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Full Length Research Paper

Investigation of *in vitro* effects of daptomycin, tigecycline and teicoplanin combinations against MRSA, VISA and VRE strains

Ahmet Yilmaz Coban[1]*, Aydin Deveci[2], Ozlem Acicbe[2], Nuriye Tasdelen Fisgin[2], Alper Ciftci[3], Basak Dokuzoguz[4] and Belma Durupinar[1]

[1]Ondokuz Mayis University, Medical School, Department of Medical Microbiology, Samsun, Turkey.
[2]Ondokuz Mayis University, Medical School, Department of Clinical Microbiology and Infectious Diseases, Samsun, Turkey.
[3]Ondokuz Mayis University, Faculty of Veterinary Medicine, Samsun, Turkey.
[4]Ankara Numune Education and Research Hospital, the First Infectious Diseases and Clinical Microbiology Clinic, Ankara, Turkey.

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Multidrug resistant (MDR) gram positive infectious agents can cause severe infections and monotherapy of these infections by current available antimicrobial agents can be problematic. Therefore, antimicrobial combination therapy may be needed for effective treatment. The study aimed to investigate *in vitro* effects of binary combinations of tigecycline (TGC), daptomycin (DPC) and teicoplanin (TP) against 4 methicillin resistant *Staphylococcus aureus* (MRSA), 2 vancomycin intermediate *S. aureus* (VISA) and 9 vancomycin resistant enterococci (VRE) species by E test method. Whilst, all binary combination of TGC, DPC and TP showed synergistic effect against standard strain of *S. aureus* and 50% of clinical MRSA isolates, all of the combinations except DPC-TP combination showed additive effect against VISA strains (Mu 3 and Mu 50). TGC-TP, DPC-TP and TGC-DPC combinations showed additive effect against 25, 60 and 50% of clinical VRE isolates, respectively. Synergistic effect was observed in combinations of TGC-TP and DPC-TGC against 2 and 1 VRE clinical isolates, respectively. Combinations of TP with any of TGC and DPC brought MIC values of TP below the level of sensitivity in all of TP resistant VRE and VISA isolates. Antagonistic effect was not observed in combinations of tested drugs against any strains. In conclusion, *in vitro* effectiveness of these drug combinations may reflect advantages in clinical practice for severe MDR gram positive bacterial infections.

Key words: Multidrug resistant, antimicrobial agents, methicillin resistant *Staphylococcus aureus*, infectious agents, vancomycin resistant enterococci.

INTRODUCTION

Incidence of infections caused by methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin intermediate *S. aureus* (VISA) and vancomycin resistant *Enterococcus* (VRE) species has been increasing by time (Arias and Murray, 2009; Rice, 2006). Methicillin resistance rate among *S. aureus* has been increasing in hospital and community acquired infections following first MRSA infection report from United Kingdom in 1961 (Nordmann et al., 2007). MRSA strains have been reported to account for 30 to 62% of nosocomial *S. aureus* bloodstream infections and 42 to 60% of *S. aureus* surgical-site infections in United States (Boyce et al., 2005). As a consequence of overuse of vancomycin, glycopeptides resistant MRSA and enterococci have

*Corresponding author. E-mail: cobanay2003@yahoo.com.tr. Tel: +90 362 3121919/3526. Fax: +90 362 4576041.

Abbreviations: MDR, Multidrug resistant; **TGC,** tigecycline; **DPC,** daptomycin; **TP,** teicoplanin; **MRSA,** methicillin resistant *Staphylococcus aureus*; **VISA,** vancomycin intermediate *Staphylococcus aureus*; **VRE,** vancomycin resistant enterococci.

been emerged and spread all over the world (Appelbaum, 2006). Main mechanism of methicillin resistance in MRSA is mediated by penicillin binding protein (PBP) 2A which has low affinity for almost all β-lactam drugs and encoded by *mecA* gene (Que and Moreillon, 2010). Following methicillin resistance in *S. aureus*, glycopeptides have been remaining the main therapeutic agents for MRSA infections until VISA strains with reduced vancomycin susceptibility was identified in Japan (Hiramatsu et al., 1997) Intermediate glycopeptide resistance arises from chromosomal mutations responsible for increased synthesis of free uncross-linked D-alanin-D-alanin of bacterial cell wall precursors, are target of glycopeptides, consequence in thickening of cell wall (Appelbaum, 2006). These precursors effectively bind and sequester glycopeptide molecules, thereby inhibiting antibacterial action as a consequence of preventing the reach of their bacterial target. Glycopeptide resistance in enterococci is acquired by gene clusters responsible for chemical alterations in bacterial cell wall structure (Walsh et al., 1996). Expression of these genes results in the synthesis of abnormal peptidoglycan precursor termination instead of D-alanin-D-alanin (Cetinkaya et al., 2000). Thereby, new effective antimicrobials are needed for the treatment of these infections because of their antimicrobial resistance. Tigecycline and daptomycin that have been developed recently, are effective in the treatment of infections related to gram positive bacteria (Entenza and Moreillon, 2009; Tsuji and Rybak, 2006; Steenbergen et al., 2009).

