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Original Research Article

In vitro and *in vivo* evaluation of antibiotic combination against colistin resistant *Acinetobacter baumannii* isolated from patients of a tertiary care hospital, Bangladesh

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ABSTRACT

Background: Emerging resistance to colistin in clinical *Acinetobacter baumannii* isolates is of growing concern as these-strain are simultaneously resistant to most antimicrobial agents. Colistin is considered as last resort for treating severe bacterial infections caused by gram negative multidrug resistant bacteria. Since current treatment option for these strains are extremely limited, different antimicrobial combinations were used to see their efficacy against colistin resistant *A. baumannii* both *in vitro* and *in vivo*.
Methods: Cross-sectional study was done in the department of Microbiology, Dhaka Medical College, Bangladesh from July, 2017 to June, 2018. Antibiotic susceptibility was performed by disc-diffusion technique and MIC. Colistin resistance genes were detected by PCR. In vitro activity of colistin, tigecycline, imipenem, amikacin and their combinations were evaluated by agar dilution method. Rat septicemic models were made using the colistin resistant A. baumannii and effectiveness of antibiotic combinations were tested in rats.
Results: Among 32 isolated *A. baumannii* 12.5% were colistin resistant. All colistin resistant *A. baumannii* were positive for *lpx*A, *lpx*C, *pmr*A, *pmr*B genes; 75% were positive for *lpx*D gene, 50% were positive for *pmr*C gene. The proportions of synergy observed in colistin-tigecycline, colistin-imipenem, colistin-amikacin and imipenem-amikacin were 75%, 50%, 50% and 25% respectively in vitro. The best *in vivo* result appeared in group treated with colistin-tigecycline combination where 100% bacterial clearance from rats was observed while colistin-imipenem combination showed 83.33% clearance.
Conclusion: Colistin plus tigecycline or colistin plus imipenem may be alternative treatment option against colistin resistant *A. baumannii* infection.

Keywords: *Acinetobacter baumannii*, Colistin resistance, Combined antibiotic therapy, *In vitro* and *in vivo* efficacy

INTRODUCTION

Acinetobacter baumannii is major nosocomial pathogen worldwide.[1] These gram-negative coccobacilli causing ventilator-associated pneumonia, urinary tract, bloodstream and surgical site infections within the healthcare settings.[2] Mortality in *A. baumannii* infection is 30% and when not treated promptly may rises upto 75%. Existance of MDR serotypes of *A. baumannii* and the high mortality and morbidity rates associated with these infections pose a universal problem.[3] Colistin resistance is increasingly reported in *A. baumannii* and pandrug-resistant *A. baumannii* isolates have been emerged.[4] Colistin activity can be enhanced when combined with some other antibiotics with different mode of action such as carbapenems, amikacin and

tigecycline.[5,6] Combination of either two or one antibiotic and an adjuvant can achieve a synergistic antibacterial effect and may be beneficial in this regard. This may provide broader spectrum coverage, decrease the emergence of resistance and also dose related toxicity.[7] To the best of knowledge, data regarding use of different antibiotic combinations on colistin registant *A. baumannii* is lacking in Bangladesh. This study has been designed to see the effectiveness of single drug and combination of colistin, tigecycline, imipenem, amikacin against colistin registant *A. baumannii* and to compare the in vitro and in vivo effect (rat model) and thus to identify effective therapeutic alternatives for patients infected with such strains.

METHODS

Study design

This cross-sectional study was carried out in the department of Microbiology of Dhaka Medical College Hospital, Bangladesh from July, 2017 to June, 2018. Informed written consent was taken from each participant and ethical clearance was obtained from the ethical review committee of Dhaka Medical College.

In vitro study

Isolation of colistin resistant A. baumannii isolates

Total 350 samples (blood, urine, wound swab and endotracheal aspirate) were collected from adult patients attending in Dhaka medical college hospital having clinically suspected infections were inoculated on MacConkey agar media and Blood agar media. *A. baumannii* isolates were identified by non-lactose fermenting colonies on MacConkey agar, Gram gram-negative coccobacilli, non-motile, catalase positive, oxidase negative, indole and urease negative, citrate positive, alkaline slant and butt without H_2S and gas in TSI agar and grew at 41^0C and 44^0C.[8,9]

