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Multidrug resistance stimulated antagonistic antibiotic interactions

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Abstract: Urinary tract infections (UTIs) are the most common bacterial infections caused by predominantly Escherichia coli. Increasing antimicrobial resistance and transmission among the strains cause a serious problem, which needs immediate consideration. Treatment options are narrowed and the success rate is decreasing because of the strong multi-drug-resistant pathogenic bacterial strains. Especially synergistic antimicrobial combination therapy is a rational option with potential benefits. However, some antibiotic combinations exhibit antagonistic interaction that hinders treatment efforts. Therefore, screening antibiotic combinations in terms of their interaction types prior to treatment is crucial. In this study, we aimed to screen the response of strong and intermediate multi-drug resistant (MDR) E. coli strains to several protein synthesis inhibitor antibiotic combinations. Experimented antibiotic combinations were found to be antagonistic along with a retrospective analysis among 20 more antibiotics of the same class demonstrated antagonistic interaction. Interestingly and most importantly, antagonism level is found to be associated with the level of drug resistance. This result suggests that stronger MDR pathogen exhibits stronger antagonistic interactions compared to the intermediate MDR strain when protein synthesis inhibitor antibiotics are combined. As a result, antibiotic combinations should be more carefully evaluated when multi-drug regimens are planned to be administered to severe infections to avoid antagonizing individual drug effectiveness.

Key Words: antibiotic, antagonism, drug interaction.

INTRODUCTION

Urinary tract infections (UTIs) are considered to be the most common bacterial infections with an estimated number of over 250 million infections and causing approximately 1.6 $ billion economic losses worldwide per year. Escherichia coli remains as the predominant (80%) pathogen of UTIs, which is usually considered as a benign illness. However, emerging antimicrobial resistance and transmission of the antimicrobial resistance determinants among the strains via horizontal gene transfer complicates the management of the infection and as a result; it increases the rate of hospitality and mortality. Moreover, in patients with underlying diseases antimicrobial therapy becomes even more complicated [1-3].

According to new ISO standard 20776 of European Society for Clinical Microbiology and Infectious Diseases. The categorization of infectious pathogens as "susceptible," "intermediate," or "resistant" to specific antibiotics will become more reliable and will be consistent throughout Europe. A bacterial strain is said to be susceptible (S) to a given antibiotic when it is inhibited in vitro by a concentration of this drug that is associated with a high likelihood of therapeutic success whereas the sensitivity of a bacterial strain to a given antibiotic is said to be intermediate (I) when it is inhibited in vitro by a concentration of this drug that is

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associated with an uncertain therapeutic effect. Finally, a bacterial strain is considered to be resistant (R) to a given antibiotic when it is inhibited in vitro by a concentration of this drug that is associated with a high likelihood of therapeutic failure [4]. Bacteria resistant to multiple drugs is defined as multi-drug resistant (MDR) bacteria and it might result to severe infections and death [5].

Antimicrobial combination therapy is a rational approach to prevent and overcome emerging antibiotic resistance, also to get benefit of potential synergistic interaction within the drugs [6]. However, combination therapies exhibit some risks; as some antibiotics show antagonistic interaction. Therefore, screening drug-drug interactions prior to the combination therapy is crucial. In this study, we aimed to examine the response of strong and intermediate multi-drug resistant (MDR) *E. coli* strains isolated from patients with UTIs to several protein synthesis inhibitor combinations. Three antibiotic pairs were chosen for comparison as controls reported previously as additive, synergistic and synergistic for ERY-CLA, CLA-CHL and ERY-CHL respectively [7]. Our hypothesis is to show the alterations of drug interactions especially among protein synthesis inhibitors in clinical intermediate and strong MDR stains. Other three antibiotic combinations were also chosen from protein synthesis inhibitors as AMK-CHL, KAN-CHL and AMK-KAN.

Experimented protein synthesis inhibitor combinations were found to be antagonistic along with retrospective analysis within 20 more same class antibiotics demonstrated same antagonistic interaction manner. More interestingly and importantly, antagonism level is found to be associated with the level of multi-drug resistance. This result suggests that stronger MDR pathogen exhibits stronger antagonistic interaction pattern within antibiotics compared to intermediate MDR strain when protein synthesis inhibitors are combined. Therefore, protein synthesis inhibitor combinations should be more carefully evaluated when multi-drug regimens are planned to be administered to clinically severe patients for avoiding antagonizing individual drug effectiveness.

