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In-vitro efficacy of polymyxin B with rifampin, colistin and doxycycline against extensively drug resistant *Acinetobacter baumannii*

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***Acinetobacter baumannii* is an opportunistic Gram negative coccobacillus that can grow easily in moist as well as dry conditions. During the last decade, *A. baumannii* emerged as one of the most resistant opportunistic pathogens responsible for nosocomial infections including ventilator associated pneumonia. The bug remains an important and difficult to treat pathogen whose pan-drug resistant nature has created a serious challenge. This has restricted the choice of treatment modalities. Currently, it appears as if all the available antibiotics are failing against this pathogen while single antibiotic therapy is certainly not working anymore. Thus, there is a strong need, thus, to explore new regimens to combat this resistant organism. A wide range of various combinations of drugs should therefore be tested for their synergistic activity against this pathogen. This study was aimed to find some effective combinations against extensively drug resistant (XDR) *A. baumannii* by combining various antibacterials. The microdilution checkerboard titration method was used for this purpose and fractional inhibitory concentrations (FICs) were calculated. *In-vitro* synergy was found in polymyxin B-colistin (n = 3; 15%) and polymyxin B-rifampin (n = 3; 15%) combinations. Only additive effect was noted with polymyxin B-doxycycline (n = 12; 60%), polymyxin B-rifampin (n = 11; 55%), and polymyxin B-colistin (n = 13; 65%). However, antagonism was detected in the polymyxin B-rifampin combination in one of the 20 strains evaluated for the purpose. Polymyxin B in combination with rifampin and colistin may be exploited against XDR *A. baumannii*. Synergy between polymyxin B and colistin have been demonstrated in only 15% of strains, this fully warrants the testing of more combinations.**

Key words: *Acinetobacter baumannii*, extensively drug resistant, fractional inhibitory concentration.

INTRODUCTION

The genus *Acinetobacter* is a ubiquitous group of micro-organisms (Giamarellou et al., 2008) and is found in the environment. *Acinetobacter baumannii* was considered to be a pathogen of low grade pathogenecity and was ignored whenever isolated from clinical specimens until the 1970s (Zarrilli et al., 2009). It has recently emerged as one of the most troublesome nosocomial pathogens globally and has become a major cause of health care-associated and community-acquired infections (Talbot et al., 2006; Davis et al., 2005). Management of MDR/XDR

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Abbreviations: *A. baumannii*, *Acinetobacter baumannii*; **API 20NE,** analytical profile index 20 Non-Enterobactericeae; **ATCC,** American type culture collection; **FIC,** fractional inhibitory concentration; **FICI,** fractional inhibitory concentration index; **MBC,** minimal bactericidal concentration; **MDR,** multi-drug resistant; **MIC,** minimal inhibitory concentration; **PDR,** pan-drug resistant; **XDR,** extensively drug resistant.

A. baumannii infections is a big challenge for physicians and clinical microbiologist. The organism's capability to survive in hospital settings and to persist for long periods of time on various surfaces makes it a frequent cause of healthcare associated infections. Another problem regarding *A. baumannii* is its ability to cause a wide spectrum of infections which include wound infections, bacteremia, pneumonia, urinary tract infections, etc (Manchanda et al., 2010). *A. baumannii* is proven to have the capability to form biofilms that is believed to play central part in the process of colonization (Pour et al., 2011).

During the early 1970s, the clinical isolates of *A. baumannii* were usually susceptible to various antibiotic classes (Bergogne-Berezin and Towner, 1996). However, since 1975, increasing resistance to almost all groups of antibacterials started appearing (Manchanda et al., 2010; Montefour et al., 2008). From the year 2000 to date, various combinations of antibiotics have been evaluated for their synergistic activity or otherwise to combat this resistant pathogen (Rodriguez-Hernandez et al., 2000; Montero et al., 2004; Saballs et al., 2006; Tan et al., 2011).

The focus of this study was to determine the synergistic effect of polymyxin B with either of the antibiotics: rifampin, colistin and doxycycline by the checkerboard microtitration technique.

MATERIALS AND METHODS

Bacterial strains

The extensively drug resistant *A. baumannii* (XDR-AB) phenotype was identified as an *A. baumannii* strain, resistant to all classes of the traditional antibiotics except tigecycline and polymyxin B using the disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI, 2012) recommendations. These strains were isolated from various clinical samples, and collected from various patients hospitalized in Lahore, Pakistan in 2012. The strains were identified by their morphological and biochemical characteristics and, later by using API 20-NE (BioMerieux, France). The selected strains were stored in microbanks at -80°C.