Daptomycin is a natural fermentation product of cyclic lipopeptide produced by *Streptomyces roseosporus* (Yao and Moellering, 2007; Johnson and Decker, 2008). It is highly active against common gram positive pathogens. It is specially preferred in the treatment of infections caused by gram-positive bacteria resistant to methicillin, vancomycin, linezolid, quinupristin/dalfopristin and tigecycline (Steenbergen, 2009). Daptomycin is not recommended for pneumonia because it is inactivated by pulmonary surfactant (Johnson and Decker, 2008). It binds irreversible to the cytoplasmic membrane of susceptible bacteria and causes membrane depolarization (Yao and Moellering, 2007). Loss of membrane potential leads to inhibition of protein, DNA and RNA synthesis resulting in bacterial death without cell lysis (Steenbergen, 2009). It can not penetrate into the outer membrane of gram-negative bacteria. Resistance can be gained against daptomycin by thicken cell wall resulting in physical barrier in enterococci and *S. aureus* like in VISA or by different resistance mechanism (Yao and Moellering, 2007).

Tigecycline is parenteral antibiotic that is a semi-synthetic minocycline derivative. It binds reversibly to 30S subunit of bacterial ribosomes and inhibits protein synthesis by the same mechanism of tetracyclines. It has higher binding affinity and, it is much less affected by efflux pumps and enzymatic modifications interfere with tetracyclines. It has a broad-spectrum antibacterial activity against gram-positive, gram negative and anaerobes although *Proteus, Morganella, Providencia* ve *Pseudomonas aeruginosa* are generally resistant (Yao and Moellering, 2007; Johnson and Decker, 2008; Entenza and Moreillon, 2009; Murray et al., 2009). Teicoplanin is a complex glycopeptide that is chemically related with vancomycin. It is used out of United Sates in several countries in the world. The primary effect of teicoplanin is inhibition of synthesis of the peptidoglycan layer in bacterial cell wall by making a complex with D-alanyl-D-alanin region which is a precursor of cell wall (Yao and Moellering, 2007). Resistance can be developed by two mechanisms; 1) substitution of D-ala-D-ala with D-ala-D-lactate in distal structure of peptidoglycan layer resulting in lower binding affinity of glycopeptides in VRE. 2) Accumulation of peptidoglycan precursors resulting in thickening of bacterial cell wall and diffusion of glycopeptides between layers of the bacterial wall is blocked in VISA (Yao and Moellering, 2007). In the present study, we investigated the combined effects of daptomycin (DPC), tigecycline (TGC) and teicoplanin (TP), act by different mechanism, against MRSA, VISA and VRE strains that have a real threat in their treatment.

MATERIALS AND METHODS

Bacterial isolates

Nine VRE clinical isolate tested in the study were supplied from Ankara Numune Education and Research Hospital. Eight was identified as *Enterococcus faecium* and one was *Enterococcus faecalis* by Vitek2 System (bioMerieux). *E. faecalis* ATCC 29212 was used as a control strain in the study. *S. aureus* isolates were four mecA positive MRSA, Mu3 (ATCC 700698) and Mu50 (ATCC 700695) VISA strain and, *S. aureus* ATCC 43300 (*mec A* positive) was used as a control strain. *Nuc* and *mec A* genes were identified by PCR in MRSA strains (Louie, 2002).