MATERIALS AND METHODS

MDR *E. coli* strains isolated from patients with UTIs obtained from Istanbul University, Faculty of Medicine, Clinical Microbiology Department, Istanbul. Antibiograms were done for screening antibiotic susceptibility by using disk-diffusion assay. Glycerol stocks of bacterial strains were prepared and stored at -20°C. Strains were plated on LB agar for colony formation prior to preparation of the starter cultures. Colonies were picked and grown in LB overnight for MIC and interaction experiments.

Drug-interaction experiments were done by using a 4 × 4 checkerboard assay. Accordingly, the concentration of each drug used in the assay increases gradually in each axis with a starting drug concentration zero (no drug). Clinically isolated multi-drug resistant (MDR) Escherichia coli strains were grown in LB media and treated with the combination of five different antibiotics namely Amikacin (AMK), Kanamycin (KAN), Chloramphenicol (CHL), Clarithromycin (CLA), Erythromycin (ERY) and with different combinatorial concentrations. End-point optical density (OD-600nm) measurements were done at the end of over-night incubation by using 96-well microplate reader. In the drug-interaction experiments, each individual drug, was used at the concentration of that was > 50% inhibition at the highest dose and < 50% inhibition at the lowest dose as described detailed in a previous study [7]. All drugs were purchased from Sigma. MICs for each drug were found by using simple two-fold dilution assay.

Interactions were quantified based on the Bliss independence model utilizing probabilistic theory to model the effects of individual drugs in a combination as independent yet competing events by using MATLAB [8]. According to Bliss independence model, two drugs, A and B, both inhibit bacteria growth: drug A at dose a inhibits Xa percent of bacteria growth and drug B at dose b inhibits Xb percent of bacteria growth. If two drugs don't work synergistically, the combined percentage inhibition Xab,P can be predicted using the complete additivity of probability theory as Xab,P=Xa+Xb-XaXb. The observed combined percentage inhibition Xab,O is then compared with Xab,P. Typically, if Xab,O > Xab,P, the combination treatment is thought to be more efficacious than expected; if Xab,O < Xab,P, the combination treatment is worse than expected; and if Xab,O =Xab,P, the combination is equal to a simple addition of two separate drugs [9]. The data obtained from 4 × 4 checkerboard assays were further evaluated by using Bliss independence model to determine drug interaction types.

RESULTS

According to the antibiogram analysis, one of the clinically isolated *E. coli* strains is found to be strong multi-drug resistant (MDR). Among common clinically used antibiotics, only amikacin and fosfomycin have shown to be effective to the MDR strain (Table 1). Second isolate is found to be intermediate MDR compared to the strong MDR strain, antibiogram results are shown in Table 1. In order to evaluate protein synthesis inhibitor combinations on both strong and intermediate MDR isolates, we used Amikacin (AMK), Kanamycin (KAN) and Chloramphenicol (CHL) pairwise combinations. In addition, one additive and two synergistic protein synthesis inhibitor drug combinations, Erythromycin – Claritromycin (ERY-CLA), Claritromycin – Chloramphenicol (CLA-CHL) and Erythromycin

- Chloramphenicol (ERY-CHL) respectively, against susceptible *E. coli* model strain reported in a previous study [7] were also screened on strong and intermediate MDR clinical isolates for comparison of drug interaction alterations. AMK-CHL bliss independence model of drug-drug interaction seen in Figure 1 shows antagonistic relationship of drugs. In the figure, X axis and Y axis show drug concentrations plotted against percentage difference of observed and model-predicted growth inhibition of the bacteria at different concentrations of drug combinations. Darker areas (Antagonism) show decreased/lower growth inhibition while drugs were used together at their certain concentrations whereas lighter areas show increased/higher growth inhibition representing synergistic drug-drug interaction. Average Bliss interaction scores for all pairwise antibiotic combinations were demonstrated in Table 2. Negative Bliss interaction scores show antagonistic drug-drug interactions and more negative Bliss interaction score represents stimulated antagonism between drug pairs

Table 1. Antibiogram results of strong and intermediate MDR E. coli isolates. R- Resistant, S-Susceptible, I-Intermediate response to antibiotics are shown for strong S-MDR and intermediate I-MDR clinical pathogenic isolates