Antibiotic susceptibility of the colistin resistant *A. baumannii* were tested by disk diffusion technique using commertially available antibiotic disks (Oxoid Ltd, Basngstoke, United Kingdom). Criteria of the United States Food and Drug Administration (FDA, 2010) was used for interpretation of zone of inhibition of tigecycline. Colistin and tigecycline resistance was determined by MIC. *Escherichia coli* ATCC 25922 was used for quality control.[10]

Molecular characterization of colistin resistance gene

Polymerase chain reaction was done to detect colistin resistance genes. To prepare bacterial pellets, a loop full of bacterial colonies from Mueller-Hinton agar media was inoculated into a microcentrifuge tube having sterile trypticase soy broth. After incubation overnight at 37^0C, incubated tube was centrifuged at 4000 g for 10 minutes. Supernatant was discarded and tube containing bacterial pellet were kept at -20^0C for DNA extraction. DNA was extracted following simple boiling method.[11] The pair of primers were used to yield PCR products shown in (Table 1).

PCR assays were performed in a thermal cycler. After initial denaturation at 94^0C for 10 minutes followed by 36 cycles that includes of denaturation at 94^0C for 30 seconds, annealing at 52^0C for 40 seconds, extension at 72^0C for one minute with a final extension at 72^0C for 10 minutes. The amplified DNA were loaded into a 2% agarose gel, electrophorosed at 100 volts for 30 minutes, stained with 1% ethidium bromide, and visualization under UV light.

Minimun inhibitory concentration

MIC of colistin (Forest Pharma Ltd), imipenem (Incepta Ltd), tigecycline (Incepta Ltd), amikacin (ACI Ltd) was determined among colistin resistant *A. baumannii*. MIC was performed by agar dilution method. MICs were performed by using dilutions of individual antibiotics incorporated into Mueller-Hinton agar (Oxoid Ltd, Basngstoke, United Kingdom). Seven doubling dilutions each antibiotic was prepared. To obtain 10^4 cfu/spot on agar surface one µl of 10 times diluted 0.5 McFarland turbidity of test inoculums were placed on Mueller-Hinton agar plates. All the inoculated plates were incubated aerobically at 37˚C overnight. The lowest concentration of antibiotic impregnated Mueller-Hinton agar showing no visible growth on agar media was considered as MIC of the drug of that strain. *Escherichia coli* ATCC 25922 was used as control organism CLSI.[10] MIC is defined as the lowest concentration of drug that inhibits the growth of organism.

Antibiotic combination testing

Four isolates of *A. baumannii* with clear resistace to colistin were isolated by susceptibility testing and selected for combination studies. Combination of colistin with tigecycline, imipenem, amikacin and imipenem with amikacin were examined by agar dilution method. Twofold serial dilutions of antibiotics were prepared from twofold higher dilutions of MICs upto fourfold lower dilutions of MIC. In evaluating the combination effect, synergy was present when there was a fourfold or greater reduction in the MICs of both antibiotics. A reduction of less than fourfold in the MICs of both antibiotics was considered additive. Indifference was found when neither drug exhibited a decrease in MIC, and an increase in the MIC was considered antagonism. Testing for synergy by agar dilution method is based on inhibitory rather than bactericidal endpoints.[12]

Table 1: Primers used in this study Colistin resistance genes.[21]

Gene	Primer-oligonucleotide sequence	Base pair
***pho*Q**	Forward-GAG CTT CAG ACT ACT ATC GA Reverse-GGG AAG ATA TGC CGC AAC AG	2500
***pho*P**	Forward-ATA CCC ACA GGA CGT CAT CA Reverse-CAG GTG TCT GAC AGG GAT TA	2800
***mgr*B**	Forward-TTA AGA AGG CCG TGC TAT CC Reverse-AAG GCG TTC ATT CTA CCA CC	248
***pmr*C**	Forward-GCG TGA TGA ATA TCC TCA CCA Reverse-CAC GCC AAA GTT CCA GAT GA	1602
***pmr*A**	Forward-GAT GAA GAC GGG CTG CAT TT Reverse-ACC GCT AAT GCG ATC CTC AA	675
***pmr*B**	Forward-TGC CAG CTG ATA AGC GTC TT Reverse-TTC TGG TTG TTG TGC CCT TC	1304
***mcr*-1**	Forward-CGG TCA GTC CGT TTG TTC Reverse-CTT GGT CGG TCT GTA GGG	309
***lpx*A**	Forward-TGA AGC ATT AGC TCA AGT TT Reverse GTC AGC AAA TCA ATA CAA GA	1180
***lpx*D**	Forward-CAA AGT ATG AAT ACA ACT TTT GAG Reverse-GTC AAT GGC ACA TCT GCT AAT	1502
***lpx*C**	Forward-TGA AGA TGA CGT TCC TGC AAA Reverse-TGG TGA AAA TCA GGC AAT GA	1164