Molecular characterization of vancomycin resistance in enterococci

Bacterial DNA's were extracted by using the boiling method. For this aim, 5 to 10 colonies of each bacterium were suspended in 500 µl distilled water. The suspensions were boiled for 5 min and centrifuged at 9000 rpm for 5 min. The supernatants were taken and used as template DNA in PCR. They kept frozen at -20°C until used (Depardieu, 2004). The determination of vancomycin resistance genotype of enterococci was performed with using *vanA-B-D-E-G* primers (Table) by multiplex PCR (m-PCR). M-PCR was carried out as described by Depardieu et al. (2004). For the reaction, 5 µL of the genomic DNA was added in a 25 µL PCR mixture containing 1 × PCR buffer, 2.5 mmol $MgCl_2$, 50 mM each dNTP, 2 U of Taq polymerase and 50 pmol of each of vanA/B/D/E/G oligonucleotide primers. The amplification thermal cycler parameters were as follows: 3 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C, with 7 min at 72°C for the final extension. DNA fragments were analyzed by electrophoresis on a 1.0% agarose gel. The gel was visualized under ultraviolet (UV) light and evaluated for bands of 732-bp (vanA), 647-bp (vanB), 820-bp (vanC) 500-bp (vanD), 430-bp (vanE) and 941-bp (vanG) (Depardieu, 2004). All the Enterococcus

Table 1. Effects of TGC and TP combination against MRSA, VISA and VRE isolates.

Strains	MIC (µg/ml)				FIC index	Outcome
	TGC	TP	TP+	TGC+		
S. aureus ATCC 43300	0.125	2	<0.016	0.016	0.008	Synergy
Mu 3 ATCC 700698	0.5	8	0.5	0.25	0.562	Additive
Mu 50 ATCC 700695	0.25	8	0.5	0.19	0.822	Additive
MRSA-3	0.125	4	<0.016	0.094	0.756	Additive
MRSA-11	0.38	1.5	0.064	0.19	0.542	Additive
MRSA-25	0.5	2	<0.016	0.19	0.388	Synergy
MRSA-37	0.38	4	0.047	0.094	0.258	Synergy
E. faecalis ATCC 29212	0.094	2	<0.016	0.016	0.178	Synergy
VRE-1	0.125	>256	0.125	0.19	1.520	Indifference
VRE-5	0.25	>256	<0.016	0.064	0.256	Synergy
VRE-6	0.125	>256	0.016	0.125	1.000	Indifference
VRE-7	0.125	>256	<0.016	0.094	0.752	Additive
VRE-8	0.064	>256	<0.016	0.064	1.000	Indifference
VRE-9	0.19	>256	<0.016	0.25	1.315	Indifference
VRE-11	0.125	>256	0.064	0.064	0.512	Additive
VRE-12	0.25	>256	0.064	0.047	0.188	Synergy

TP+: MIC of TP in the presence of TGC, TGC+: MIC of TGC in the presence of TP.

strains tested for vancomycin resistance genotype were given as 732 bp band and characterized as vanA genotype.

Determination of DPC, TGC and TP MIC's and effects of combination by E test

DPC, TGC and TP E test strips were supplied from AB BIODISK (Solna, Sweden). MIC's were determined according to manufacturer recommendations. Briefly, bacterial suspension was prepared from fresh over night incubation, with 0.5 McFarland turbidity inoculated onto the surface of Mueller Hinton agar plates. Following placement of E test strips on the agar plates, they were incubated at 35°C for 24 h. The MIC of tested drug was determined by the point where the elliptical zone of growth inhibition intersected the MIC scale on the E test strip after incubation time (AB BIODISK, 2007).

Binary combinations of TP, DPC and TGC were evaluated for determining of combination effects. Combination test was performed according to manufacturer recommendations. Briefly, bacterial suspension was prepared from fresh overnight incubation with 0.5 McFarland turbidity was swabbed on the surface of Mueller Hinton agar plate. E test strip of one drug of combination were placed on the plate and kept at the room temperature for 1 h. Following E test strip was replaced with the other drug of combination on the same place of first one; plates were incubated at 35°C for 24 h. It was paid attention that antibiotics gradients were similar. After 24 h incubation, elliptical inhibition growth zone intersection with E test strip recorded as MIC of second drug in the presence of first drug (AB BIODISK, 2007). Fractional inhibitory concentration (FIC) index was calculated by using MIC values of drugs alone and in combination as fallows:

FIC index = $MIC_{AB}/MIC_A+MIC_{BA}/MIC_B$.

In formula MIC_A; MIC value of drug A alone, MIC_B; MIC value of drug B alone, MIC_{AB}; MIC value of drug A in the presence of drug B, MIC_{BA}; MIC value of drug B in the presence of drug A. Calculated FIC index ≤ 0.5 is interpreted as synergy, a FIC index > 0.5 and ≤ 1.0 as additive effect, > 1 and ≤ 4.0 as indifference and > 4.0 as antagonistic effects (AB BIODISK, 2007).