Antibiotics	*E.coli* (S-MDR)	*E.coli* (I-MDR)
Ampicillin	R	R
Cefazolin	R	R
Gentamicin	R	S
Tobramycin	R	S
Ampicillin / Sulbactam	R	S
Cefuroxime - Axetil	R	R
Sefuroxime - Sodium	R	R
Cotrimoxazole	R	R
Amoxicillin / Clavulanic acid	R	S
Imipenem	I	S
Meropenem	I	S
Ertapenem	R	S
Amikacin	S	S
Cefoxitin	R	S
Cefotaxime	R	R
Ceftazidime	R	R
Cefepime	R	R
Ciprofloxacin	R	S
Norfloxacin	R	S
Nitrofurantoin	I	S
Fosfomycin	S	S
Piperacillin / Tazobactam	R	S

Table 2. Average of Bliss scores for each antibiotic pair, negative scores show antagonistic type drug-drug interaction

Antibiotic Pairs	INT-MDR Avg. Bliss Scores	STR-MDR Avg. Bliss Scores
AMK-CHL	**-26**	**-33**
CLA-CHL	**-12**	**-17**
ERY-CHL	**-12**	**-15**
KAN-CHL	**-31**	**-37**
ERY-CLA	**-0,5**	**-13**
AMK-KAN	**-15**	**-36**

especially at some specific concentrations in their mixture. AMK is known to be working by blocking the bacteria's 30S ribosomal subunit, which in turn blocks protein synthesis, whereas CHL binds to specific residues in the 23S rRNA of the 50S ribosomal subunit, which is blocking peptide bond formation and prevents protein synthesis. Although, each drug has different targets, which both resides in ribosome's different locations, two drugs antagonize each other on both strong and intermediate MDR strains.

AMK – KAN drug interaction was also found to be antagonistic as shown in Figure 2. AMK works by inhibiting the 30S ribosomal subunit as KAN works on blocking again the 30S ribosomal subunit. However, two antibiotics are similar; amikacin can evade attacks by most of the antibiotic-inactivating enzymes that are responsible for antibiotic resistance in bacteria compared to kanamycin. The antagonistic interaction between AMK - KAN might be resulted from their competition for the same target on the ribosome.

Another protein synthesis inhibitor combination, KAN – CHL was also found to be antagonistic (Fig. 3). Kanamycin blocks the 30S subunit of ribosome, which confers a different target unlike to 50S ribosomal subunit blocker chloramphenicol.

Interaction between CHL – CLA was found antagonistic on both strong and intermediate MDR strains. As seen in Figures 1-4, Table 2, strong MDR strain shows more strong antagonism between same pairwise drug combinations compared to intermediate resistant isolate. CLA binds to 23S rRNA, which is a part of 50S subunit of ribosome and inhibits peptide translation. CHL acts in a similar way as CLA does. So that, the antagonistic interaction between CLA – CHL is predictable resulting from their competition for the same target on the ribosome.

Erythromycin another protein synthesis inhibitor drug, prevents tRNA transfer by interfering with aminoacyl translocation were combined with CLA and CHL. As shown in Figure 5, ERY – CHL and ERY – CLA pairwise interactions were found antagonistic. According to experiments, all interactions between protein synthesis inhibitors are antagonistic on strong and intermediate clinical isolates even if their targets are different.

In order to support this observation, we analyzed recently published data involving 20 more protein synthesis inhibitor pairwise combinations [7]. As shown in Figure 6, antibiotics blocking protein synthesis, are tend to show more antagonistic interaction pattern in their pariwise combinations. Positive alpha scores shown in the Figure 6 represent antagonistic way of interaction besides negative alpha scores demonstrate synergistic way of interaction. Thus, the avarage of alpha scores of pairwise combinations is 0.551 along with the median value of 0.432 that represent more antagonistic interaction

