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Comparative Time-Kill Study of Doxycycline, Tigecycline, Cefazolin and Vancomycin Against Several Clones of *Staphylococcus Aureus*

Melina Herrera[1], Liliana Mobilia[1,2], Graciela Posse[1], Adriana Limansky[3], Viviana Ballerini[3] and Carlos Bantar[1,2,*]

[1]*Universidad Adventista del Plata, Villa Libertador San Martín, Entre Ríos,* [2]*Laboratorio Domingo I Nanni, Paraná, Entre Ríos,* [3]*IBR (CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Santa Fe. Argentina*

Abstract: *Background*: We present herein, a comparative study assessing the bactericidal kinetics of tigecycline, doxycycline, cefazolin and vancomycin against several methicllin-susceptible (MSSA) and –resistant (MRSA) *Staphylococcus aureus* isolates recovered from patients of 24 different cities in Argentina.

Methods: After genotypic characterization, 20 strains (10 MRSA and 10 MSSA) were selected for time-kill studies.

Results: Vancomycin showed bactericidal effect (i.e., $\geq$ 3-$\log_{10}$ CFU/mL decrease) against 50% and 10% of the MRSA strains at 4 x Minimal Inhibitory Concentration (MIC) and 2xMIC, respectively, after 24 h of incubation and displayed bactericidal activity against all MSSA isolates at 4xMIC. Cefazolin was bactericidal against 30% of MSSA strains at the higher concentration (4xMIC) and against 10% at 2 x MIC and MIC dose concentrations. The bactericidal magnitude of cefazolin observed after 24 h of incubation was lower than the vancomycin one. Albeit bacteriostactic, tigecycline at 2xMIC exerted a -1 to2-log decrease in the viable cell counts after 24-h incubation against 19 of the 20 *S. aureus* strains. Doxycycline was the least inhibitory of the antibiotics tested against both MRSA and MSSA, displaying no bactericidal activity in any of the cases and showing regrowth after 24 h of incubation at MIC level.

Conclusion: Vancomycin at high concentrations showed the best activity. Cefazolin did not show the activity expected for a beta-lactam antibiotic against MSSA. Tigecycline may be a useful option in infections caused by MRSA, where bactericidal activity is not an exclusive requirement and doxycycline does not seem an attractive alternative in serious infections.

Keywords: Cefazolin, doxycycline, *S.aureus,* tigecycline, vancomycin.

INTRODUCTION

Staphylococcus aureus is one of the pathogens more frequently involved in both nosocomial and community infections. Methicillin-resistant *S. aureus* (MRSA) has been increasing its resistance profile over recent years and this represents a growing concern in public health worldwide. Vancomycin has been considered for over 50 years the choice for treatment of MRSA infections. However, an increasing number of reports describing MRSA isolates with reduced susceptibility, or even resistant, to vancomycin have been published in the last years [1-3]. Hence, the need for new therapeutic options tailored toward MRSA infections becomes evident [2].

Tigecycline is the first in a new family of antimicrobial, the glycylcyclines, a 9-t-butylglycylamido derivative of minocycline [4]. It has shown a broad antibacterial spectrum against gram-positive, gram-negative, aerobic, anerobic, and atypical bacteria, including several multiresistant organisms, such as MRSA, vancomycin-resistant enterococci and staphylococci and *Enterobacteriaceae* producing extended spectrum β-lactamases [5,6]. It lacks activity against *Pseudomonas aeruginosa; Proteus, Morganella , Serratia, Providencia and Legionella species* [7].

Tigecycline is currently approved for treatment of complicated skin and soft tissue infections, complicated intra-abdominal infections [8-10] and community-acquired pneumonia [25]. It has been also tested in clinical trials for the treatment of multidrug resistant pathogens, diabetic foot and osteomyelitis [11-13]. However, pharmacodynamic data against *S. aureus* are still scarce [14]. We present herein, a comparative study assessing the bactericidal kinetics of tigecycline, doxycycline, cefazolin and vancomycin against several methicillin-susceptible and -resistant *S. aureus* isolates recovered from patients of 24 different cities in Argentina. Tigecycline was selected as a newest option active against MRSA, doxycycline as an old drug active against either MRSA or methicillin-susceptible *S. aureus* [MSSA], cefazolin as one of the primary options against MSSA and vancomycin as the primary option against MRSA.

