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# *In Vitro* Activity of Colistin and Vancomycin or Azithromycin Combinations on Extensively Drug Resistant *Acinetobacter baumannii* Clinical Isolates

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## Abstract

**Background:** Extensively drug resistant *Acinetobacter baumannii* (XDR-AB) presents an increasing challenge to health care in Egypt as they are among the most common bacteria isolated in hospital setting. Treatment of such infections usually involves the use of antimicrobial agents in combination. Various combinations have been proposed, with colistin serving as the backbone in many of them even for colistin resistant isolates. **Aim:** The study was conducted in order to test the *in vitro* combined effects of colistin and vancomycin or azithromycin against (XDR-AB) causing infections at Alexandria Main University Hospital in Egypt, in an attempt to detect the possibility of a beneficial combination therapy. **Material/Methods:** Thirty XDR-AB clinical isolates were included in the study. Antibiotic susceptibility testing was performed using automated Vitek 2 compact system and disc diffusion method. Colistin antibiotic disc diffusion test was compared with broth microdilution method. Organisms were also tested against colistin and vancomycin or azithromycin in combination using checkerboard synergy test and FICI (Fractional Inhibitory Concentration Index) was calculated. Synergy was defined as a FICI of ≤0.5. **Results:** On comparing the two methods used to detect susceptibility to colistin to broth microdilution for MIC (minimum inhibitory concentration) determination, as a reference method, the Vitek showed 100% categorical agreement (CA), on the other hand, the disc diffusion showed CA of 93% with very major errors. Synergy was detected for all isolates (100%) when combining colistin with vancomycin (FICI mean = 0.08). As for azithromycin, 21 strains had FICI range from 0.7 to 1.001, denoting indifference; the remaining 9 strains showed synergy with FICI range from 0.06 to 0.241. The mean colistin/azithromycin FICI was 0.71 for the 30 isolates. **Conclusion:** These findings suggest that regimens containing vancomycin may confer therapeutic benefit for infection due to XDR-AB; however, other methods (time-kill assay) should be used to confirm such synergy. Furthermore, the optimal combination treatment for serious XDR-AB infection should be addressed in a prospective clinical trial.

**Keywords**

Colistin Resistance, Antibiotic Combination, Checkerboard, Synergy

## 1. Introduction

Extensively-resistant *Acinetobacter baumannii* (XDR-AB) presents an enormous challenge to health care, particularly in intensive care units (ICU) [1] [2]. The presence of strains resistant to all available antibiotics has led to reliance on the polymyxins as the last resort. This group of antibiotics was used in the 1950's, but due to their neurotoxicity and nephrotoxicity, there was a massive decline in their use [3].

Another problem with colistin especially with multidrug resistant isolates is heteroresistance that has been observed *in vitro* and has also developed during therapy [4] [5], raising issues about colistin being used alone as a monotherapy which may lack sufficient killing activity and aid in selection of resistant subpopulation [6].

Polymyxins work through disrupting the integrity of the Gram-negative bacterial membrane, increasing its permeability to substances that are usually excluded, thus increasing the activity of hydrophobic antibiotics, which otherwise had no effect [7] [8] [9]. One of these antibiotics is the glycopeptide vancomycin, where due to its large size and hydrophobicity; it lacks the power to exert its action against Gram-negative bacilli. Using colistin could improve the penetration of glycopeptides through bacterial membrane. Also azythromycin, the most commonly prescribed antibiotic in the U.S., is never recommended for clinical treatment of serious Gram negative infections because of poor or absent *in vitro* activity by standard *in-vitro* testing. However, antibacterial activity of azithromycin was found to be enhanced in tissue culture media vs. bacteriologic media, prompting closer examination of its interaction with drug resistant Gram negative bacilli [10] [11].

In order to explore the potential usefulness of such antibiotics, we attempted to evaluate the presence of *in-vitro* activity (synergy) of colistin in combination with vancomycin or azithromycin against XDR-AB, in an attempt to find out the possibility of such combination therapy, to minimize the toxic effects and to prevent the development of resistance when using colistin alone.