Antimicrobial agents and minimal inhibitory concentration determination

The base materials of antimicrobial agents used in combinations were: polymyxin B (Glaxosmith Kline pharmaceuticals), rifampin (Pacific pharmaceuticals), colistin (Forest pharmaceuticals) and doxycycline (Pfizer Global pharmaceuticals). Stock solutions of antibiotics were prepared in their respective solvents (water for polymyxin B, colistin and doxycycline, methanol for rifampin) according to the CLSI 2012 guidelines and stored at -20°C for one week. Minimal inhibitory concentrations (MICs) of all strains for each antibiotic were determined by a standard agar dilution method. Bacterial inoculum equivalent to 0.5 McFarland ($5x10^8$) was prepared and diluted 1:10 to achieve the final concentration of $5x10^7$ CFU/ml. The concentration range of various antibiotics was prepared; 0.125 to 8.0 µg/ml (polymyxin B and colistin), 0.5 to 64 µg/ml (doxycycline), 0.06 to 64 µg/ml (rifampin) in Mueller Hinton agar. The plates were inoculated with the bacterial suspensions using multipoint inoculators (MAST Diagnostics UK). The same was incubated for 24 h at 37°C. Lowest concentration at which bacterial growth was inhibited was noted after incubation (CLSI 2012). *Escherichia coli* ATCC 25922 was used as a control strain. MIC results were read and interpreted according to the CLSI breakpoint criteria for *A. baumannii*. Since there are no CLSI interpretation criteria of rifampin, available, relevant to *A. baumannii*, the breakpoints for this antibiotic were based on the MIC standards of CLSI for Gram positive bacteria (CLSI, 2012).

Synergy testing

The synergistic activity of the antibiotic combinations was determined using the microdilution checkerboard titration method. The range of concentrations was chosen according to the previously determined MIC of each antibiotic for each isolate. Concentrations used ranged from 0.06xMIC to 8xMIC for each antibiotic. The interpretation of the checkerboard synergy testing results was determined by the method of Orhan et al. (2005). FICs and FICI were calculated for each antimicrobial combination using the formulas below:

$$\Sigma FIC \text{ or } FICI = FIC\ A + FIC\ B$$

Where,

$$FIC\ A = \frac{\text{MIC of drug A in the combination}}{\text{MIC of drug A alone}}$$

and

$$FIC\ B = \frac{\text{MIC of drug B in the combination}}{\text{MIC of drug B alone}}$$

The combination was considered synergistic when the ΣFIC was ≤0.5, additive when the ΣFIC was >0.5 to ≤1.0 indifferent when the ΣFIC was >1.0 to <2, and antagonistic when the ΣFIC was ≥2 (Orhan et al., 2005).

RESULTS

The detail of the various clinical materials from which *A. baumannii* was originally isolated is shown in Figure 1. The highest number of *A. baumannii* strains were isolated from central venous catheter tips (n=9; 45%) followed by pus (n=8; 40%), urine (n=1; 5%), high vaginal swab (n=1; 5%) and body fluids (n=1; 5%). Major isolation from CVC tips is mainly due to the capability of *A. baumannii* to survive on dry as well as moist conditions and also grow well on tubings of catheters and ventilators. The second highest source was pus which indicates its ability to colonize open wounds and from where it can invade into the blood stream to cause life threatening bacteremia. All isolates were resistant to all the classes of antimicrobials except polymyxin B (100% susceptible) and doxycycline (85% susceptible). However, all were resistant to colistin irrespective of their susceptibility or otherwise to polymyxin B and doxycycline. Their susceptibility patterns are shown in the Figure 2.

MIC_{90} for polymyxin B, colistin, doxycycline and rifampin was 1, 8, 64 and 2 µg/ml respectively. Their MIC ranges and susceptibility rates are shown in Table 1. MIC for ATCC *E. coli* 25922 for polymyxin B, colistin, doxycy-