*Address correspondence to this author at the Hospital San Martín, Perón 450, (3100) Paraná, Entre Ríos, Argentina; Tel: 54-343-4310783; Fax: 54-343-4310783; E-mails: cbantar@arnet.com.ar, cbantar@labnanni.com.ar

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MATERIALS AND METHODS

Organisms

Forty *S. aureus* isolates were collected between 2003 and 2007 from clinical specimens belonging to patients from 24 cities in Argentina. The sources of the isolates were as follows: respiratory secretions, surgical and trauma wounds, skin and soft tissues, blood, puncture fluid, abscess and catheter. Isolates were identified according to the standard procedures following the schedule proposed in Manual of Clinical Microbiology [15]. They were subjected to genotypic characterization and susceptibility testing and finally, 20 isolates (10 MRSA and 10 MSSA) were selected for the study. No VISA isolates were included, as this kind of resistance has not emerged in Argentina. The organisms were stored as frozen suspensions (-70ºC) in tryptic soy broth containing 20% glycerol until use.

Clonal Relationship Analysis

Bacterial DNA was purified from cells treated with lysozyme/proteinase K. Then polymerase chain reaction (PCR) assay with degenerate oligonucleotide primers (DO-PCR) [16] was performed by using primer 19, as previously described [17, 18]. *S. aureus* isolates were considered different when their amplification profiles differed in more than 2 bands. Differences in the band intensities at a same position between two isolates were not considered as significant.

Agar Dilution Test

It was performed following the Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards [NCCLS]) recommendations [16]. Laboratory standard antibiotic powders of tigecycline (Wyeth S.A., Buenos Aires, Argentina), doxycycline (Chemo S.A., Lugano, Switzerland), vancomycin (Laboratorios Northia., Buenos Aires, Argentina), and cefazolin (Laboratorios Northia, Buenos Aires, Argentina) were obtained and reconstituted according to the respective manufacturer's instructions. Stock solutions and media were prepared on the day of the tests; serial 2-fold dilutions were performed and added to molten Mueller-Hinton agar (Laboratorios Britania, Buenos Aires, Argentina) warmed at 45ºC. The organisms were incubated overnight on trypticase soy agar (Laboratorios Britania, Buenos Aires, Argentina) at 35°C. Inocula were prepared by suspending a cell paste in 0.9% NaCl saline solution to equal the turbidity of a 0.5 McFarland standard. A 1:10 dilution was prepared before inoculating into a Steers replicating device. The organisms were applied to the plates at a final concentration of 1×10^4 to 5×10^4 CFU/1 µl-spot. Plates without antibiotics were inoculated before and after each set of drug-containing plates as growth controls. After overnight incubation at 35°C, the MIC (minimal inhibitory concentration) was interpreted. *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 were included as controls.

Time-kill Studies

They were carried out in duplicate as previously described [19]. In case of differences <0.5 $\log_{10}$ CFU/ml between duplicates, the first test was taken into account. Otherwise, a third test was performed. The drugs and media were prepared on the day of the experiment at four different concentrations as follows: tigecycline, doxycycline, cefazolin and vancomycin; 4 x MIC, 2 x MIC, 1 x MIC and 1/2 x MIC. Briefly, tubes containing Mueller-Hinton broth (Laboratorios Britania) with and without (growth control) antibiotic were seeded with a log-phase inoculum of roughly 5×10^5 CFU/ml to a final volume of 10ml. Inoculated broths were incubated in an ambient atmosphere at 35°C. At 0 and at 2-, 4-, 6-, 8- and 24-h time intervals after inoculation, a 0.1-ml portion was removed from each tube and was subjected to a 10-fold serial dilution. Then, 0.1ml of every dilution was spread onto trypticase soy agar. By using the viable counts determined at each time point, a 24-h time-kill curve was constructed for each isolate. Bactericidal activity was defined as a ≥3-$\log_{10}$ CFU/ml decrease in the cell counts with respect to the original inoculum. Inhibitory activity was defined as <3-$\log_{10}$ CFU/ml decrease in viable cell counts. Any viable cell count higher than the starting inoculum was considered as overgrowth. Carryover of antibiotics was avoided by 10-fold serial dilutions before plating. The lowest limit of detection was 100 CFU/ml and this value was assigned to any count below the limit.