## 2. Material & Methods

### 2.1. Clinical Isolates

Thirty XDR-AB clinical isolates were included in this study. Isolates were collected from urinary, blood, pus and respiratory samples received at the diagnostic Microbiology Lab of Alexandria Main University Hospital over a period of one year starting January till December 2015. *Acinetobacter* initial identification was done via conventional biochemical methods (oxidase negative, citrate positive, non-motile and triple sugar iron agar negative) and confirmed to the species level by (Vitek 2 compact, bioMérieux, France) [12] [13].

XDR was defined according to European Centre for Disease Prevention and Control

(ECDC) and the Centers for Disease Control and Prevention (CDC) as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (*i.e.* bacterial isolates remain susceptible to only one or two categories). Antibiotic susceptibility was interpreted according to CLSI recommendations [14].

## 2.2. Antimicrobial Susceptibility Testing of *A. baumannii* Isolates

### 2.2.1. Automated Vitek 2 Compact System

The Vitek 2 susceptibility card (GN222 AST card) (bioMérieux, France) including a colistin susceptibility test was used. Interpretive breakpoints (MIC ≤ 2 μg/ml, susceptible, and MIC ≥ 4 μg/ml, resistant) were considered for the Vitek 2 susceptibility testing according to the manufacturer's instructions. *A. baumannii* ATCC 19606 was used as a control strain.

The GN222 AST card tests susceptibility against the following antibiotics besides colistin (CST): ticarcillin (TIC), ticarcillin/clavulanic acid (TIC/CA), piperacillin (PIP), piperacillin/tazobactam (TZP), ceftazidime (CAZ), cefepime (FEP), Aztreonem (ATM), imipenem (IMP), meropenem (MEM), amikacin (AMK), gentamicin (CN), tobramycin (TOB), ciprofloxacin (CIP), pefloxacin (PEF), minocycline (MN), rifampicin (RP) and trimethoprim/sulphamethoxazole (SXT).

### 2.2.2. Disc Diffusion Test

To cover the panel of antibiotics suggested by CLSI, disc diffusion was used for ampicillin/sulbactam (SAM), levofloxacin (LVX), tetracycline (TC), doxycycline (DC), cefotaxime (CTX) and ceftriaxone (CRO) according to CLSI 2015 guidelines. As for tigecycline (TGC) breakpoints suggested by Piewngam *et al.* were used in disc diffusion testing where a zone diameter of ≥17 mm was considered sensitive, while a diameter of ≤12 mm was considered resistant [15].

Besides testing colistin susceptibility by Vitek 2 compact system, the disc diffusion method using 10 μg disc was also done (≤ 12 mm = resistant and ≥ 14 mm = susceptible) [16]. Since neither the current CLSI nor EUCAST provided disc diffusion zone diameter breakpoints for colistin against *Acinetobacter spp.*, and since both recommended that colistin testing should be performed by dilution method and interpreted according to MIC, colistin susceptibility disc diffusion results were compared against broth microdilution method (as a reference method) [17] [18].

Colistin Categorical agreement (CA) was defined as the percentage of isolates classified in the same susceptibility category by broth microdilution method and the disc diffusion or Vitek. Very major errors (VMEs) denoted a false-susceptible result, and major errors (MEs) denoted a false-resistant result, while minor errors (MinE) were intermediate zone diameters that had susceptible or resistant MIC, or intermediate MIC with a susceptible or resistant zone diameter. Acceptable performance was evaluated according to criteria established by the International Organization for Standardization: ≥90% for category agreement and ≤3% for VMEs or MEs [19].

## 2.3. Checkerboard Synergy Test

The checkerboard method was performed according to the method described by Schwalbe (2007) [20].

#### 2.3.1. Preparation of Checkerboard Antibiotic Microdilution

Antibiotic powders used were colistin sulfate salt, vancomycin, and azithromycin (Sigma-Aldrich, USA). The concentration of antibiotic prepared prior to dilution was four times higher than the highest concentration to be tested. For example, if the initial concentration for antibiotic A was 256 µg/ml and for antibiotic B was 8 µg/ml, then it was necessary to start with a concentration of 1024 µg/ml for antibiotic A and 32 µg/ml for antibiotic B.

