negative lactose non-fermenter test cards was uesd for non-conclusive isolates.

The isolates were subjected to antimicrobial sensitivity test (AST) using eight antimicrobial disks, and double-disk synergy test was carried out to detect synergism between seven antimicrobial combinations [27, 28]; using overnight culture of *P. aeruginosa* at 37 ºC in Mueller Hinton broth suspended to equivalent of 0.5 McFarland standard. The diffusion tests were carried out on Mueller Hinton agar. The antibiotic disks (Mast Diagnostics, UK) were; ticarcillin-clavulanate 75/10 µg, ceftazidime 30 µg, meropenem 10 µg, polymyxin B 300 unit, gentamicin 10 µg, netilmicin 30 µg, levofloxacin 5µg, and ciprofloxacin 5µg.

For double-disk synergy test, the petri dish surface was marked for the center of the disks to be placed. Ticarcillin-clavulanate disk was placed at the center of 3 or 4 other disks and the distance between the central disk and other disks was calculated as sum of

half cut points diameter of inhibition zone of the two disks plus 5 mm to detect synergism clearly. The plates were incubated at 35±2 ºC for 18 hours. *P. aeruginosa* ATTC 27853 was used as quality control strain [27, 28]. The results were read visually with the aid of a vernier *caliper*.

For quantitative determination of biofilm formation we used microtiter assay [29]. Three to five *P. aeruginosa* fresh overnight cultured colonies were suspended in 10 mL of Brain Heart infusion broth supplied with 0.25 % glucose and incubated for 18 hours at 39°C in a shaking incubator. After incubation, the stationary-phase culture was vortexed and diluted 1:100 in BHIB. From this, 200 µL of solution was transferred into 96-well microtiter plates in triplicates for each sample, and incubated for 18 hours at 37°C. After the incubation period, the media with suspended bacteria was removed. The microtiter plate was carefully washed four times with tap water and air-dried before staining with 200 µL of 0.9% crystal violet solution for 15 min. After removing the dye solution and washing with water, the attached dye was solubilized with 95% ethanol and the optical density of the adherent biofilm was measured at wave length of 570 nm with a spectrophotometer (Thermo Multiskan MCC). BHIB with 0.25% glucose and *P. aeruginosa* ATCC 27853 (non-biofilm-producing) was used as negative controls (background absorbance). The adherence capabilities of the test strains were classified into four categories: non-adherent (0), weakly adherent (+), moderately adherent (++), or strongly (+++) adherent, based upon the ODs of bacterial films. The cut-off optical density (OD) is defined as three standard deviations above the mean OD of the negative controls [30].

All media, solution, test reagents, and stains were prepared according to the manufacturer's instructions.

Statistical analysis

We used Microsoft Excel for data entry and used both Microsoft Excel and Jamovi software version 0.9.5.15 for statistical analysis.

RESULTS

One hundred clinical isolates of *Pseudomonas aeruginosa* were tested. Twenty-two isolates were from private laboratories, and 78 were from hospital laboratories. The source specimens were urine (34 isolates), tissue from burn wounds (32 isolates), blood (13 isolates), and other sources, Table 1. Seventy-six isolates showed pigments formation, while 24 isolates produced no visible pigment. For biofilm formation, 32 isolates failed to from biofilm, while 68 isolates formed biofilm of various degrees, Table 1.

The antimicrobial susceptibility pattern of *P. aeruginosa* isolates is shown in Figure 2. Polymyxin B was the most effective agent (92%) followed by meropenem (65%) and ceftazidime (60%). Most of the isolates (75%) were resistant to ticarcillin-clavulanate, followed by netilmicin (59%), levofloxacin (56%) and ciprofloxacin (54%).

The results of double-disk synergy revealed that a total of 65 synergism reactions were observed among 700 tested combinations (10.7 %). Table 2 shows the number of synergism reactions.

Among the 65 individual synergism observed, 40 synergisms (61.54%) were between ticarcillin-clavulanate & netilmicin, 12 synergisms (18.46%) were between ticarcillin-clavulanate & meropenem and 6 synergisms (9.23%) between ticarcillin-clavulanate & gentamicin. No synergism was observed between ticarcillin-clavulanate & ciprofloxacin or ticarcillin-clavulanate & polymyxin B. No antagonism reaction was observed.

Non-statistically significat relations were found between synergy to biofilm formation (P = 0.311), pigment formation (P= 0.488), and source of the isolates (P= 0.832), Table 3.

The mean response to antimicrobial agents in regard to isolates source (hospital or community), biofilm formation, and pigment formation was analyzed using independent student's t-test (Table 4) and all relations were found to be statistically insignificant.

Table 1. The source of specimens, and biofilm formation by the *Pseudomonas aeruginosa* isolates (*n*=100) according the source laboratory.