Data Analysis

A time-kill curve was constructed for every pair of isolate-drug with values of viable cell counts ($\log_{10}$ CFU/ml) obtained at the different times of incubation (i.e., 0, 2, 4, 6, 8

Table 1. MIC Inhibiting 50% and 90% of the Strains (MIC50 and MIC90, Respectively) and MIC range of four Antibiotics Against 20 Isolates of Methicillin-resistant (MRSA) and 20 -Susceptible *Staphylococcus aureus* (MSSA)

Antibiotic	Values (µg/ml) Corresponding to:					
	MRSA			MSSA		
	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range
Tigecycline	0.25	0.25	0.125-0.25	0.25	0.25	0.25-0.25
Doxycycline	0.06	0.25	0.06-4	0.06	0.25	0.06-1
Vancomycin	1	1	0.5-1	1	1	0.5-1
Cefazolin	NA	NA	NA	0.5	1	0.25-4

NA: not applicable

and 24 h) and an average time-kill curve was constructed with MSSA and MRSA for every drug.

RESULTS

MIC and Clonal Analysis

Values (μg/ml) of MIC_{50}, MIC_{90} (MIC inhibiting 50% and 90% of the isolates, respectively) and range for tigecycline, doxycycline, cefazolin and vancomycin, obtained against the 20 MSSA and 20 MRSA isolates are given in Table **1**. Respective MIC values (μg/ml) for *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 were as follows: tigecycline, 0.25 and 0.125; doxycycline, 0.125 and 0.25; cefazolin, 0.5 and 0.5; vancomycin, 0.5 and >256. Results of DO-PCR showed 9 different clones of MRSA: A (3 isolates), B (3 isolates), C (1 isolate), D (3 isolates), E (1 isolate), F (1 isolate), G (1 isolate), H (6 isolates) and I (1 isolate); whereas 8 different clones were observed among the MSSA isolates: A (3 isolates), B (1 isolate), C (1 isolate), D (4 isolates), E (1 isolate), F (5 isolates), G (3 isolates), H (2 isolates). Amplification profiles are given in Fig. (**1**). No relationship between clone type and susceptibility pattern was observed.

Time-kill Studies

Ten strains of MRSA and ten of MSSA representing 9 and 8 distinctive clones or different susceptibility patterns within the same clone were selected for time-kill studies. The extent of bacterial killing was estimated by the number of these strains showing a decrease that ranged from 1 to 3 log_{10} CFU/ml in viable cell counts at the different times after incubation. Data are given in Tables **2** and **3**. For all antibiotics tested against both MRSA and MSSA, it was observed that, although the bactericidal velocity was not concentration dependent, the kill extent was so after 24 h of incubation. No bactericidal activity (i.e., ≥ 3-log_{10} CFU/mL