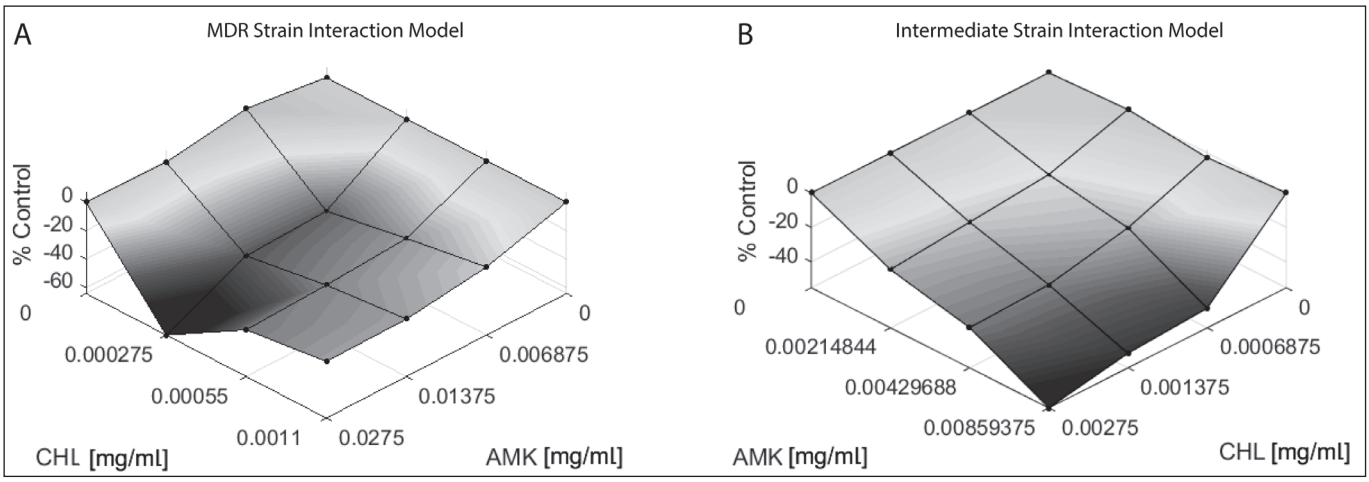

Figure 1. Bliss independence model of drug-drug interaction of CHL – AMK combination used on (A) strong MDR and (B) intermediate MDR isolates of E. coli. Darker areas (More negative Bliss interaction scores) of the surface are demonstrating more antagonistic interactions compared to lighter areas (More positive Bliss interaction scores) representing more additive/synergistic interactions.

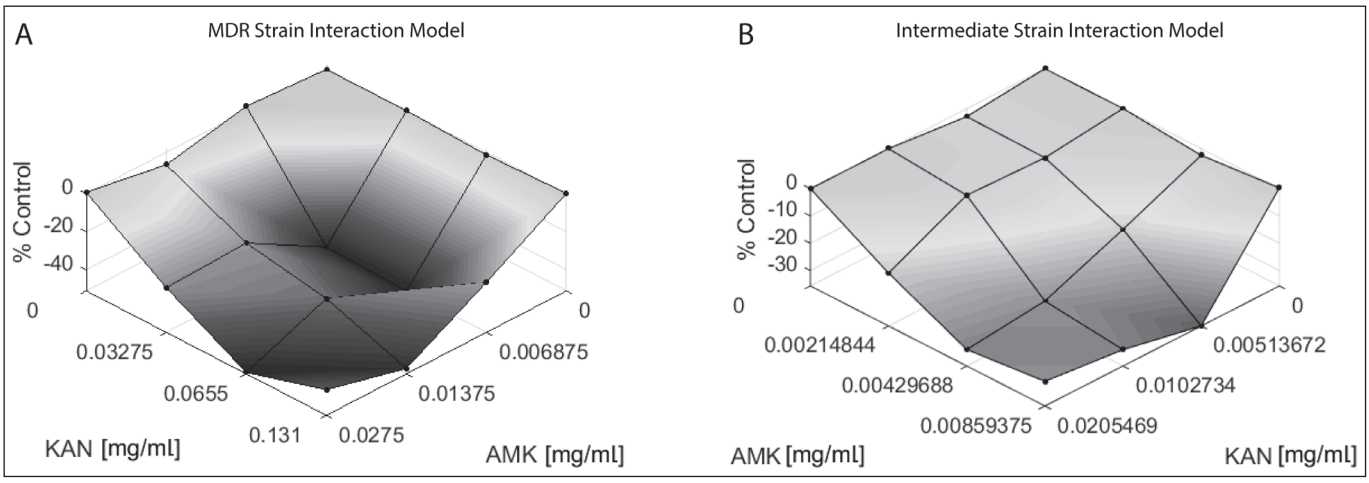

Figure 2. Bliss independence model of drug-drug interaction of KAN – AMK combination used on (A) strong MDR and (B) intermediate MDR E. coli. Darker areas of the surface are demonstrating more antagonistic interactions compared to lighter areas representing more additive/synergistic interactions.