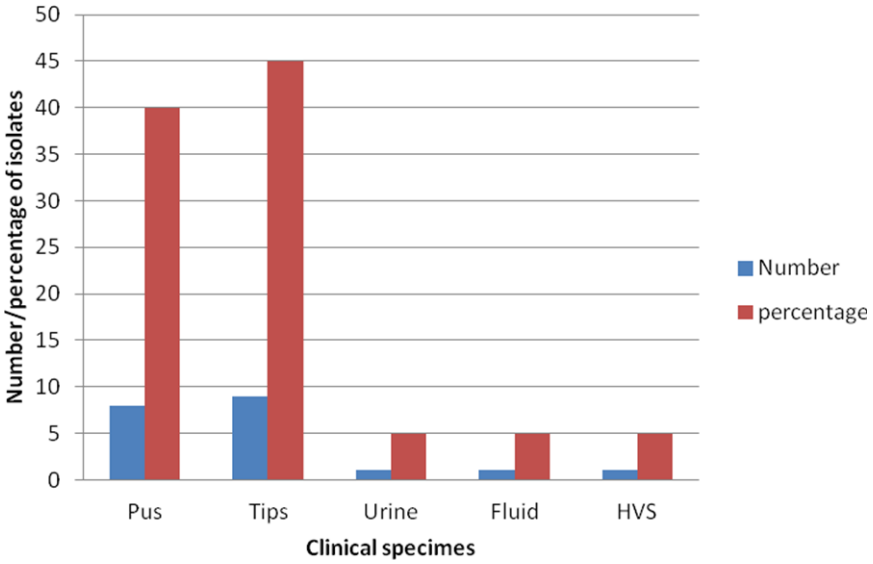

Figure 1. Sources of XDR *A. baumannii* isolates.

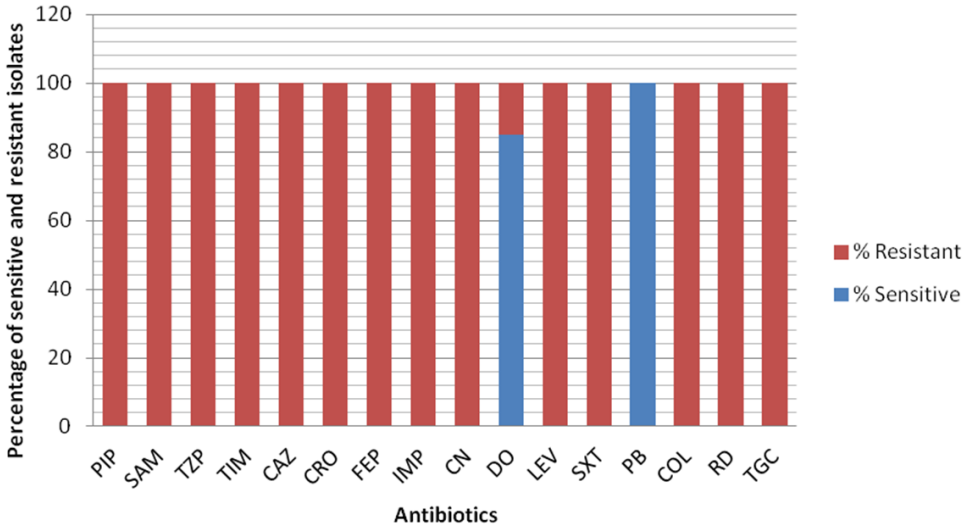

Figure 2. Susceptibility of *A. baumannii* isolates to various antibiotics. PIP: piperacillin, SAM: ampicillin-sulbactam, TZP: piperacillin-tazobactam, TIM: ticarcillin-clavulanic acid, CAZ: ceftazidime, CRO: ceftriaxone, FEP: cefepime, IMP: imipenem, CN: gentamicin, DO: doxycycline, LEV: levofloxacin, SXT: cotrimoxazole, PB: polymyxin B, COL: colistin, RD: rifampin, TGC: tigecycline.

cline, and rifampin are also shown in Table 1. MICs of XDR *A. baumannii* for four tested antibiotics by broth microdilution method are shown in Table 2.

The results of the microtitration checkerboard method are shown in Table 3. Three isolates (15%): AB-02, AB-14, and AB-20 strains showed synergistic effect in polymyxin B-rifampin combination while 55% additive effect was seen in this combination. In the case of AB-02 and AB-20, the MIC of polymyxin B in combination was reduced to one fourth as compared to the individual MICs against these isolates. The MIC of rifampin was reduced to one eighth and one fourth for AB-02 and AB-20 respectively. For the isolate AB-14, the MIC of polymyxin B and rifampin was reduced to one eighth and one fourth respectively.