In vivo study

The experiments were performed in immunocompetent male and female rats (long-Evans species) weighing 55-65 grams obtained from the International Centre for Diarrheal Disease Research, Bangladesh (icddr,b) breeding house, Dhaka, Bangladesh. Rats were infected by intra-peritoneal injection of 170 µl of approximately 10^8cfu/ml bacterial inoculum (equivalent to 0.5 McFarland's standard) using a 26-gauge needle in the lower right abdomen.[12] Bacterial inoculums were obtained through a 24 hours subculture of one out of 4 colistin resistant *A. baumannii* in MacConkey agar media at 37^0C. The animals were observed for 72 hours and the survival of rats were recorded every 12 hours. Blood samples were taken as detailed below. All the blood samples were processed for microbiological studies.

Antibacterial treatment

To evaluate the effectiveness of the different antibiotic regimens, 40 rats were divided into 7 groups (A, B, C, D, E, F and G). Group A, B, C, D, E and F contain 6 rats in each group and group G contain 4 rats. Group A, B, C, D, E and F were inoculated with bacterial inoculums. But group G was not inoculated with bacterial inoculums and was regarded as negative control group. Group F was inoculated with bacterial inoculums but did not receive antimicrobial treatment and was regarded as positive control group. Group A, B, C, D and E received antimicrobial treatment 4 hours after inoculation of the organism in following treatment regimens twice daily over 72 hours. Group A - only colistin, 50000 IU/kg/ day, intraperitoneally, Group B - colistin plus tigecycline, colistin 50000 IU/kg/day, intraperitoneally; tigecycline: 20 mg/kg/day, intraperitoneally, Group C - colistin plus imipenem, 50000 IU/kg/day, intraperitoneally; imipenem:120 mg/kg/day, intramuscularly, Group D - colistin plus amikacin, colistin: 50000 IU/kg/day, intraperitoneally; amikacin: 15 mg/kg/day, intramuscularly, Group E - amikacin plus imipenem, 120 mg/kg/day intramuscularly for imipenem, 15 mg/kg/day intramuscularly for amikacin.

The first dose of every antibiotic was administered 4 hours after inoculation of the organism. In order to confirm that these drugs were not toxic to the animal, another group of four uninfected rats (Group G) were given each antibiotic for 72 hours (uninfected treat group/negative control). The animals were observed for 72 hours of treatment and the cumulative survival rates were recorded every 12 hours. Blood samples were taken as described below.[13]

Microbiological study

After 72 hours of antibiotic treatment, blood samples were collected from rats by cardiac puncture aseptically. At first, upper part of the chest was shaved by razor, then washed with chlorhexidine. After palpating the cardiac pulsation with the finger pulp, the area was washed with povidone iodine, then a syringe was introduced through the skin in the heart of rat blindly. For blood culture 1.5 ml of each rat blood was collected and then inoculated in sterile conical flask with 15 ml of TSB and incubated for 24 hours at 37^0C. Subculture was done in Blood agar and MacConkey agar media and incubated for 24 hours at 37^0C. Then the incubated plates were observed for positive or negative growth.[13]

RESULTS

In vitro tests

Out of total 350 samples 32 were *A. baumannii* isolates. Among thirty-two isolated *A. baumannii*, 4 (12.50%) colistin reistant strains were detected by MIC by agar dilution method of which, 3 (75%) were from endotracheal aspirates and none were isolated from urine and blood samples.

Table 2: Antimicrobial resistance pattern among colistin resistant *A. baumannii* (n=4).

Antimicrobial drugs	Resistance, N (%)
Imipenem	4 (100)
Amoxiclav	4 (100)
Ceftriaxone	4 (100)
Ceftazidime	4 (100)
Cefotaxime	4 (100)
Cefepime	4 (100)
Gentamicin	4 (100)
Amikacin	3 (75)
Meropenem	3 (75)
Piperacillin	3 (75)
Tigecycline	3 (75)

Most colistin resistant *A. baumannii* were resistant to most of the cephalosporin, aminoglycosides and carbapenem group of antibiotics (Table 2). Among the colistin resistant *A. baumannii*, 100% were positive for *lpx*A, *lpx*C, *pmr*A and *pmr*B genes; 75% were positive for *lpx*D gene and 50% were positive for *pmr*C gene detected by PCR. *pho*P, *pho*Q, *mgr*B and *mcr*-1 genes were not found. MIC of colistin, tigecycline, imipenem and amikacin among the colistin resistant *A. baumannii* were ranged from ≥256 µg/ml-64 µg/ml, ≥16 µg/ml-2µg/ml, ≥64 µg/ml-32 µg/ml and ≥2048 µg/ml-16 µg/ml respectively.