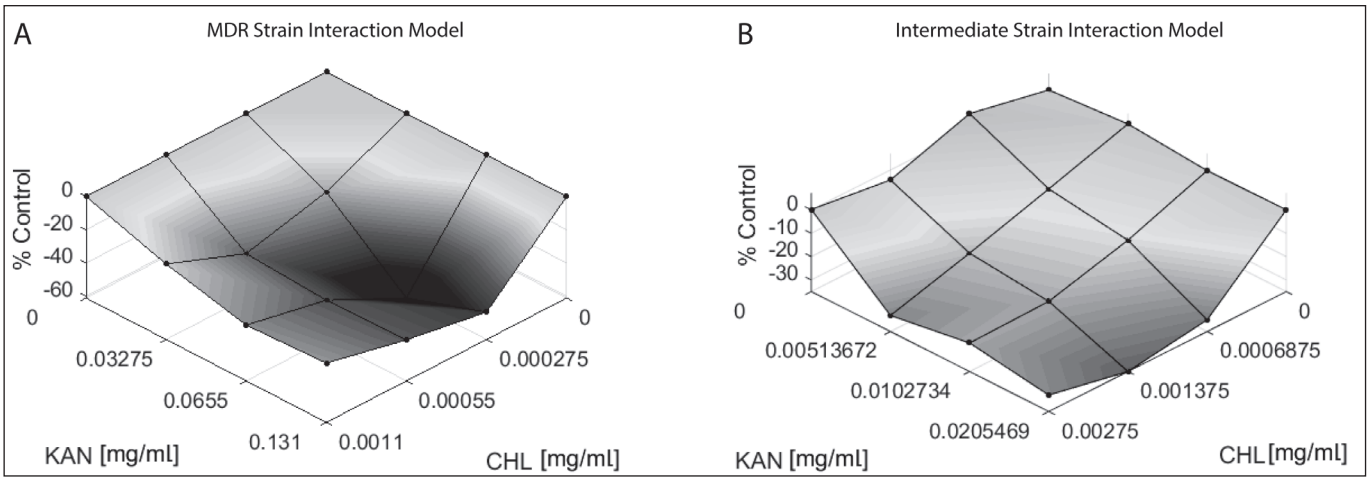

Figure 3. Bliss independence model of drug-drug interaction of KAN – CHL combination used on (A) strong MDR and (B) intermediate MDR E. coli. Darker areas of the surface are demonstrating more antagonistic interactions compared to lighter areas representing more additive/synergistic interactions.