	Community Lab. *n*=22	Hospital Lab. *n*=78	Total
Specimen			
Urine	14	20	34
Burn Tissue	0	32	32
Blood	1	12	13
Surgical Wound	1	8	9
Sputum	3	5	8
Ear pus	2	0	2
Diabetic	0	1	1
Pleural Fluid	1	0	1
Biofilm formation			
No biofilm	6	26	32
Weak	11	30	41
Moderate	4	15	19
Strong	1	7	8

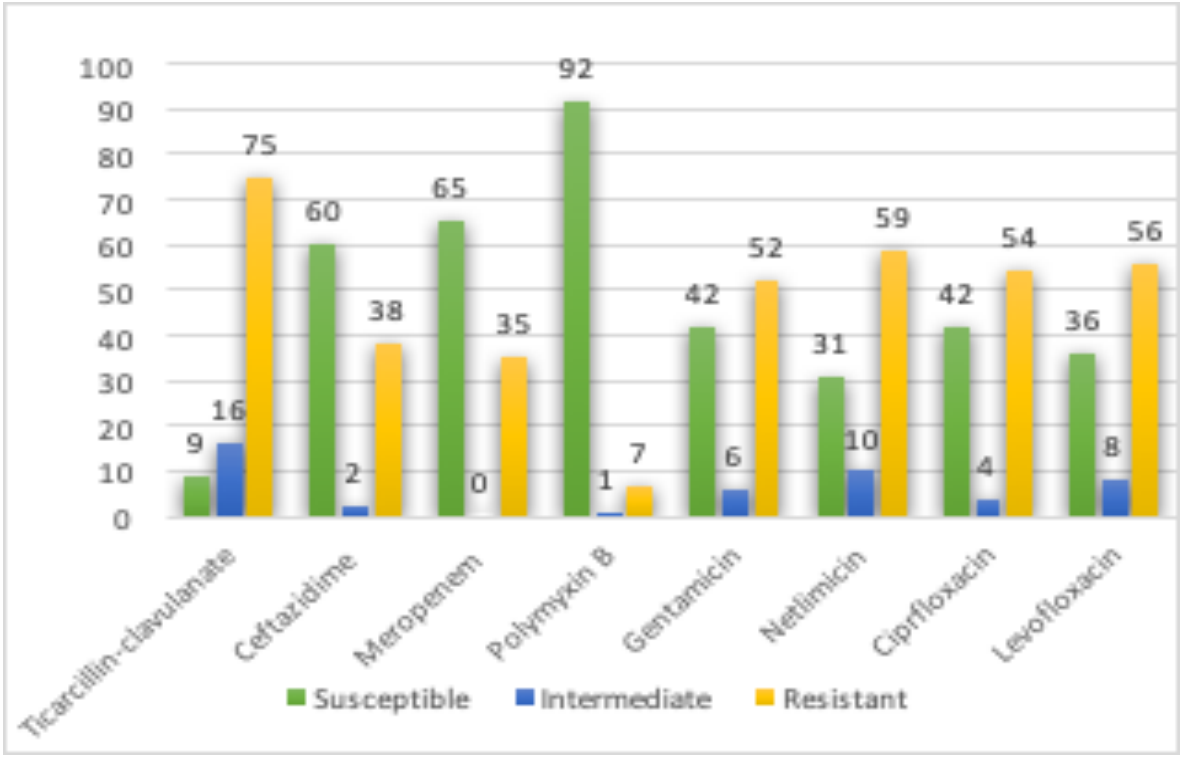

Figure 2. The antimicrobial susceptibility of *Pseudomonas aeruginosa* isolates (*n*=100) to eight antimicrobial agents, tested by disk diffusion test.

Table 2. The observed synergism reactions to seven combinations of antimicrobials agents among *Pseudomonas aeruginosa* isolates tested by double-disk synergy.

Antimicrobial combination	No.	%
Ticarcillin-clavulanate & netilmicin	40	61.54
Ticarcillin-clavulanate & meropenem	12	18.46
Ticarcillin-clavulanate & gentamicin	6	9.23
Ticarcillin-clavulanate & ceftazidime	5	7.69
Ticarcillin-clavulanate & levofloxacin	2	3.08
Ticarcillin-clavulanate & ciprofloxacin	0	0
Ticarcillin-clavulanate & polymyxin B	0	0
Total	65	100

Table 3. Relation between synergism and biofilm formation, and the source of the isolates.

	No Synergism No.	Synergism No.	Total No.	P value
Biofilm formation				
No Biofilm	13	19	32	0.311
Biofilm	35	33	68	
Total	48	52	100	
Source of the isolate				
Community Lab.	11	11	22	0.832
Hospital Lab.	37	41	78	
Total	48	52	100	

Table 4. The mean diameter response in millimeters to eight antimicrobial agents in relation to isolates source (hospital or community), biofilm formation.