Checkerboards were prepared by doubling dilutions of vancomycin, or azithromycin (0 to 256 µg/ml) in the horizontal wells and colistin sulfate (0 to 8 µg/ml) in the vertical wells using the format shown in Table 1.

#### 2.3.2. Preparation of Inoculum

The inoculum was prepared using a 1:100 dilution of a half McFarland bacterial suspension from an overnight culture (approximately $5 \times 10^5$ CFU/ml of bacteria).

#### 2.3.3. Inoculation and Incubation of Checkerboard Panel

50 µl was inoculated into each well of a 96-well microtiter plate. The plates were covered and incubated at 35˚C for 18 - 24 h.

#### 2.3.4. Reading the Results

1) The wells without visible signs of growth were identified visually against a dark background. Then the MICs (lowest concentration showing inhibition of growth) for the individual antibiotics in the checkerboard method were recorded.

2) The lowest fractional inhibitory concentration index (FICI) was used to define synergy.

#### 2.3.5. Data Analysis

FICI was used to analyze data from the checkerboard assay. The FICI was calculated using the following equation: the FIC of antibiotic = MIC of antibiotic in combination/MIC of antibiotic alone and FICI = FIC of colistin + FIC of Vancomycin or azithromycin. The results of the FICI was interpreted as follows: synergy, FICI ≤ 0.5; antagonism, FICI > 4; indifference, 0.5 < FICI ≤ 4 [21].

Table 1. The checkerboard format used in the study to test *in-vitro* synergy of colistin and vancomycin/azithromycin (where colistin dilutions were distributed horizontally in rows (B to H) while Vancomycin or azithromycin dilutions were distributed vertically in columns (2-12).

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Growth control | 256 µg/ml | 128 µg/ml | 64 µg/ml | 32 µg/ml | 16 µg/ml | 8 µg/ml | 4 µg/ml | 2 µg/ml | 1 µg/ml | 0.5 µg/ml | 0.25 µg/ml |
| B | 8 µg/ml | | | | | | | | | | | |
| C | 4 µg/ml | | | | | | | | | | | |
| D | 2 µg/ml | | | | | | | | | | | |
| E | 1 µg/ml | | | | | | | | | | | |
| F | 0.5 µg/ml | | | | | | | | | | | |
| G | 0.25 µg/ml | | | | | | | | | | | |
| H | 0.12 µg/ml | | | | | | | | | | | Sterility control |

## 3. Results

The 30 XDR-AB were isolated from respiratory; broncho-alveolar lavage and sputum (20/30), Pus (6/30), Urine (2/30), CSF (1/30) and Blood (1/30) samples delivered to AMUH microbiology lab. Intensive care units being the source of 73.3% (22/30) of *A. baumannii* isolates in our study.

### 3.1. Susceptibility Testing Results

The susceptibility patterns of the strains isolated are shown in Table 2. Twenty eight isolates were sensitive to colistin MIC ≤ 2 μg/ml, only 2 were found resistant with MIC ≥ 4 μg/ml by broth microdilution method (reference method) and by the Vitek 2 system, and these two strains were pan-resistant, isolated at different periods during the study. However, disc diffusion failed to detect these 2 resistant strains, thus identified 100% of the isolates as susceptible.

Table 2. Distribution of antimicrobial profile of the 30 XDR *A. baumannii* strains.

| Number of strains | Sensitive | Intermediate | Resistant | Departments | |
|---|---|---|---|---|---|
| | | | | ICU | Non-ICU |
| 13 strains | CST, TGC | - | PIP, TIC, TIC/CA, SAM, TZP, CAZ, CRO, CTX, FEP, ATM, IPM, MEM, AMK, CN, TOB, CIP, LVX, PEF, MN, DC, TC, SXT, RP | 9 | 4 |
| 5 strains | CST, DC | - | PIP, TIC, TIC/CA, SAM, TZP, CAZ, CRO, CTX, FEP, ATM, IPM, MEM, AMK, CN, TOB, CIP, LVX, PEF, MN, TC, TGC, SXT, RP | 3 | 2 |
| 10 strains | CST | TGC | PIP, TIC, TIC/CA, SAM, TZP, CAZ, CRO, CTX, FEP, ATM, IPM, MEM, AMK, CN, TOB, CIP, LVX, PEF, MN, DC, TC, SXT, RP | 8 | 2 |
| 2 strains | - | - | PIP, TIC, TIC/CA, SAM, TZP, CAZ, CRO, CTX, FEP, ATM, IPM, MEM, AMK, CN, TOB, CIP, LVX, PEF, MN, DC, TC, TGC, SXT, RP, CST | 2 | 0 |