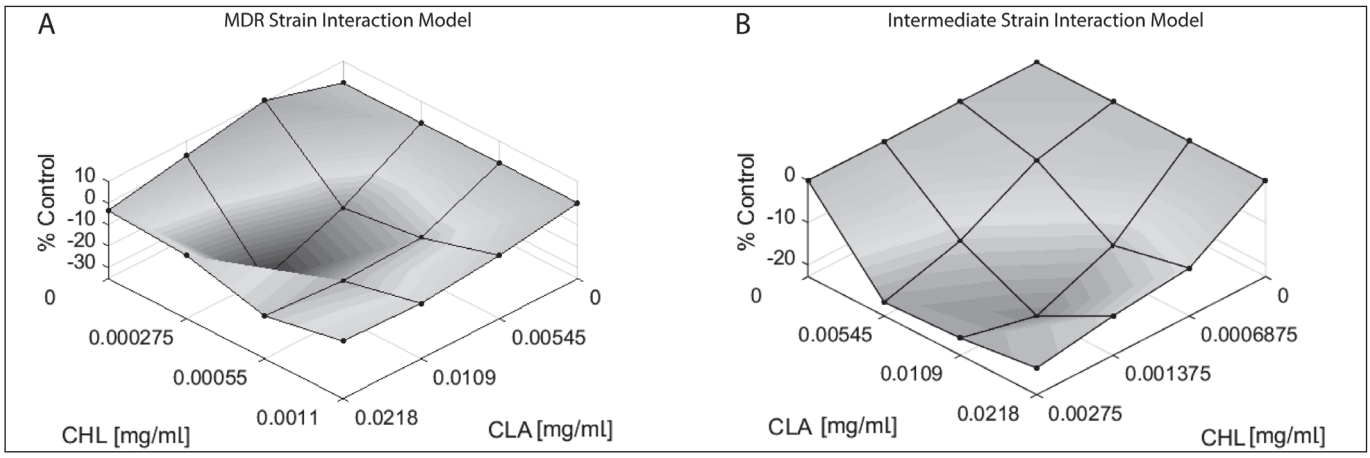

Figure 4. Bliss independence model of drug-drug interaction of CLA – CHL combination used on (A) strong MDR and (B) intermediate MDR E. coli. Darker areas of the surface are demonstrating more antagonistic interactions compared to lighter areas representing more additive/synergistic interactions.

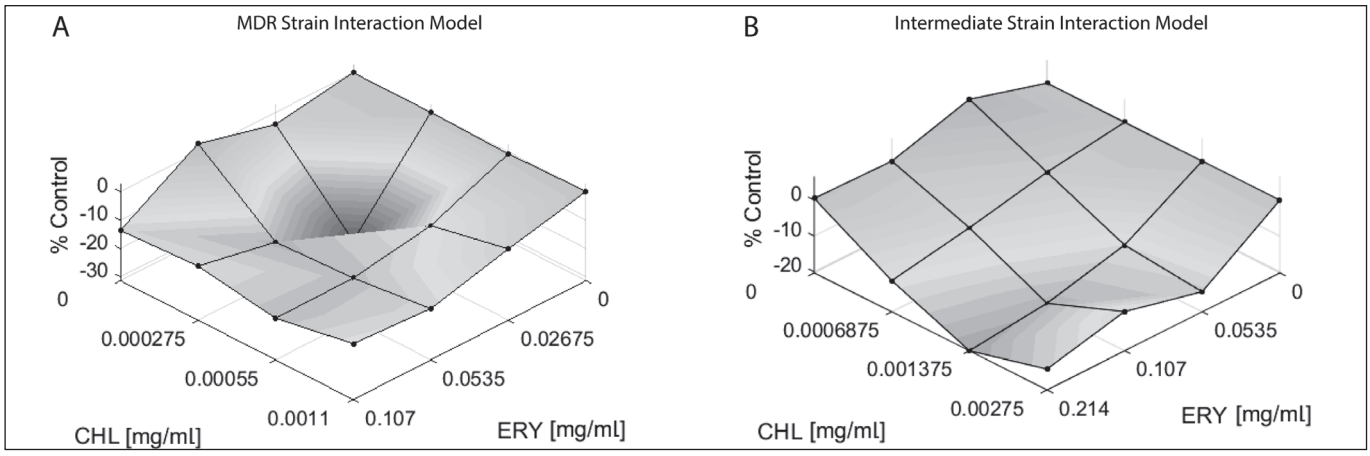

Figure 5. Bliss independence model of drug-drug interaction of ERY – CHL and ERY – CLA combination used on (A-C) strong MDR and (B-D) intermediate MDR E. coli. Darker areas of the surface are demonstrating more antagonistic interactions compared to lighter areas representing more additive/synergistic interactions.

