Effective in Vitro Synergy of Piperacillin/Tazobactam Plus either Netilmicin or Aztreonam against Metallo- β -lactamase-producing *Pseudomonas Aeruginosa*

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Abstract This study was designed to compare the activity of piperacillin/tazobactam (TZP) in combination with either netilmicin (NET) or aztreonam (ATM) against metallo- β lactamases (M β L) *Pseudomonas aeruginosa*. Out of 46 nosocomial *Ps. aeruginosa* isolates, 38 (82.6%) isolates were carbapenem-resistant by disc diffusion susceptibility testing. These were then screened by imipenem + EDTA combined disc test for M β L production. Thirty (78.9%) M β L-producing isolates detected were further tested for antibiotic synergy against NET-TZP and ATM-TZP combinations by Etest synergy and Etest/agar dilution tests. Neither combination showed antagonism. The synergistic effect of NET-TZP combination was detected in [27 (90%) and 28 (93.3%), respectively] and ATM-TZP combination in [24 (80%) and 25 (83.3%), respectively] of the tested isolates by Etest synergy and Etest/agar dilution tests, respectively. Thus, we have determined the synergistic effect of these two combinations for treating M β L-producing *Ps. aeruginosa*. Significance and Impact of the Study: The present study has shown valuable information on the combination treatment of *Ps. aeruginosa* and recommends their use for treatment of resistant *Ps. aeruginosa*.

Keywords *Pseudomonas Aeruginosa*, MBL, Synergy Tests, Netilmicin, Aztreonam, Piperacillin/Tazobactam

1. Introduction

Pseudomonas aeruginosa is an important opportunistic pathogen that plays a serious role in many infections especially in immuno-compromised patients [1]. Infections by this organism are characterized as severe and resistant to treatment with drugs [2]. Metallo- β -lactamase (M β L) production by *Ps. aeruginosa* is a major challenge because

it confers multidrug resistance (MDR) to β -lactam agents, as well as co-resistance to fluoroquinolones and aminoglycosides [3]. Continued emergence and rapid spread of MDR *Ps. aeruginosa* strains have resulted in increased morbidity, mortality and cost [1,4]. This increased the need for alternative strategies such as combination of anti-pseudomonal antibiotics as an alternative strategy in the management of these cases.

Although aminoglycosides are among the oldest antibiotics to treat serious infections, they have severe side effects such as nephrotoxicity, ototoxicity (vestibular and auditory) and, rarely, neuromuscular blockade and hypersensitivity reactions. They are concentration-dependent, bactericidal agents that have certain limitations including the age of the patient, serum creatinine level and the duration of exposure [5,6]. This and the appearance of new and safer antibiotics resulted in the dramatic drop in the use of aminoglycosides since 1970s. However, their use is being reconsidered again due to the development of MDR among different microorganisms [6]. Netilmicin (NET) is an effective bactericidal drug with a little toxic effect rather than other aminoglycosides which favors its use with severe infections in critical cases that cannot withstand severe toxicity associated with other aminoglycosides [7].

It is well known now that a β -lactam plus an aminoglycoside is the standard for treating severe *Ps. aeruginosa* infections [8]. This synergy could be explained by the theory that exposure of bacterial cell to β -lactam drugs lessen the barriers, leading to increase the susceptibility to aminoglycosides [9]. The effective *in vitro* synergistic activity of NET plus β -lactams against resistant *Ps. aeruginosa* has been documented by several studies with variable degrees. These combinations included NET plus penicillin [10], piperacillin [11], carbenicillin [12], aztreonam, 3rd generation cephalosporins as cefotaxime,

cetazidime, ceftriaxone and cefoperazone [14]. Some of these combinations showed better results than gentamicin when combined with the same β -lactams [11,12,13].

On the contrary, aztreonam (ATM) is a monobactam which has a strong activity against Gram-negative rods with no remarkable toxicity [8,14]. It may be similar in efficacy to aminoglycosides as gentamicin and tobramycin. It could be an effective alternative to aminoglycosides in serious Gram-negative infections [15].