Antimicrobial agents	Isolate source			Biofilm formation		
	Community Mean Dia.	Hospital Mean Dia.	P value	No Biofilm Mean Dia.	Biofilm Mean Dia.	P value
Gentamicin	11.4	11.4	0.965	11.8	11.3	0.713
Meropenem	21.8	22.4	0.841	22.1	22.4	0.901
Ciprofloxacin	14.5	15.6	0.678	15	15.6	0.802
Ceftazidime	15.5	16.5	0.609	15.9	16.5	0.764
Levofloxacin	11.7	12.6	0.651	12	12.6	0.726
Polymyxin B	14.2	13.4	0.134	13.7	13.5	0.768
Netilmicin	11.1	10.5	0.666	10.4	10.7	0.854
Ticarcillin-clavulanate	12	11.4	0.765	9.88	12.4	0.096

DISCUSSION

In a location of extensive use of antibiotics such as Kurdistan, Iraq, *Pseudomonas aeruginosa* became one of the main pathogens causing community and hospital-acquired infection. The simple nutritional requirements and activity over a wide range of temperatures give this organism a significant competitive favor, this beside its ability to develop resistance to antimicrobial and antiseptics [31, 32]. Our isolates were from all ages, mainly from hospital-acquired infections but also community infections. *P. aeruginosa* were isolated from a wide range of nosocomial infections including urinary tract infection; burn wound infection, surgical site infection, pneumonia, and sepsis [33]. It's reported that the isolates were mainly urinary tract infection, burn wound infection, blood and surgical site infections and other infections outside hospital environment. Patients with cystic fibrosis are excluded since there are no specialized centers to deal with cystic fibrosis in Kurdistan.

Despite the wide range of anti-pseudomonal antimicrobials agents, infections with *P. aeruginosa* are still difficult to treat, mainly due to the ability of the organism to develop resistance. To address resistance problem and especially in *P. aeruginosa*, regular and repeated AST will be useful to detect wide range resistance, multi- drug resistant strains, and to detect development of resistance during treatment. A previous local study done in 2014 [34], showed nearly similar results for resistance to meropenem and ciprofloxacin but higher resistance to ceftazidime and gentamicin were reported, indicating that resistant strains of *P. aeruginosa* is relatively, prevalent in this area, which would change the antibiotics prescription strategy by the local medical practitioners [35]. A study from India showed that *P. aeruginosa* isolates were more sensitive to meropenem, levofloxacin, gentamicin and ciprofloxacin. These differences might reflect the pattern of antibiotic use and resistance development to more frequently used drugs [36].

We have tested susceptibility to the drugs that are commonly used and considered to be effective for *P. aeruginosa* as it was Found. The anti-pseudomonal agent; ticarcillin-clavulanate was the least effective agent, probably due over use and due to the intrinsic resistance of *P. aeruginosa* to most β-lactams [17] while, polymyxin B was most effective agent which is the last-resort drug for treating infections caused by this bacteria, despite its undesirable side effects [37]. Using antibiotic combinations, may overcome resistance and prevent its development, and if synergism was observed in laboratory, it should be taken in concern [38].

In this study, the focus was on the synergistic activity of ticarcillin-clavulanate with seven other antimicrobial agents, Table 2. The results showed that combination of ticarcillin-clavulanate & netilmicin or combination of ticarcillin-clavulanate & meropenem give synergistic effects more than other Combinations. These combinations are recommended to overcome drug resistance before choosing polymyxin B with its side effects. No *in vitro* synergism was found between ticarcillin-clavulanate & polymyxin B unlike that observed by Berditch in 2015 that showed combining polymyxin B with meropenem, amikacin, or rifampin was partially effective against extremely drug-resistant *P. aeruginosa* [37]. According to our results, the more effective polymyxin B still can be reserved in case other safer combinations were ineffective clinically. Selection and development of different drug combinations with stronger activity than the current treatment to eradicate *P. aeruginosa* infections are necessary [39]. So, if clinical decision favors combination therapy, it is better to depend on double-disk synergy data rather than suggested empirical combinations.

Regarding biofilm formation, 68 % of the isolates revealed this ability, which adds difficulty to treatment and justify the demand of intensive or combination therapy. Little is known about the relationship between biofilm formation and the antibiotic resistance of the bacteria [40], but biofilm formation helps bacteria within a biofilm to become more resistance to antimicrobials [40, 41].The results revealed no statistically significant differences between the relation of biofilm formation and its strength with antimicrobial synergism by the bacterial strains. This might be due to biofilm formation not occur properly on conditions provided by culture environment for disk synergy test.

In conclusion, ticarcillin-clavulanate was least effective while polymyxin B was more effective against clinical isolates of *P. aeruginosa*. Double-disk synergy revealed synergism with ticarcillin-clavulanate & netilmicin combination, disk synergy results can aid in deciding combination therapy. Biofilm formation was common in *P. aeruginosa* but was not found to affects disk synergy.

Acknowledgment

We express our thanks to the management and the

bacteriology staff in Sulaymaniyah city hospitals and community health laboratories, for their cooperation in providing the bacterial isolates and their technical support.

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