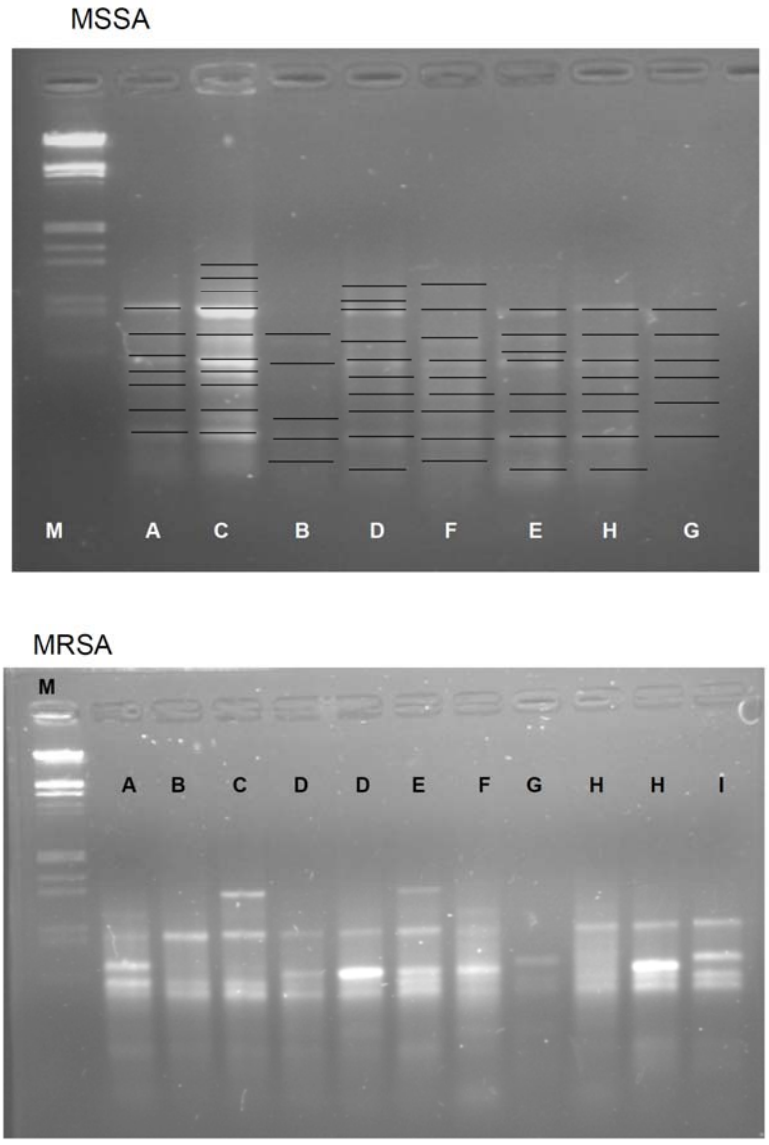

Fig. (1). Amplification profiles by polymerase chain reaction (PCR) assay with degenerate oligonucleotide primers for methicillin-susceptible (MSSA) and –resistant (MRSA) *Staphylococcus aureus* isolates.

Table 2. Extent of Bacterial Killing Exerted by Several Antibiotics Over Time Against 10 selected Methicillin-resistant *Staphylococcus aureus* Isolates

Drug and Concentration (µg/ml)	Nº of Isolates Showing the Following Log_{10} CFU/ml Decrease at the Designated Incubation Time[a]:														
	2h			4h			6h			8h			24h		
	-1	-2	-3	-1	-2	-3	-1	-2	-3	-1	-2	-3	-1	-2	-3
Tigecycline															
4 MIC	1	0	0	3	0	0	6	0	0	7	0	0	3	7	0
2 MIC	0	0	0	2	0	0	5	0	0	3	0	0	7	3	0
MIC	0	0	0	2	0	0	4	0	0	3	0	0	5	2	0
½ MIC	1	0	0	1	0	0	0	0	0	1	0	0	5	0	0
Vancomycin															
4 MIC	0	0	0	2	0	0	7	0	0	9	0	0	1	3	5
2 MIC	0	0	0	1	0	0	6	0	0	7	0	0	2	5	1
MIC	0	0	0	0	0	0	4	0	0	6	0	0	3	3	0
½ MIC	0	0	0	0	0	0	2	0	0	3	0	0	1	0	0
Doxycycline															
4 MIC	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0
2 MIC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MIC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
½ MIC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a]-1, -2 and -3-log CFU/ml decrease represent 90%, 99% and 99.9% of killing, respectively.