β -lactam/ β -lactamase inhibitor drugs, represented by piperacillin/tazobactam (TZP) is particularly effective against *Ps. aeruginosa* [16]. It is effective in the treatment of wide range of infections including moderate to severe polymicrobial infections [17].

In certain conditions, such as severe infection in patients with poor renal functions or immuno-compromised patients who need prolonged courses of treatment, it is preferable to use dual β -lactam drugs rather than β -lactam-aminoglycoside combination. The mechanism of synergy between β -lactams includes the action of different particles at different penicillin binding proteins and subsequent interference of cell wall synthesis. Another mechanism is the synergistic effect of the inhibition by β -lactamases [18].

The goal of this study was to compare the activity of TZP in combination with either NET or ATM against M β L-producing *Ps. aeruginosa* using two methods; Etest synergy and Etest/agar dilution.

2. Material and Methods

2.1. Bacterial Isolates and Growth Conditions:

This study included 46 nosocomial *Ps. aeruginosa* isolates obtained from Microbiology Laboratory of Infection Control Unit. Pure cultures were preserved on 15% glycerol in screw top tubes at -70°C until use. Tubes were transported immediately to the Microbiology Laboratory of Microbiology and Immunology Department, Faculty of Medicine for further processing. To recover the bacteria: the frozen surface of the culture was scraped with a sterile inoculating loop, the bacteria that adhere to the loop were streaked onto the surface of plates and the frozen culture was returned to storage at -70°C [19]. Blood agar and MacConkey's agar plates were used for subculture with incubation at 37°C for 24 hours. Any growth was identified as *Ps. aeruginosa* by conventional microbiology methods; colony morphology, Gram film and biochemical reactions [20].

Isolates Data were collected retrospectively from patients fulfilling criteria of Centre for Disease Control (CDC) for nosocomial infections [21] and admitted between January 2015 to July 2015 in Tanta University Hospitals, Egypt. These 46 nosocomial *Ps. aeruginosa* isolates were collected from patients admitted to emergency ICU, pediatric ICU

and surgery wards (18, 17 and 11 respectively).

2.2. Antibiotic Susceptibility Testing:

Antibiotic susceptibility tests were performed using the Kirby-Bauer disc diffusion method and interpreted according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2015) [22]. Amikacin (30 μg), gentamicin (10 μg), NET (30 μg), ciprofloxacin (5 μg), piperacillin (100 μg), TZP (110 μg), ceftazidime (30 μg), cefepime (30 μg), imipenem (10 μg), meropenem (10 μg), ATM (30 μg), polymyxin-B (300 U) and colistin (10 μg) discs (Oxoid, UK) were used for testing. *Ps. aeruginosa* ATCC 27853 was used as control strain.

2.3. Screening Test for M β L:

Ps. aeruginosa isolates resistant to imipenem or meropenem or both were subjected to the screening test for M β L production by imipenem (Oxoid, UK) + EDTA (Zhonglan Industry Co, China) combined disc test as described previously by Yong *et al.* [23]. Isolates with enhancement of zone size of ≥ 7 mm between imipenem + EDTA disc compared with imipenem disc alone were considered as M β L-positive. One *Ps. aeruginosa* strain producing IMP and another one producing VIM that were confirmed by PCR and provided by our laboratory, were used as MBL-positive controls [24]. *Ps. aeruginosa* ATCC 27853 was used as a M β L -negative control.

2.4. Antibiotic Synergy Testing

In this study, we chose to use the Etest synergy to measure the in vitro synergistic activity between antibiotic combinations. It is an easy and rapid method with low cost, and it has a high agreement with the time-kill assay test [25,26]. In addition, Etest/agar dilution was used to confirm results obtained by the Etest synergy technique [27,28].