Indifference was detected in 25% of the isolates while AB-08 showed antagonism (5%) in this combination of antimicrobials. The MIC of polymyxin B was increased

Table 1. Minimal inhibitory concentrations (MICs), susceptibility rates and quality control (QC) ranges of XDR *A. baumannii* (n=20) and *E. coli* ATCC 25922 versus 4 different antibiotics.

Antibiotic	XDR *A. baumannii* (n=20)						*E. coli* ATCC [a] 25922 (n=1)	
	MIC (µg/ml)			Susceptibility rate (%) [b]			MIC (µg/ml)	MIC QC Ranges[d] (µg/ml)
	Range[c]	MIC_{50}	MIC_{90}	Susceptible	Intermediate	Resistant		
Polymyxin B	0.5 to 1.0	0.5	1.0	100	-	-	0.5	0.25-2.0
Rifampin	1.0 to 8.0	2.0	2.0	-	-	-	8.0	4.0-16
Colistin	4.0 to 8.0	4.0	8.0	-	-	100	1.0	0.25-2.0
Doxycycline	1.0 to 64	1.0	64	85	-	15	1.0	0.5-2.0

a: American Type Culture Collection; b: susceptibility was interpreted according to Clinical Laboratory Standard (CLSI) 2012 guidelines. c: Susceptibility range given by CLSI 2012 guidelines. d: Quality control ranges provided by CLSI 2012 guidelines against ATCC reference strain.

Table 2. Minimal inhibitory concentrations (MICs) of XDR *A. baumannii* (n=20) versus 4 different antibiotics by broth microdilution method.

Isolate no.	MIC (µg/ml)			
	Polymyxin B	Colistin	Doxycycline	Rifampin
AB-01	0.5	1.0	0.5	1.0
AB-02	0.5	2.0	32	2.0
AB-03	1.0	2.0	0.5	1.0
AB-04	0.5	1.0	0.25	1.0
AB-05	0.5	1.0	0.25	1.0
AB-06	0.5	2.0	0.5	1.0
AB-07	1.0	1.0	0.5	1.0
AB-08	0.5	2.0	0.25	1.0
AB-09	0.5	1.0	0.5	2.0
AB-10	0.5	1.0	0.5	1.0
AB-11	0.5	2.0	0.25	2.0
AB-12	0.5	4.0	0.5	1.0
AB-13	0.5	2.0	0.5	1.0
AB-14	0.5	2.0	32	8.0
AB-15	0.5	2.0	0.5	2.0
AB-16	1.0	2.0	0.25	2.0
AB-17	0.5	2.0	0.25	1.0
AB-18	0.5	2.0	0.25	1.0
AB-19	0.25	2.0	0.5	1.0
AB- 20	1.0	2.0	64	8.0

four times for AB-08 in combination with rifampin and showed antagonism. In polymyxin B-colistin combination, three strains: (15%) AB-06, AB-19, and AB-20 showed synergistic effect. In all the three isolates showing synergism, MIC of colistin was reduced by one fourth in combination with polymyxin B. For AB-19, MIC was dropped to one eighth, while for the rest of the two isolates it was reduced to one fourth in combination.

Additive effect in this combination was found to be 65%, and indifference was 20% while there was no antagonism detected in this combination. In the case of polymyxin B-doxycycline combination, no synergism or antagonism was shown. Additive effect was 60% while the remaining 40% was indifference shown in this combination.

DISCUSSION

A. baumannii has become a major challenge due to its multiple drug resistance. In recent years, more and more cases of mortality and morbidity due to MDR/XDR *A. baumannii* have come to light (Queenan et al., 2012; Karaiskos et al., 2013).

Several studies have been done, both *in-vitro* and *in-vivo*, to demonstrate the synergism of two or more antibiotics in combination against resistant pathogens (Gunderson et al., 2003; Lim et al., 2011; Fiori and Van

Table 3. Synergy test results of antibiotic combination by checkerboard microtitration method.