Table 3. Extent of bacterial killing exerted by several antibiotics over time against 10 selected Methicillin-susceptible *Staphylococcus aureus* isolates.

Drug and Concentration (µg/ml)	Nº of Isolates Showing the Following Log_{10} CFU/ml Decrease at the Designated Incubation Time[a]:														
	2h			4h			6h			8h			24h		
	-1	-2	-3	-1	-2	-3	-1	-2	-3	-1	-2	-3	-1	-2	-3
Tigecycline															
4 MIC	0	0	0	0	0	0	7	0	0	8	0	0	5	4	1
2 MIC	0	0	0	0	0	0	4	0	0	7	0	0	8	1	0
MIC	0	0	0	0	0	0	4	0	0	4	0	0	7	0	0
½ MIC	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0
Vancomycin															
4 MIC	1	0	0	5	0	0	7	1	0	9	1	0	0	0	10
2 MIC	1	0	0	2	0	0	5	1	0	8	1	0	1	4	5
MIC	1	0	0	1	0	0	5	0	0	7	1	0	2	4	2
½ MIC	0	0	0	0	0	0	3	0	0	4	0	0	0	0	0
Doxycycline															
4 MIC	0	0	0	0	0	0	0	0	0	2	0	0	3	0	0
2 MIC	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
MIC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
½ MIC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cefazolin															
4 MIC	0	0	0	2	0	0	3	1	0	8	0	1	1	2	3
2 MIC	0	0	0	1	0	0	3	1	0	6	0	1	2	2	1
MIC	0	0	0	0	0	0	2	0	0	4	1	0	1	0	1
½ MIC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1

[a]-1, -2 and -3-log CFU/ml decrease represent 90%, 99% and 99.9% of killing, respectively.