Etest Synergy Test:

Minimum inhibitory concentration (MIC) test strips (Liofilchem®, Italy) were used with the following concentrations ($\mu\text{g}/\text{ml}$): NET, 0.016–256; ATM, 0.016–256; TZP, 0.016–256. Synergy test was measured by Etest synergy method that was determined in triplicate according to Pankey and Ashcraft [25] on Muller-Hinton agar (MHA) (Oxoid, UK) plates. The following antibiotic combinations were tested: NET-TZP and ATM-TZP.

Etest/Agar Dilution Test:

NET (Merck Schering Plough, Nestpan-Cipla, USA) was incorporated into MHA at concentration of 0.06 mg/L as previously described [22,29]. Plates were inoculated with 0.5 McFarland suspensions of each isolate, then TZP Etest strips were applied. After 24hrs incubation at 37°C , the strips were read and the Etest MICs were compared to a series performed in the absence of TZP. The same was done

with ATM (Bristol Mayers squibb, Princeton, USA) using concentration of 0.5 mg/L and then TZP Etest strips were applied. In this method, we used the least concentration of the drug to be added to the MHA in case of *Ps. aeruginosa* [22,29].

The FIC was calculated for each antibiotic in each combination by using the following formula:

FIC of drug A = (MIC of drug A in combination)/
(MIC of drug A alone)

FIC of drug B = (MIC of drug B in combination)/
(MIC of drug B alone)

Σ FIC = FIC of drug A + FIC of drug B.

Synergy was defined by a **Σ FIC** of ≤ 0.5 . Antagonism was defined by a **Σ FIC** of > 4 . Interactions represented by a **Σ FIC** of $\Sigma 0.5$ but ≤ 4 were termed indifferent.

MIC required to inhibit 50% and 90% of the isolates (MIC₅₀ and MIC₉₀, respectively) were calculated using Microsoft Σ ® Excel® 2007 using Etest strips. *Ps. aeruginosa* ATCC 27853 was used as control strain.

2.5. Ethical Consideration

It was approved by the Ethical Committee of the Faculty of Medicine, Tanta University, Egypt.

2.7. Statistical Analysis

Data were analyzed using statistical package of social science (SPSS) version 21. Qualitative data were expressed as frequency and percentage. Chi-square test was used to examine the relation between qualitative variables. *P*-value less than 0.05 was considered significant.

3. Results

Out of 46 *Ps. aeruginosa* isolates tested for their antibiotic susceptibility, eight isolates (17.4%) were carbapenem sensitive while thirty eight (82.6%) isolates were carbapenem-resistant, among which there were only 30 MβL producers. According to specimen type, 20(43.5 %) of MβL isolates were from endotracheal aspirates, 7(15.2%) from wound swabs, and 3(6.5%) from urine (**Table 1**).

By disc diffusion method, all MβL isolates were sensitive for both colistin and polymyxin B, while (23.3%, 10%) were sensitive for ATM and NET, respectively. Intermediate sensitivity was (43.3%) for piperacillin and (33.3%) for both TZP and ATM. Absolute resistance (100%) was found with all other tested antibiotics (**Table 2**). These results identified our choices for the preferable combinations to be tested.

The MIC ranges for NET, ATM and TZP tested against MβL-producing *Ps. aeruginosa* strains, and their MIC₅₀ and MIC₉₀ values are shown in (**Table 3**).

Table 1. Distribution of isolated *Ps. aeruginosa* according to department and specimen type.

Number(46)	Carbapenem sensitive <i>Ps. aeruginosa</i> (8)	Carbapenem resistant <i>Ps. aeruginosa</i> (38)	
Department		MβL positive	MβL negative
Emergency ICU	3	12	3
Pediatric ICU	3	11	3
Surgery wards	2	7	2
Specimen			
Endotracheal aspirate	1	20	2
Wound	3	7	3
Urine	4	3	3

ICU: intensive care unit, MβL: metallo beta lactamase. These isolates were collected from different departments of Tanta University Hospitals, Egypt

Table 2. Susceptibility pattern of 30 MβL-producing *Ps. aeruginosa* isolates by disc diffusion method