Using different antibiotic combination against colistin resistant *A. baumannii* resulted varying fold in reduction of MIC. While combining colistin and tigecycline among four colistin resistant *A. baumannii* isolates, 75% showed synergistic effect (fourfold reduction of MIC), 12.50% showed additive effect (twofold reduction of MIC). In case of colistin and imipenem combination 50% showed synergistic effect, 25% showed additive effect and 25% showed indifferent effect (no reduction of MIC). While combining colistin and amikacin 50% showed synergistic effect, 25% showed additive effect and 25% showed indifferent effect. In case of imipenem and amikacin combination 25% showed synergistic effect, 50% showed additive effect and 25% showed indifferent effect. None of the combination showed antagonism.

In vivo tests

All the rats in the positive control group were bacteraemic and all the rats in the negative control group were blood culture negative. In the group treated with colistin, 16.67% were culture negative. In the group treated with colistin and tigecycline combination, 100% were culture negative. In the group treated with colistin and imipenem combination, 83.33% were culture negative. In the group treated with colistin and amikacin combination, 66.67% were culture negative. In the group treated with imipenem and amikacin combination, 50% were culture negative.

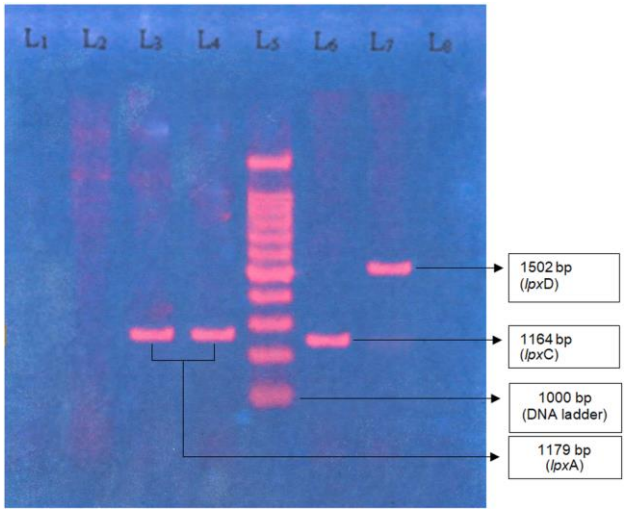

Figure 1: Photograph of gel electrophoresis: negative control without DNA (TE buffer) (lane one), negative control Escherichia coli ATCC 25922 (lane 2), amplified DNA of 1179 bp for lpx A gene (lane 3, 4), one kbp DNA ladder (lane 5), DNA of 1164 bp for lp C gene (lane 6), DNA of 1502 bp for lpx D gene (lane 7), negative sample (lane 8).

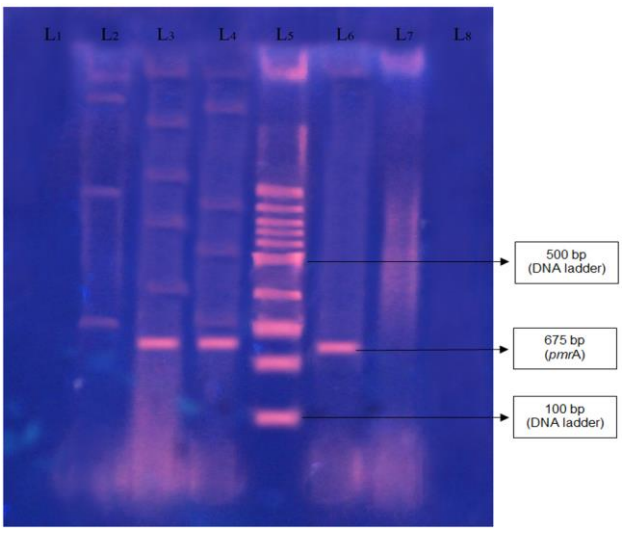

Figure 2: Photograph of gel electrophoresis: negative control without DNA (TE buffer) (lane one), negative control Escherichia coli ATCC 25922 (lane 2), amplified DNA of 675 bp for pmrA gene (lane 3, 4, 6), hundred bp DNA ladder (lane 5), negative sample (lane 7, 8).