RESULTS

It was determined that all of tested 4 MRSA isolates had *mec A*. It was also confirmed that these isolates were *S. aureus* by determination of *nuc* gene by PCR. Vancomycin resistance characterization of 9 VRE (1 *E. faecalis* and 8 *E. faecium*), were tested in the study, showed that all of them had vanA type resistance by PCR. Combination effects and MIC values of DPC, TGC and TP are shown in Table 1 to 3. TGC and TP combination showed synergistic effect against *S. aureus* ATCC 43300 and 2 MRSA clinical isolates. Additive effect was observed against MU3, Mu50 and 2 MRSA clinical isolates. Whilst TGC and TP combination showed synergistic effect against *E. faecalis* ATCC 29212 standard strain and 2 clinical VRE isolates, it showed indifference effect against 4 VRE isolates and additive effect against 2 VRE isolates (Table 1).

Whilst, it was observed synergistic effect with DPC and TP combination against *S. aureus* ATCC 43300 and 2 MRSA isolates, it was observed that additive effect against Mu50 and 1 clinical MRSA isolate and, indifference effect against Mu3 strain and 1 clinical MRSA isolate. Whilst, it was determined that there was synergistic effect against *E. faecalis* ATCC 29212 standard strain and 1 clinical VRE isolate, there was additive effect in 5 VRE isolates and indifference effect in 2 VRE isolates (Table 2). It was observed that synergistic effect against *S. aureus* ATCC 43300 and 2 MRSA

Table 2. Effects of DPC and TP combination against MRSA, VISA and VRE isolates.

Strains	MIC (µg/ml)				FIC index	Outcome
	TP	DPC	TP+	DPC+		
S. aureus ATCC 43300	2	0.5	0.064	0.016	0.064	Synergy
Mu 3 ATCC 700698	8	0.25	0.25	0.25	1.031	Indifference
Mu 50 ATCC 700695	8	1	0.5	0.5	0.562	Additive
IST-MRSA-3	4	0.125	0.016	0.25	2.004	Indifference
IST-MRSA-11	1.5	0.5	0.064	0.064	0.170	Synergy
IST-MRSA-25	2	0.125	0.032	0.094	0.768	Additive
IST-MRSA-37	4	2	0.032	0.125	0.070	Synergy
E. faecalis ATCC 29212	2	1	0.125	<0.016	0.076	Synergy
VRE-1	>256	0.75	0.5	2	2.601	Indifference
VRE-5	>256	2	2	1.5	0.757	Additive
VRE-6	>256	1.5	1.5	1.5	1.005	Indifference
VRE-7	>256	3	1.5	2	0.671	Additive
VRE-8	>256	2	4	1.5	0.765	Additive
VRE-9	>256	2	1.5	1	0.505	Additive
VRE-11	>256	1.5	0.064	1	0.666	Additive
VRE-12	>256	2	0.125	0.125	0.062	Synergy

TP+: MIC of TP in the presence of DPC, DPC+: MIC of DPC in the presence of TP.

Table 3. Effects of DPC and TGC combination against MRSA, VISA and VRE isolates.

Strains	MIC (µg/ml)				FIC index	Outcome
	TGC	DPC	DPC+	TGC+		
S. aureus ATCC 43300	0.125	0.5	<0.016	0.016	0.160	Synergy
Mu 3 ATCC 700698	0.5	0.25	0.125	0.19	0.880	Additive
Mu 50 ATCC 700695	0.25	1	<0.016	0.125	0.516	Additive
IST-MRSA-3	0.125	0.125	0.094	0.047	1.128	Indifference
IST-MRSA-11	0.38	0.5	0.064	0.094	0.375	Synergy
IST-MRSA-25	0.5	0.125	<0.016	0.25	0.628	Additive
IST-MRSA-37	0.38	2	0.094	0.064	0.215	Synergy
E. faecalis ATCC 29212	0.094	1	<0.016	0.094	1.016	Indifference
VRE-1	0.125	0.75	0.094	0.094	0.877	Additive
VRE-5	0.25	2	0.25	0.125	0.625	Additive
VRE-6	0.125	1.5	0.19	0.125	1.126	Indifference
VRE-7	0.125	3	0.19	0.125	1.063	Indifference
VRE-8	0.064	2	0.125	0.064	1.062	Indifference
VRE-9	0.19	2	0.047	0.125	0.680	Additive
VRE-11	0.125	1.5	0.125	0.125	1.083	Indifference
VRE-12	0.25	2	0.125	0.19	0.812	Additive

DPC+: MIC of DPC in the presence of TGC, TGC+: MIC of TGC in the presence of DPC.

strains with DPC and TGC combination. Beside that, there was additive effect against 2 VISA and 1 MRSA isolates and indifference effect against 1 MRSA isolate. Whilst, it was not determined synergy against VRE isolates, it was determined additive effect against 4 VRE isolates and indifference against *E. faecalis* ATCC 29212 standard strain and 4 VRE isolates (Table 3). Finally, it was not determined an antagonistic effect with binary combination of 3 tested antibiotics.