Drug	Sensitive	Intermediate	Resistant
Amikacin	0 (0%)	0 (0%)	30(100%)
Netilmicin	3(10%)	0 (0%)	27 (90%)
Gentamicin	0(0%)	0 (0%)	30(100%)
Ceftazidime	0(0%)	0 (0%)	30(100%)
Cefepime	0(0%)	0 (0%)	30(100%)
Ciprofloxacin	0(0%)	0 (0%)	30(100%)
Colistin	30(100%)	0 (0%)	0 (0%)
Polymyxin B	30(100%)	0 (0%)	0 (0%)
Aztreonam	7(23.3%)	10(33.3%)	13(43.3%)
Piperacillin/Tazobactam	0(0%)	10(33.3%)	20(66.7%)
Piperacillin	0(0%)	13(43.3%)	17(56.7%)
Imipenem	0(0%)	0 (0%)	30(100%)
Meropenem	0(0%)	0 (0%)	30(100%)

Table 3. MIC values for NET, ATM and TZP antibiotic tested against 30 M β L- producing *Ps. aeruginosa* isolates

Drug	MIC range μ g/ml	MIC ₅₀ μ g/ml	MIC ₉₀ μ g/ml
NET	0.5 -256	3	32
ATM	1-32	2	8
TZP	0.5 -256	1.5	24

NET: netilmicin, ATM: aztreonam, TZP: piperacillin/tazobactam, MIC: minimal inhibitory concentration.

Table 4. Effects of antibiotic combinations tested against 30 M β L-producing *Ps. aeruginosa* isolates.

Method	Drug combination	Synergism	Indifference	Antagonism	P-value
Etest synergy	NET- TZP	27(90%)	3(10%)	0(0%)	0.424
	ATM- TZP	24(80%)	6(20%)	0(0%)	
Etest/agar dilution	NET- TZP	28 (93.3%)	2(6.7%)	0(0%)	0.227
	ATM- TZP	25 (83.3%)	5(16.7%)	0(0%)	

NET : netilmicin , ATM : aztreonam , TZP: piperacillin/tazobactam , p<0.05 significant estimated by chi -square test.

The effects of antibiotic combinations tested against the 30 M β L-producing *Ps. aeruginosa* isolates were assessed using the Σ FIC index criteria. There was (90%, 93.3%) synergy between NET-TZP and (80%, 83.3%) synergy between ATM-TZP by using Etest synergy and Etest/agar dilution test, respectively and there was no antagonism by either two methods (**Table 4**).

4. Discussion

Infection with *Ps. aeruginosa* is a very serious problem due to difficulties associated with treatment options for resistant isolates and poor response rates of patients. In addition, it is usually recruited from immuno-compromised patients with nosocomial infections. Therefore, special care should be taken while determining its antibiotic susceptibility pattern and prescribing the suitable antibiotic therapy. During the last decades, many resistance enzymes had been detected to be produced by *Ps. aeruginosa*, including Extended-spectrum β -lactamases (ESBL), AmpC and M β L. M β L enzymes confer resistance to all β -lactams (except ATM) and usually to aminoglycosides [30]. M β L-producing *Ps. aeruginosa* is an emerging threat as it can hydrolyze carbapenems which are given as a last resort to the patient having infection with ESBL and AmpC-lactamase producing *Ps. aeruginosa* [31].

In this study, 30 out of 38 (78.9%) carbapenem resistant *Ps. aeruginosa* isolates were positive for M β L production by imipenem + EDTA double disk synergy test. This is a relatively high ratio when compared with finding of Abd El-Baky *et al* [31] where only 31(53.4%) of their 58 *Ps. aeruginosa* isolates were M β L producers, and that of Asghar study [32] who reported that M β L-producing *Ps. aeruginosa* were identified in only 76 (15.9%) of their 478 isolates. While a close result was obtained by Toval *et al.* [33] where their M β L production ratio was 102 (81.6%) out of 125 carbapenem resistant *Ps. aeruginosa* isolates. This high ratios of carbapenem resistance and M β L-producing

Ps. aeruginosa in our institution may be due to absence of antibiotic policy and the misuse of antibiotics especially β -lactams and carbapenems.