Strain no.	PB - RD				PB - COL				PB - DO			
	Conc. of PB	Conc. of RD	∑ FIC	Activity	Conc. of PB	Conc. of COL	∑ FIC	Activity	Conc. of PB	Conc. of DO	∑ FIC	Activity
AB-01	MIC	1/2 MIC	1.5	ID	1/2 MIC	1/2 MIC	1.0	ADD	1/2 MIC	MIC	1.5	ID
AB-02	1/4 MIC	1/8 MIC	0.375	S	1/4 MIC	1/2 MIC	0.75	ADD	1/2 MIC	1/4 MIC	0.75	ADD
AB-03	1/2 MIC	1/4 MIC	0.75	ADD	1/2 MIC	MIC	1.5	ID	1/2 MIC	1/4 MIC	0.75	ADD
AB-04	MIC	1/2 MIC	1.5	ID	1/2 MIC	1/4 MIC	0.75	ADD	1/2 MIC	MIC	1.5	ID
AB-05	1/2 MIC	1/8 MIC	0.625	ADD	1/2 MIC	1/2 MIC	1.0	ADD	1/2 MIC	MIC	1.5	ID
AB-06	1/2 MIC	1/4 MIC	0.75	ADD	1/4 MIC	1/4 MIC	0.49	S	1/2 MIC	1/2 MIC	1.0	ADD
AB-07	1/2 MIC	1/4 MIC	0.75	ADD	1/2 MIC	MIC	1.5	ID	MIC	1/2 MIC	1.25	ID
AB-08	4 MIC	1/2 MIC	4.5	AG	1/2 MIC	MIC	1.5	ID	1/2 MIC	MIC	1.5	ID
AB-09	1/8 MIC	1/2 MIC	0.62	ADD	1/2 MIC	MIC	1.5	ID	1/2 MIC	1/2 MIC	1.0	ADD
AB-10	1/2 MIC	1/4 MIC	0.75	ADD	1/2 MIC	1/4 MIC	0.75	ADD	1/2 MIC	1/4 MIC	0.75	ADD
AB-11	MIC	1/8 MIC	1.125	ID	1/2 MIC	1/2 MIC	1.0	ADD	1/4 MIC	1/2 MIC	0.75	ADD
AB-12	MIC	1/4 MIC	0.75	ADD	1/8 MIC	1/2 MIC	0.62	ADD	1/8 MIC	1/2 MIC	0.62	ADD
AB-13	1/2 MIC	1/4 MIC	0.75	ADD	1/8 MIC	1/2 MIC	0.62	ADD	1/2 MIC	1/2 MIC	1.0	ADD
AB-14	1/8 MIC	1/4 MIC	0.37	S	1/2 MIC	1/2 MIC	1.0	ADD	1/4 MIC	1/2 MIC	0.62	ADD
AB-15	1/8 MIC	1/2 MIC	0.62	ADD	1/2 MIC	1/4 MIC	0.75	ADD	1/4 MIC	MIC	1.25	ID
AB-16	1/16 MIC	1/2 MIC	0.56	ADD	1/2 MIC	1/2 MIC	1.0	ADD	1/4 MIC	MIC	1.25	ID
AB-17	MIC	1/4 MIC	1.25	ID	1/2 MIC	1/4 MIC	0.75	ADD	1/8 MIC	MIC	1.125	ID
AB-18	MIC	1/4 MIC	1.25	ID	1/2 MIC	1/4 MIC	0.75	ADD	1/2 MIC	1/2 MIC	1.0	ADD
AB-19	1/2 MIC	1/2 MIC	1.0	ADD	1/8 MIC	1/4 MIC	0.37	S	1/2 MIC	1/4 MIC	0.75	ADD
AB- 20	1/4 MIC	1/4 MIC	0.5	S	1/4 MIC	1/4 MIC	0.5	S	1/2 MIC	1/8 MIC	0.62	ADD

AB: *A. baumannii*, S: synergism, ID: indifference, ADD: additive effect, AG: antagonism, PB: polymyxin B, RD: rifampin, COL: colistin, DO: doxycycline, FIC: fractional inhibitory concentration, Conc.: concentration.

Dijck, 2012). Antimicrobial resistance in *A. baumannii* has considerably increased in the recent past (Lockhart et al., 2007). In our study, 15% of XDR *A. baumannii* strains showed resistance to doxycycline. In a study done in 2006, doxycycline resistance was reported to be 22% for *A. baumannii* (Elmanama, 2006).