Combining colistin with tigecycline showed 75% synergistic effect *in vitro* and 100% synergistic effect *in vivo*, combining colistin with imipenem showed 50% synergistic effect *in vitro* and 83.33% synergistic effect *in vivo*, combining colistin with amikacin showed 50% synergistic effect *in vitro* and 66.67% synergistic effect *in vivo* and combining imipenem with amikacin showed 25% synergistic effect *in vitro* and 50% synergistic effect *in vivo*.

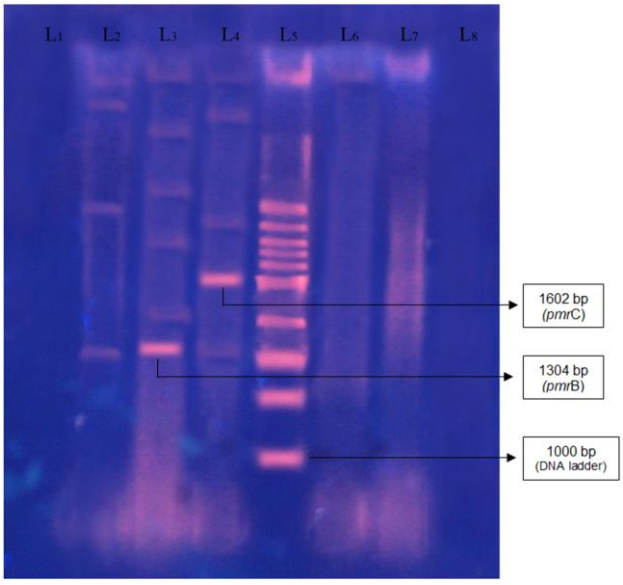

Figure 3: Photograph of gel electrophoresis: negative control without DNA (TE buffer) (lane one), negative control Escherichia coli ATCC 25922 (lane 2), amplified DNA of 1304 bp for pmrB gene (lane 3), DNA of 1602 bp for pmrC gene (lane 4), one kbp DNA ladder (lane 5), negative sample (lane 6, 7, 8).

DISCUSSION

Colistin resistance is emerging among *A. baumannii*, leaving limited therapeutic options for the management of serious infections. Prompt and rapid detection of colistin resistance will prevent their spread and combination therapy may be good options for treatment of infection caused by them. In consistent with other reports from Bangladesh and India 12.5%.[14,15] *A. baumannii* were resistant to colistin in the present study. There are two main hypotheses of the colistin resistance mechanism. The first is the loss of LPS hypothesis and it involves inactivation (amino acid substitutions, frameshifts or truncation) of a lipid A biosynthesis gene.[16] An LPS-deficient colistin-resistant strain with a less negative charge might be the reason for a loss of affinity to colistin.[17] The second is the PmrAB two-component system mediated hypothesis and it involves mutations in the genes *pmr*A and *pmr*B are linked to colistin resistance in *A. baumannii.*[18] *A. baumannii* is lacking the biosynthesis genes for LAra4N, therefore it uses PEtN addition as the main colistin resistance mechanism, which is mediated by the chromosomally encoded *pmr*CAB operon.[19] Plasmid mediated colistin resistance gene, mcr-1 has also been reported in Enterobacteriaceae.[20] The present high level of colistin resistance might be due to use of colistin in ICU and severely ill patients. In aggrement with other study from Bangladesh 75% of colistin resistant *A. baumannii* were isolated from tracheal aspirates in ICU patients.[21] As Acinetobacter is ubiquitous organisms and important nosocomial pathogens critically-ill patients acquire infection during their stay in ICU, patient contact with health care personnel and length of exposure to invasive procedures. All these may explain the present high-level resistance of colistin in ICU.