**Table 3.** Comparison of colistin susceptibility by disc diffusion and broth microdilution test for the 30 XDR *A. baumannii* strains.

| Disk diffusion results | Broth microdilution results (reference method) | | | | | |
|---|---|---|---|---|---|---|
| | <0.25 ug/ml | 0.5 ug/ml | 1 ug/ml | 2 ug/ml | 4 ug/ml | 8 ug/ml |
| Sensitive | 3 | 8 | 11 | 6 | | 2 VME |
| Intermediate | | | | | | |
| Resistant | | | | | | |
| CA agreement based on interpretation | 28/30 = 93% | | | | | |
| Minor Errors | 0 | minor errors based on interpretation/Total strains tested x100 | | | | |
| Major Error | 0 | major errors based on interpretation/Total susceptible strains x100 | | | | |
| Very major error | 2/2 = 100% | very major errors based on interpretation/Total resistant strains x100 | | | | |

On comparing the two methods used for detection of susceptibility to colistin to broth microdilution MIC reference method, the Vitek showed 100% CA with no errors among the 30 isolates tested, on the other hand the disc diffusion although showed CA = 93%, it failed to detect the 2 resistant strains thus showing very major errors (Table 3).

## 3.2. Checkerboard Synergy Test

The checkerboard synergy test was performed for each isolated *A. baumanniii* strain against, colistin/vancomycin and colistin/azythromycin combinations. All strains were found resistant to vancomycin and azithromycin (mic ≥ 256 μg/ml) by broth microdilution. However, when colistin was added in the checkerboard format and the lowest (FICI) was used to analyse the outcome, synergy was detected for all isolates (100%) with vancomycin (FICI ranged from 0.03 to 0.0241 *i.e.* ≤0.5), mean FICI = 0.08. As for azithromycin 21 strains had FICI range from 0.7 to 1.001 denoting indifference; 0.5 < FICI ≤ 4, the remaining 9 strains showed synergy with FICI range from 0.06 to 0.241. The mean colistin/azithromycin FICI = 0.71 for the 30 isolates.

When combining colistin and vancomycin, there was a reduction in colistin MIC level from 8 μg/ml to ≤0.5 μg/ml for all isolates, where 100% of the isolates showed decreased MIC level below the susceptible breakpoints (≤2 μg/ml) of them 80% (24/30) showed an MIC level of 0.12 μg/ml after combination. As for vancomycin, all isolates (100%) showed reduction in MIC level from ≥256 μg/ml to 0.25 μg/ml.

On the other hand, the colistin MIC levels showed a decrease from 8 μg/ml to ≤2 μg/ml for all isolates when combined to azithromycin; 80% (24/30) of the isolates' colistin MICs were ≤1 μg/ml. The azithromycin MIC decreased from ≥256 μg/ml to 0.25 μg/ml for all isolates.

## 4. Discussion

*Acinetobacter* drug resistance is increasing, reducing the treatment options available for managing such infections. *A. baumannii* are considered as major infectious threat especially in intensive care units (ICU), where it has been implemented in the treat-

ment of various nosocomial infections especially pneumonia, urinary tract infections and bloodstream infections [22] [23]. Our study has similar findings to these data, ensuring that *acinetobacter* ICU infections are a global trend. Also respiratory specimens had the highest rate of *A. baumanniii* isolation, constituting (20/30) 66.7% of all specimens, similar findings were found by Al Bshabshe *et al.* 2016 [22].