According to antibiotic susceptibility pattern, we noticed that all M β L positive *Ps. aeruginosa* isolates were sensitive to polymyxin B and colistin. All 30 isolates showed absolute resistant (100%) to amikacin, gentamicin, ceftazidime, cefepime and ciprofloxacin. While, number of resistant isolates to NET was 27(90%), TZP 20(66.7%), piperacillin 17(56.7%) and ATM 13(43.3%). These results supported the measurement of the synergistic effect of TZP combined with either NET or ATM on M β L-positive isolates.

TZP is a β -lactam/ β -lactamase inhibitor combination that has a broad spectrum activity against most Gram-positive and Gram-negative aerobic and anaerobic bacteria [34]. The addition of tazobactam enhances the antimicrobial activity of piperacillin against most β -lactamase-producing organisms [35]. As regard drug-drug interaction, when aminoglycosides are administered in combination with piperacillin in patients with end-stage renal disease requiring hemodialysis, the concentrations of the aminoglycosides (especially tobramycin) may be significantly altered and should be monitored. Probenecid if administered concomitantly with TZP, it prolongs the half-life of piperacillin by 21% and that of tazobactam by 71%. In addition, coagulation parameters should be monitored regularly during simultaneous administration of high doses of heparin and oral anticoagulants [36]. TZP is generally safe, well tolerated and of low cost. This renders it to be a reliable option when used in combination with other drugs for the early empiric treatment of moderate-to-severe infections [34,37]. On the other hand, NET is an ethyl derivative of dehydrogenated C1a gentamicin and is active against many gentamicin-resistant organisms. It has the least nephrotoxicity and ototoxicity among the other aminoglycosides [38,39]. Although, some studies did not find NET to be useful against gentamicin-resistant *Ps. aeruginosa* [40,41]. Others like

Biswal *et al.*, [42] detected less resistance of *Ps. aeruginosa* to NET (58.62%) when compared with amikacin and gentamicin (81.03% for each), which is consistent with our findings.

In the current study, synergy was detected between TZP and NET (90-93.3%) by Etest synergy and Etest /agar dilution respectively with no antagonism. Several studies measured the synergy between TZP and aminoglycosides like tobramycin, gentamicin and amikacin. As far as we know, this is the first attempt for detecting the in vitro synergy between TZP and NET as one of the aminoglycosides. An early study was performed on the combination between carbenicillin and NET which detected a synergy rate of (45.65%) in *Ps. aeruginosa* isolates [40]. Consistent with our study, a clinical trial noted the effectiveness of NET-TZP combination as a safe excellent early empiric therapy for high-risk febrile neutropenia in pediatric cancer patients [43]. Studies on the other aminoglycosides detected synergy between TZP plus either; tobramycin (50%) [44], amikacin (95.9%, 42%) [44,45] respectively, arbekacin (100%) [3] and gentamicin (33.33%) [46].

On the other hand, less synergy was detected between TZP and ATM (80-83.3%) in this study by Etest synergy and Etest /agar dilution respectively, with no antagonism. Fujimura *et al.* [3] tested this combination in vitro and reported synergy rate (89.9%) with no antagonism, which is similar to our results. Also investigators supported the *in vivo* and *in vitro* synergy between ATM and piperacillin alone, even without the tazobactam addition [45,47]. Nevertheless, Pinheiro *et al.* [48] did not recommend the ATM-TZP combination as the best combination in empirical or definitive treatment against *Ps. aeruginosa*.

No statistically significant differences in synergy rates were found between the two combinations using both methods (*P-value* = 0.227 and 0.424). This may refer to the effectiveness of both combinations to overcome MBL-producing *Ps. aeruginosa*.

5. Conclusions

This study determines that NET-TZP and ATM-TZP combinations were effective against MBL-producing *Ps. aeruginosa*. This may confer better choices in empirical therapy, with less toxicity especially in critically ill patients.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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