Similarly, for the same reason the prevalence of tigecycline resistance in *A. baumannii* in ICU is more. In the present study, among colistin resistant *A. baumannii* 75% were resistant to tigecycline. It was found that 20% colistin reistant *A. baumannii* isolates were resistant to tigecycline.[22] Another study from North India found 16% of the carbapenem resistant MDR strains were resistant to both tigecycline and colistin.[23] The higher tigecycline resistance rate among the isolated *A. baumannii* might be due to the fact that the patients from ICU were more resistant to tigecycline than those isolated from non-ICU patients.[24] The long-term use of colistin and tigecycline could result of resistant strains effected through hetero-resistance and hyper expression of the efflux pump.[25-27] However, involvement of an efflux pump mechanism is mainly associated with the tigecycline resistance in *A. baumannii* with increased levels of AcrAB.[27] As tigecycline is one of the last resorts of the antimicrobials in colistin resistant clinical isolates, this high resistance rate to tigecycline is worrisome and should be further investigated.[28]

In accordance with other study 100% colistin resistant *A. baumannii* were resistant to third and fourth generation cephalosporins, aminoglycosides and imipenem and 75% were resistant to meropenem and piperacillin in the present study.[22] Coexistance of carbapenem and colistin resistance has also been found in other studies.[29]

This findings further explores the well-known ability of *A. baumannii* to become resistant to commonly used antimicrobials.[30] It has also been reported that Acinetobacter can develop antimicrobial resistance even during treatment.[31]

Colistin was observed to be the most common constituent of antimicrobial combinations that were active against colistin-resistant *A. baumannii*. Non-colistin-based combinations were not very active against these strains.[32] A few studies evaluated the *in vitro* synergism of antimicrobial combinations against colistin-resistant *A. baumannii.*[33] In the present study, while combining colistin with tigecycline against colistin resistant *A. baumannii*, it showed 75% synergistic effect.

Li et al reported that the highest synergy rate of 67.4% was observed with tigecycline-colistin combinations.[34] Colistin exerts a bactericidal effect on gram-negative bacteria based on its strong affinity for lipopolysaccharide in the outer membrane and tigecycline inhibits bacterial protein synthesis by reversibly binding to the 30S ribosomal subunit.[35,36] As colistin disrupt cell membrane could result in increased uptake of tigecycline, which demonstrated higher synergy rates while combining with colistin and tigecycline. The colistin and tigecycline synergistic interaction could therefore have an impact in clinical practice by reducing the therapeutic dosage of colistin, and the risk of collateral effects which currently represent a major limitation to its clinical use.[37]

In the present study, among the colistin resistant *A. baumannii* 50% synergism was observed with the combination of colistin plus imipenem. A study by Rodriguez et al showed that colistin combined with imipenem were synergistic against heteroresistance isolates of *A. baumannii*.[5] In the present study, however, we could not confirm heteroresistance of *A. baumannii*. The higher percentage of synergism between colistin and imipenem antibiotics in the present study might be due to the fact that electrostatic binding of colistin to the outer membrane of A. baumannii causing permeability change, whereas β-lactam antibiotics inhibit the final stages in the cell wall synthesis process. In this study, while colistin combine with amikacin, showed 50% synergistic effect. This may be because colistin increase uptake of amikacin into the cell, resulting in an intracellular concentration sufficient to inhibit protein synthesis. A study Chung et al showed that antibiotic combination against *A. baumannii* isolates based on colistin (colistin + amikacin, colistin+imipenem) were more effective by removing the persisters cell.[38] It was supposed that persister cells might be one reason for antibiotic treatment failure and might contribute to the evolution of antibiotic resistance.[39] Persister formation is an intrinsic feature with respect to isolates and antibiotics, and the molecular mechanism to form persister cells against colistin is unknown, however, the partial disruption of the cell membrane by colistin could be associated with persister cell formation.[40] While combining imipenem with amikacin in the present study 25% showed synergistic effect. In a study reported that 15% synergistic effect against MDR *Enterobacter* infection which is closed to the present findings.[41] This may be due to carbapenems disrupts cell walls and helps amikacin to act on bacteria.[42]

We performed animal experiment to determine whether colistin-tigecycline, colistin-imipenem, colistin-amikacin and imipenem-amikacin combinaton may present any benefit regarding treatment. The best *in vivo* result appeared in the group treated with colistin-tigecycline combination. *In vivo* combination of colistin and tigecycline showed 100% synergism, colistin and imipenem showed 83.33% synergism, colistin and amikacin showed 66.67% synergism and imipenem and amikacin showed 50% synergism. However, no report was found to compare these results of *in vitro* and *in vivo* study against colistin resistant *A. baumannii*.

CONCLUSION

As colistin monotherapy is unable to prevent resistance, combination therapy might be the best option against colistin resistant *A. baumannii.* From this *in vivo and in vitro* experiments, it shows that colistin and tigecycline is the best effective combination for colistin resistant *A. baumannii.* The second best *in vivo and in vitro* effective combination is colistin and imipenem. Another colistin based combination and combinations of more than two drugs can also be evaluated.

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