In yet another study, 8% strains showed resistance to doxycycline (Timurkaynak et al., 2006). All strains were susceptible to polymyxin B while all of them were resistant to colistin. The susceptibility of XDR *A. baumannii* to polymyxin B is found to be 100% in other studies as well (Kuo et al., 2012; Lim et al., 2011). Colistin resistance has been reported from various regions of the world. Colistin resistance was found to be 40.6% in Spain (Arroyo et al., 2009). In Kuwait, colistin resistance was found to be 12% (Al-Sweih et al., 2011). In a study done by Chang et al. (2012) 10.4% colistin resistance was found. In another study colistin resistance was found to be 7.1% (Rodriguez et al., 2010). Although the incidence of colistin resistance is low worldwide in contrast to our findings, it has been proved through *in-vitro* experiment that the rate of resistance development to colisitin is rapid among *Acinetobacter* (Tan et al., 2007). Colistin is being used against MDR and XDR Gram negative organisms especially *Pseudomonas* and *A. baumannii* due to its relatively low neurotoxicity

and nephrotoxicity as compared to polymyxin B and aminoglycosides. So far no heteroresistance has been reported in polymyxin B, this could be the main reason behind the 100% susceptibility to it (Mammina et al., 2012; Adams et al., 2009; Li et al., 2006) . In this study we intentionally took XDR strains of *A. baumannii* because these organisms have become a major problem and are untreatable in our setup. These isolates were resistant to colistin, however this might not represent the actual percentage of colistin resistance in our setup, as we did not take random isolates. In addition to their resistance to various antimicrobials, all XDR *A. baumannii* were resistant to tigecycline as well. Decreased susceptibility to tigecycline is linked with efflux pumps, over expressed by MDR/XDR *A. baumannii* (Ruzin et al., 2007). Tigecycline resistance among MDR *A. baumannii* was found to be 78% by Li et al. (2007) and 66% by Navon-Venezia et al. (2007). Al-Sweih et al. (2011) in contrast, reported 13.6% tigecycline resistance among 250 *Acinetobacter* isolates. The most probable reason for these contrasting results to our study could be the XDR strains in comparison with the susceptible and MRD strains which were used in the above mentioned studies. It is reported that *A. baumannii* showing resistance to multiple antimicrobial agents are notorious for reduced susceptibility to tigecycline (Ruzin et al., 2007).

Rifampin has been reported in various studies to have synergistic activity with different antibiotics against *A. baumannii* (Pachon-Ibanez et al., 2010). Thus synergistic activity of polymyxin B-rifampin in combination was found to be 15% in our study, although these strains were resistant to rifampin alone. Tan et al. (2011) reported 19% synergism in polymyxin B-rifampin combination against *A. baumannii* by the checkerboard microtitration method and 56% by the time kill assay. These results are in accordance with our study when compared with the checkerboard microtitration method. Lim et al. (2011) found the highest synergistic activity in a polymyxin B-rifampin combination out of all the tested combinations (41.9%) by time kill assay. Carl et al. (2010) reported 60% bactericidal activity in polymyxin B-doripenem-rifampin triple combination against MDR *A. baumannii* in a time kill assay. All MDR *A. baumannii* strains were resistant to carbapenems and rifampin when tested alone (Urban et al., 2010). Manikal et al. (2000) found a 50% synergistic effect of polymyxin B-rifampin combination against *A. baumannii* and a 50% additive effect by the checkerboard microtitration method. Antagonism was also noted in the polymyxin B-rifampin combination and was found to be 5%, however the additive effect was found to be 55%. In contrast to our study, none of the above mentioned studies reported antagonism of polymyxin B with rifampin (Tan et al., 2011; Lim et al., 2011; Urban et al., 2010).

Polymyxin B-colistin combination also showed 15% synergism. To our knowledge, a polymyxin B-colistin combination has not yet been tested against XDR *A. baumannii*. The highest additive effect was noted in this combination (65%). Although both the antibiotic agents have the same site of action, more extensive research is needed to find an effective combination showing higher rates of synergism against XDR *A. baumannii*. In the case of the polymyxin B-doxycycline combination, only additive effect/indifference (60/40%) was found. Our results are in accordance with another study in which doxycycline in combination with other antibiotics against *A. baumannii* showed either additive effect or indifference (Timurkaynak et al., 2006). There are several reports about the synergistic activity of polymyxin B when used in combination therapies with imipenem, rifampin and azithromycin (Yoon et al., 2004; Wareham and Bean, 2006). It is noted that the results of synergy tests are highly strain and method dependent and *in vitro* synergy may or may not translate into *in-vivo* benefit (Pankey and Ashcraft, 2009).

It is concluded that polymyxin B-rifampin and polymyxin B-colistin combinations have demonstrated synergism against XDR *A. baumannii* by the method used, that is, checkerboard microtitration. However, the gold standard method for synergy testing is time-kill assay, that is, our study limitation. More antibiotic combinations should be tested e.g., tigecycline in order to find more effective combinations against XDR *A. baumannii*.

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