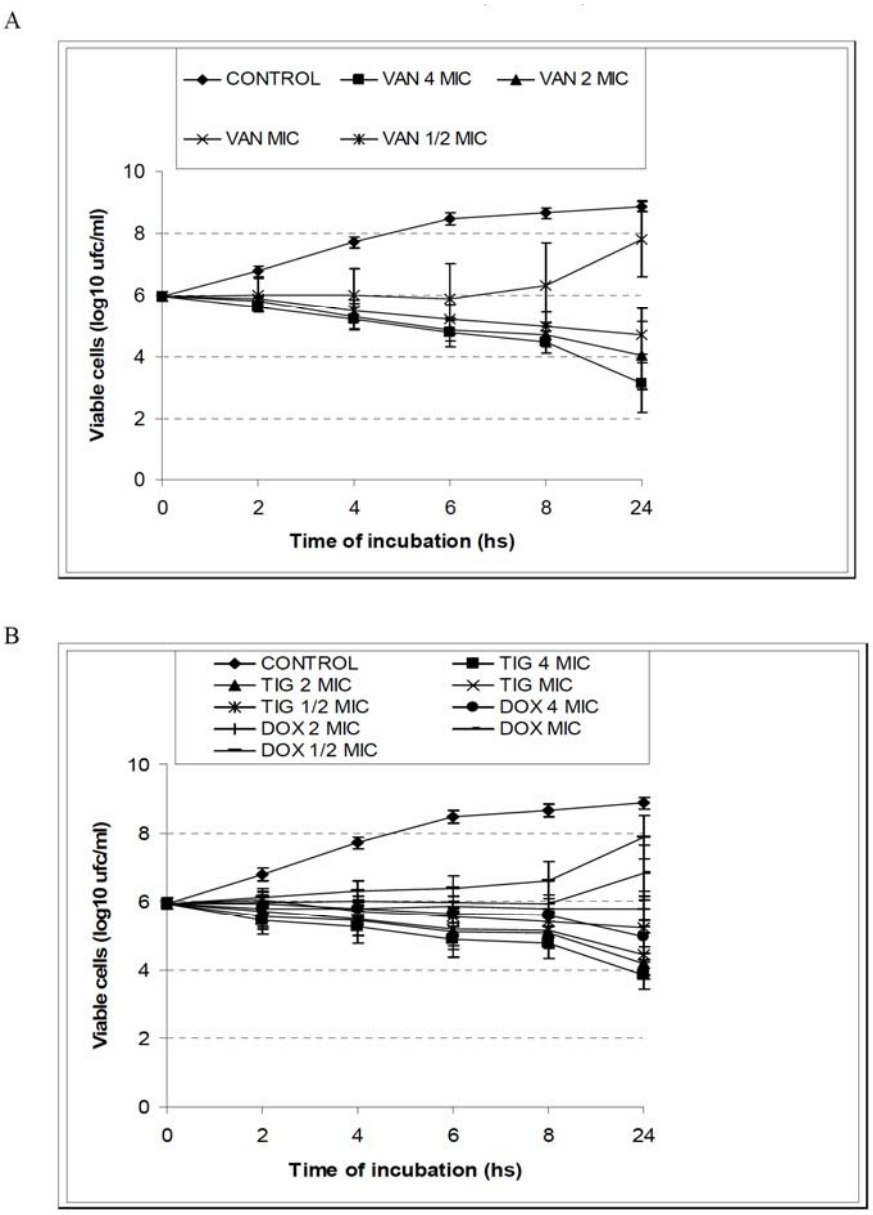

Fig. (2). Average time-kill curve of vancomycin (VAN) (**A**) and tigecycline (TIG), doxycycline (DOX) (**B**) against 10 isolates of Methicillin-Resistant *Staphylococcus aureus.* Data are means ± standard deviation (error bars).

decrease) was observed with any of the tigecycline concentrations against MRSA strains. Such a bactericidal activity was displayed against 1 isolate of the 10 MSSA strains with the higher concentration (i.e., 4 x MIC), preventing regrowth at 1/2xMIC. Vancomycin showed bactericidal effect (i.e., $\geq$ 3-$\log_{10}$ CFU/mL decrease) against 5and 1of the 10 MRSA strains at 4xMIC and 2xMIC, respectively, after 24 h of incubation. Bactericidal activity was displayed against all MSSA strains at 4xMIC and 2 ones with the MIC concentration, while significant regrowth occurred at 1/2xMIC after 24 h of incubation. Doxycycline was the least inhibitory of the antibiotics tested against both MRSA and MSSA, displaying no bactericidal activity in any of the cases and showing regrowth after 24 h of incubation at MIC concentration. Cefazolin was bactericidal against 3 of 10 MSSA strains at the higher concentration (4xMIC) and against 1strainat 2 x MIC and MIC concentrations. The bactericidal magnitude observed after 24 h of incubation was lower than the vancomycin one.

For illustrative purposes, a time-kill curve was constructed for every drug with the average of the 10 MRSA and the 10 MSSA strains. Results are displayed in Figs. (**2** and **3**). Among MSSA, the bactericidal magnitude of cefazolin was lower than the vancomycin one. Albeit bacteriostatic, tigecycline at 2xMIC exerted a decrease in the viable cell counts after 24-h incubation against 19 of the 20 *S. aureus* strains. Doxycycline was the least inhibitory of the antibiotics tested against both MRSA and MSSA, displaying no bactericidal activity in any of the cases and showing regrowth after 24 h of incubation at MIC level.