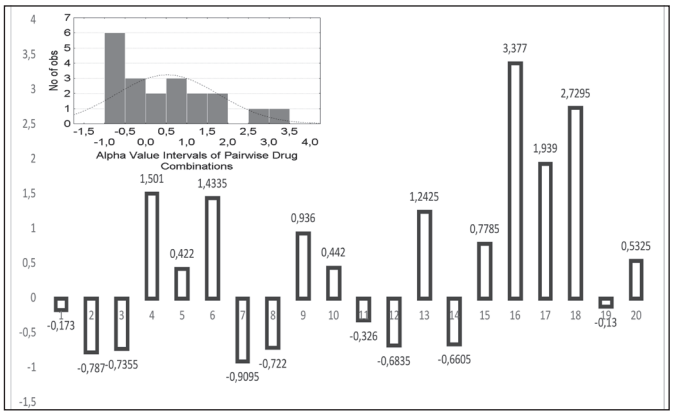

Figure 6. Alpha scores of 20 protein synthesis blocking antibiotic combinations. Positive alpha scores represent antagonistic way of interaction where negative alpha scores represent synergistic way of interaction. 1-20 numbers represent antibiotic combinations namely CLA (Clarithromycin) – ERY (Erythromycin), ERY – TET (Tetracycline), CHL (Chloramphenicol) – ERY, ERY- TOB (Tobramycin), ERY – GEN (Gentamicin), AMK (Amikacin) – ERY, CLA – TET, CHL – CLA, CLA – TOB, CLA – GEN, AMK – CLA, CHL – TET, TET – TOB, GEN – TET, AMK – TET, CHL – TOB, CHL – GEN, AMK – CHL, GEN – TOB, AMK – TOB respectively. The histogram demonstrates number of individual antibiotics with their alpha score intervals.

manner. The histogram in the Figure 6 demonstrates the number of observations between different alpha score ranges. Only six of the combinations show slight synergistic pattern.

DISCUSSION

Emerging resistance of bacteria against antibiotics become a prominent problem. Although antimicrobial combination therapy is widely used clinically to reduce treatment time, to interfere antibiotic resistance and broaden the spectrum of pathogens [10], this study shows that the stronger bacterial resistance to individual antibiotics results to stronger antagonistic interactions when drugs are combined and applied to MDR *E. coli* strains. The pairwise antibiotic interaction results were also compared with those previously demonstrated in a susceptible *E. coli* strain [7]. Antibiotic class of protein synthesis inhibitors were chosen for the study. KAN – CHL, CHL – AMK, KAN – AMK drug pairs were found antagonistic as previously shown in some studies conducted with different species [11, 12]. We supported the previous results by using a different clinically isolated

pathogenic MDR Escherichia coli strains. An Interesting observation is that strong MDR strain exhibits more strong antagonistic interactions between CHL and aminoglycosides compared to intermediate MDR isolate as shown in Figures 1-3. Boosting antagonism between drug pairs needs more serious caution when they are to be used in a combinatorial way. Experiments for CHL – CLA, CHL – ERY, ERY – CLA combinations were done and antagonistic interactions were also demonstrated for this three protein synthesis inhibitors. These three pairs which were previously shown as additive, synergistic and synergistic for ERY – CLA, CHL – CLA, CHL – ERY respectively in a non-resistant/susceptible laboratory (*E. coli*) strain, were found antagonistic for the resistant strains in this study (Figs 1-3). Interaction scoring and batch differences might result to this alteration but still this evidence supports our hypothesis that more resistant strains tend to show more antagonistic interactions among drugs. Strong MDR *E. coli* exhibits stronger antagonism for other experimented combinations (AMK - KAN, KAN - CHL, AMK - CHL) as well (Figs 4-5, Table 2). All protein synthesis inhibitor combinations subjected in this study were shown antagonistic and more interestingly, for all combinations, antagonism level within pairs was found increased, demonstrated with more negative Bliss interaction scores (Table 2). This suggests that combination therapies against MDR strains should be carefully evaluated prior to administration to avoid interfering present limited efficacy of antibiotics against MDR pathogens. There are numerous studies showing synergistic and antagonistic interactions of antibiotic combinations in numerous pathogenic species [10, 12-14]. However, evaluation of antibiotic interactions on clinically isolated, especially MDR pathogens are crucial to understand better options of treatment. Protein synthesis inhibitor combinations were not specifically studied to our knowledge. Partially there were studies showed some of the interactions between protein synthesis inhibitors [11, 12] especially in common non-clinical wild type laboratory strains. We showed antagonistic interactions between 6 experimented protein synthesis inhibitor combinations in clinically isolated MDR pathogens. In addition, we evaluated 20 more different protein synthesis inhibitor antibiotic combinations based on the previous study [7]. Accordingly, protein synthesis inhibitor antibiotic combinations tend to show antagonistic/additive interaction patterns even if each individual partner has different targets in a different location on ribosome (Fig. 6).

CONCLUSION

Although limited number of MDR strains were used in this study, to our knowledge, it is the first time that antagonism level is shown to be associated with the level of multi-drug resistance. This suggests that stronger MDR pathogens should be more carefully evaluated when multi-drug regimens are planned to be administered to clinically severe patients for avoiding antagonizing individual drug effectiveness.

Conflict of interest. The authors declare that there is no conflict of interest.

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