The last resort for treatment of drug resistant *A. baumannii* is colistin where it is often the only agent with *in vitro* activity as shown in our results; 28/30 XDR-AB strains were found sensitive to colistin, however, clinical experience of its use for the treatment of resistant strains as a monotherapy has not always been successful resulting in poor outcomes [24]. However, the optimal method for testing susceptibility of *A. baumannii* to colistin is still controversial. The drug diffusion through the agar is inconsistent and poor, making errors with disc diffusion common when compared to other methods [25]. The controversy comes from the absence of guidelines by the CLSI for disc diffusion. During our work we got to study three colistin susceptibility testing methods against *A. baumannii* clinical isolates with XDR patterns [26]. In the current study the disc diffusion although showing CA of 93% yet it failed to detect the two resistant *A. baumanniii* detected by the broth microdilution (reference method) giving VMEs, however, basing our findings on only these resistant strains is inconclusive but in the absence of verified guidelines, disc diffusion method should not be used alone in judging colistin susceptibility in *A. baumanniii*. Our finding is supported by others who reported disc diffusion to be an unreliable method for detecting polymyxins susceptibility [16] [27]. As regards Vitek 2 AST, we found it to be reliable and easy with 100% CA, Lee *et al.* 2013 tested 213 isolates, including 13 colistin resistant *Acinetobacter* strains. Vitek 2 showed excellent CA with 0.9% VMEs and no ME [28].

In addition, another problem is that trends are showing elevated colistin MICs globally, emphasizing not only the importance of accurate colistin susceptibility testing, but also the importance of appropriate combination therapy to prevent the emergence of resistance to colistin by achieving synergy [25]. Also when a clinical strain is resistant to all antibiotic the achievement of synergy might not be a reachable option by combination therapy, yet any antibiotic combination activity would be preferable to the inactivity of a single drug. Thus an additive or subadditive effect would be welcomed. This can also be achieved when having a single active agent boosted by an inactive agent. In this case, prevention of resistance to the active agent may be possible [29].

Our *in vitro* study demonstrated the possibility for a vancomycin-colistin combination, where synergy was demonstrated for all isolates tested, however, concern exists regarding its clinical application. But Gordon *et al.* hypothesized, that since synergistic combinations decreased the dose of colistin required to inhibit *Acinetobacter*, it may be possible to produce synergy *in vivo* by using lower-than-normal doses of colistin, a strategy similar to the use of low-dose aminoglycosides in combination with $\beta$-lactams in the treatment of streptococcal endocarditis [30]. They also supported the explanation for the mechanism behind this synergy by electron microscopy imaging that revealed disruption of membranes of colistin exposed bacteria in comparison to those of unexposed controls.

Regarding testing combination of colistin and azithromycin using the checkerboard

method and although azithromycin on its own shows negligible effect against MDR-GNRs. Yet, we found that when combined with colistin it did exhibit an activity against drug resistant *acinetobacter* although this combination only produced a synergistic effect on 30% of our isolates, similarly a small number of studies demonstrated the same activity for AZM against Gram negative bacilli with no explanation or suggestion about the mechanism of synergy [31] [32]. Until Lin *et al.* 2012 tested this combination in eukaryotic cell media and *in vivo* murine models of infection and concluded that AZM entry and activity is synergistically enhanced when the bacterial outer membrane was disrupted by colistin [11]. Buyck *et al.*, 2012 demonstrated that a mutation of the *oprM* efflux pump system in *P. aeruginosa* lead to an increased AZM susceptibility, and on entry AZM reduced *oprM* gene expression and protein synthesis, further enhancing the entry of AZM in eukaryotic media and in synergy with colistin that may initiate a positive feedback loop to increase effective intracellular levels of the antibiotic [10].

## 5. Conclusion

In conclusion, this study opens the door to further explore antibiotics, not known to act on Gram negative bacilli as vancomycin and azythromicin for adjunctive therapy in MDR infections. Potentially, other antibiotics not considered due to large molecular size deterring penetration through Gram-negative bacterial membranes in standard MIC testing, should be considered with colistin, allowing a lower dose and reducing the side effects. The findings of the present study suggest that regimens containing vancomycin may confer therapeutic benefit for infection due to XDR *A. baumannii*. However, other methods (time-kill assay) should be used to confirm such synergy. Furthermore, the optimal combination treatment for serious XDR-AB infection should be addressed in a prospective clinical trial.

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