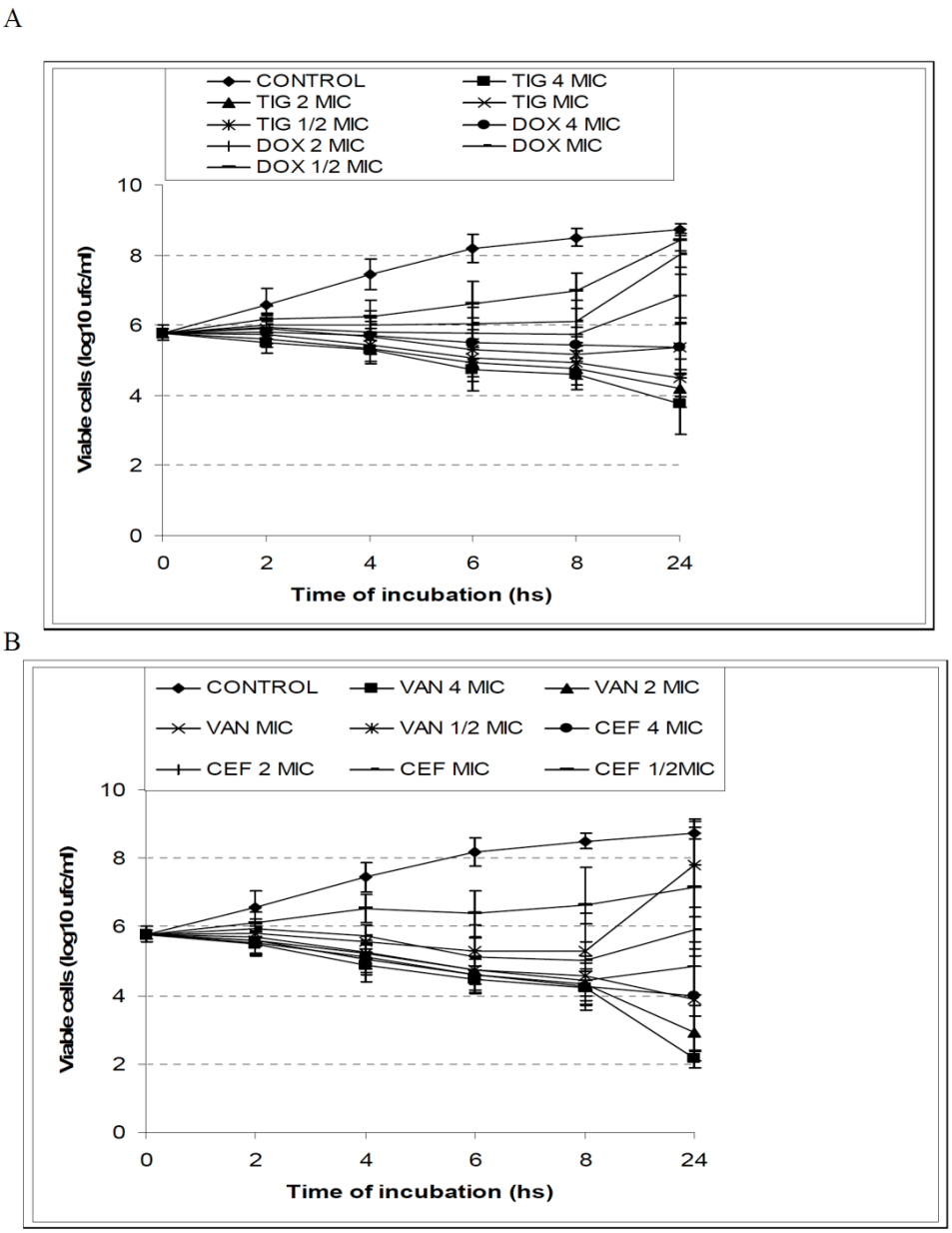

Fig. (3). Average time-kill curve of tigecycline (TIG), doxycycline (DOX) (**A**) and cefazolin (CEF), vancomycin (VAN) (**B**) against 10 isolates of Methicillin-Susceptible *Staphylococcus aureus*. Data are means ± standard deviation (error bars).