DISCUSSION

Antimicrobial drug combinations are frequently used empirically for broad antimicrobial spectrum in clinical settings until the causative pathogen is identified and

antimicrobial susceptibility tests are completed. However, *in vitro* synergy tests are rarely performed in clinical practices. Therefore, it has an important aspect for recognizing the effects of antibiotic combinations before usage of empiric antibiotic combinations. If there would be any identified antagonism between antibiotics, it would not be convenient for using these antibiotics in combinations (Petersen et al., 2006). Some antimicrobials in combination can antagonize each other by induction of genes responsible for synthesis of enzymes, degrade antimicrobials in combination. For example, cefoxitin antagonizes other β-lactam drugs by induction of β-lactamases (Sanders et al., 1982). Macrolides and lincosamide also create antagonism in *S. aureus*, has an inducible mechanism of resistance to the macrolides (Acar, 2000). Bacteriostatic antimicrobials such as tetracycline, chloramphenicol and erythromycin antagonize bactericidal effect of the β-lactams (Johansen et al., 2000). Studies had showed that TGC was effective *in vitro* against gram positive, gram negative and many anaerobic bacteria (Petersen et al., 2006). However, it is also observed that there is an increase in issue of its combinations with other antibiotics in literature. Effects of TGC alone and in combination with DPC and TP were investigated by E test method against MRSA, VISA and VRE that are cause of nosocomial severe infections in this study.

Petersen et al. (2006) tested TGC and vancomycin (VAN) against 11 *E. faecalis* isolates and, they had found indifference effect with this combination. Beside that, they had defined synergy with TGC and VAN combination against 40% of 10 *E. faecium* isolates. It had been reported that synergy between TGC and VAN against 10% of 10 *S. aureus* isolates in the same study. Vouillamoz et al. (2008) had defined synergy between TP and TGC against 17% of 6 *E. faecalis*, whilst, they defined indifference effect against all isolates with VAN combination. They had observed indifference effect against 5 *E. faecium* isolates with TGC and VAN combination. In addition, they had defined indifference effect against all of 6 *S. aureus* isolates by combination of TGC and any of TP and VAN. Whilst, Mercier et al. (2002) had determined synergy between TGC and VAN against 2 *E. faecium* isolates; they had determined indifference effect against 4 *S. aureus* isolates.

In their study, it was determined that TGC and TP combination had synergistic effect against *E. faecalis* ATCC 29212 standard strain and 2 clinical VRE isolates, an additive effect against 2 VRE isolates and indifference against 4 VRE isolates. Whilst synergistic effect was observed against *S. aureus* ATCC 43300 and 2 MRSA clinical isolates, additive effect was observed against Mu3, Mu50 and 2 MRSA clinical isolates. Jenkins (2007) reported a case of endocarditis due to linozolid and VAN resistant *E. faecium* treated successfully with a TGC and DPC combination for 70 days. The issue is that, it was not seen *in vitro* in antagonism between both antimicrobial agents in our study, but it shows that they can be used in combination in clinical practice. It had been determined that DPC and VAN combination had shown indifference/additive effect against 1 hGISA and 1 GISA isolates (Tsuji and Rybak 2006). DPC and TP combination effects were investigated against VISA (Mu3 and Mu50) in our study and additive/indifference effect was determined. Debbia et al. (1988) had determined that combination of LY146032 (DPC) with any of VAN and TP had shown mostly, indifference effect against 35 *Staphylococcus* spp. and 15 *Enterococcus* spp. isolates in their study. In our study, MIC value of TP in the presence of TGC and DPC fell down below the level of sensitivity in all TP resistant VRE isolates and VISA isolates. Furthermore, antagonistic effect was not seen between investigated antibiotic combinations in the study. However, combination of these antibiotics can be an effective treatment option in life-threatening infections due to MDR gram positive pathogens.

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