DISCUSSION

S. aureus is one of major pathogens causing infections such as bacteremia, lower respiratory tract infections and skin and soft-tissue infections [20]. Although MRSA causes mainly nosocomial infections, it is currently present in community-acquired infections, becoming a major public health problem worldwide [1,2]. Vancomycin has largely remained as the preferred option to treat MRSA infections. However, the clinical efficacy of this drug has recently been argued [22]. Thus, the assessment of additional options seems to be a rational issue. In our study, vancomycin was bactericidal against 50% of the MRSA strains only at 4xMIC. It should be noted that, in many diseases caused by *S. aureus*, such as skin infections, respiratory tract infections, endocarditis and meningitis, vancomycin penetration concentrations in the respective site of infection may be poor [21,22]. This fact may be responsible, in part, for some of the unexplainable clinical failures attributable to this drug, observed with vancomycin-susceptible MRSA isolates causing pneumonia or endocarditis [22]. On the other hand, vancomycin was bactericidal against 100% of the MSSA strains at 4xMIC and against 50% at 2xMIC. This result is in the agreement with that described by Lin and Appelbaum [26], who described bactericidal activity by vancomycin at 2xMIC after 24-h incubation in 6 of 8 *S. aureus* isolates. By contrast, cefazolin was bactericidal only against 30% of the MSSA strains, even at 4xMIC and against 10% at the other tested concentrations. Our findings are opposite to the evidence yielded by a clinical experience comparing vancomycin against cefazolin in patients undergoing hemodyalisis, in

which higher clinical failure was reported for vancomycin, despite mean serum levels of this drug were acceptable (i.e. 17 μg/mL) [23].

Clinically significant decreased activity of cefazolin against certain MSSA strains (i.e. producing type A ß-lactamase) has been well described [27]. Furthermore, the frequency of such strains among MSSA isolates from several regions, including our country, has been reported to be 19% [28]. Recently, Livorsi *et al* [29] found the bla Z gene in 77% of 185 MSSA isolates causing bacteremia and demonstrated a ≥4-fold increase in the cefazolin MIC from a standard to a high inoculum in 27% of them. Thus, it may be possible that some strains harboring ß-lactamase could have been included in our study, as we neither characterized ß-lactamase, nor asserted the prevalence of blaZ gene types among our isolates, because of it was beyond the aim of the study. Furthermore, we failed to perform time-kill assays with different inoculum size. In any event, we consider that the results suggesting the notion that vancomycin may display higher bactericidal activity than cefazolin against some MSSA strains is noteworthy and should be taken into account in the setting of an unexplainable clinical failure with this first-generation cephalosporin during the treatment of a MSSA infection.

In the hope to find useful alternative options with better tissue and intracellular penetration, we evaluated both an old (doxycycline) and a new drug (tigecycline). Albeit bacteriostactic, tigecycline at 2xMIC exerted a -1 or 2-log decrease in the viable cell counts after 24-h incubation against 19 of the 20 *S. aureus* strains. This result suggests that tigecycline may be an attractive option in cases where bactericidal activity is not required. In fact, a $2xMIC_{90}$ tigecycline concentration (i.e. 0.5 μg/ml) represents a half of the mean serum level of this drug after standard dosage and it is largely overcame in most human tissues [14, 24]. Our experience is in the agreement with that of others authors [26]. In addition, tigecycline was bactericidal against 10% of the MSSA strains and prevented the regrowth at the lowest tested concentration. By contrast, doxycycline proved poor inhibitory effect and unlike the other antibiotics, bacterial regrowth was observed at the MIC level. A subsequent susceptibility testing on the survival cells showed resistance selection. Unfortunately, this is not a surprising finding, as we [30] have previously described this phenomenon for doxycycline in a pharmacodynamic study against *Streptococcus pneumoniae*. Results from the present study with this drug shed doubt on the recommendation of oral doxycycline for the treatment of community-acquired methicillin-resistant *S. aureus* infections given by some authors [31].

In summary, vancomycin at high concentrations showed the best activity. However, such concentrations may not be achievable in certain infection sites. Cefazolin did not show the activity expected for a beta-lactam antibiotic against MSSA. Tigecycline may be a useful option in infections caused by MRSA, where bactericidal activity is not an exclusive requirement and doxycycline does not seem an attractive alternative in serious *S. aureus* infections. Nevertheless, some caution should be exerted in patients with ventilator-associated pneumonia, as concerns regarding increased mortality with this drug have been reported [32].

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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