

EFFECT OF LINEZOLID ALONE AND IN COMBINATION WITH OTHER ANTIBIOTICS, ON METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

By

HODA YEHIA¹, MANAL EL SAID², MAGDA AZMY¹, MOUSHIRA BADAWY¹,
SOHEIR MANSY¹, HAMIDA GOHAR³ AND NADIA MADANY³

Departments of Electron Microscopy¹ and Microbiology², Theodor Bilharz Research Institute, Imbaba, PO Box: 30, Giza, 12411 and Faculty of Medicine, Cairo University³

Abstract

The prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) strains has presented a new challenge in antimicrobial medication. Linezolid is a new drug with potent activity on Gram-positive pathogens such as MRSA. The aim of the study was to investigate the in vitro activity of linezolid alone and in combination with imipenem, vancomycin or rifampicin to determine the most active therapy against MRSA strains. Twenty clinical MRSA strains were isolated from patients admitted to inpatient departments and outpatient clinics of Theodor Bilharz Research Institute. Standard strain MRSA ATCC 43300 was included as a control. The MICs of MRSA strains to linezolid, vancomycin, imipenem and rifampicin were evaluated using E test. Time-kill curve were used to assess the in vitro activity of linezolid (at 8x MIC) alone and in combination with imipenem (at 32x MIC), vancomycin or rifampicin (at 8x MIC). Scanning and transmission electron microscopy were performed to compare bacterial morphological alterations owing to the different combinations. Time-kill studies showed synergistic effect when linezolid combined with imipenem was tested against all the MRSA strains. Linezolid plus vancomycin or rifampicin combinations did not display any synergism or antagonism. Scanning and transmission electron microscopy observations confirmed the interactions observed in time kill experiments. Linezolid in combination with sub-inhibitory concentrations of imipenem can be bactericidal against MRSA strains and appears to be a promising combination for the treatment of MRSA infections. No synergistic activity was seen when the linezolid and vancomycin or rifampicin were combined. Linezolid could prevent the emergence of mutants resistant to rifampicin

Keywords: MRSA- linezolid- time kill- electron microscopy

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most prevalent and notorious examples of an antibiotic-resistant pathogen (DeLeo and Chambers, 2009). MRSA can cause a wide spectrum of diseases, including bacteremia, pneumonia, osteomyelitis and cellulitis, endocarditis, and septic shock and other infectious diseases (Shi *et al*, 2014). The main impact of this microorganism is that MRSA strains were traditionally limited to the hospital environment and have become important pathogens of the community (Cunha, 2005). MRSA are associated with higher rates of treatment failure by the limited availability of antibiotics showing activity *in vivo*. Due to increased resistance to antibiotics there is an imminent need to search for new therapeutic

options (Daniela *et al*, 2014).

Linezolid is one of the few treatment options that are highly active against Gram-positive pathogens (Kishore *et al*, 2014). Linezolid is a synthetic antibiotic possessing a novel mode of action, inhibiting bacterial protein synthesis by binding to the domain V region of 23S rRNA at an early step, and prevents the formation of N-formylmethionyl-tRNA-mRNA-70S ribosomal tertiary complex (Alicem *et al*, 2014). Oral administration results in 100% bio-availability and thus, oral and parental administration of the drug are bioequivalent (Joel *et al*, 2014). However, linezolid resistant strains have been increasingly reported worldwide (Livermore *et al*, 2009; Lincopan *et al*, 2009; Almeida *et al*, 2013). Although the incidence of linezolid resistance among Gram-

positive organisms remains low, the emergence of linezolid resistant strains is still of great concern (Tian *et al*, 2014).

The bactericidal activity is considered important in the treatment of severe infections, so the use of linezolid as monotherapy appears to be problematic and the use of combinations is recommended (Tsiodras *et al*, 2001; Jacqueline *et al*, 2005). Also, linezolid monotherapies were not able to eradicate adhering bacteria (Baldoni *et al*, 2009). Linezolid may be combined with other antimicrobial agents to enhance antibiotic activity, reduce the duration of the process as well as the duration of the administration of drugs, to avoid the emergence of resistance and to obtain a synergy between both antimicrobial agents (Sahuquillo *et al*, 2006).

The present work investigated the in vitro activity of linezolid alone and in combination with imipenem, vancomycin or rifampicin in order to determine the most active therapy against MRSA strains. Time-kill curve experiments were used to assess those activities of antimicrobial combinations in vitro. In addition, scanning and transmission electron microscopy were performed to compare bacterial morphological alterations owing to the different combinations.

Materials and Methods

The current study was conducted on different clinical specimens that were referred to the Microbiology Laboratory at Theodor Bilharz Research Institute during the period from October 2012 through March 2013. Twenty MRSA isolates were recovered during this period; 6 (30%) were isolated from pus, 5 (25%) from urine, 3 (15%) from sputum, 3 (15%) from wound swabs, 2 (10%) from blood and 1 (5%) from pleural fluid. They were 15 (75%) males and 5 (25%) females. Their ages ranged from 32-71 with mean age of $51.6 \pm SD 13.7$ years. Standard strain MRSA ATCC 43300 was included as a control. They were identified to the species level by conventional methods (colony morphology, gram stain characteristics, coagulase reactions). Rapid slide latex agglutina-

tion test (Staphaurex Plus, Murex Diagnostics Ltd) was performed for the simultaneous detection of clumping factor, staphylococcal protein A and group-specific antigens on *S. aureus* cell surface. To confirm fermentation of mannitol, growth of yellow colonies on Mannitol salt agar (Oxoid, Cambridge, UK) surrounded by yellow zones after 24 hours of incubation at 37°C indicated a positive result. *S. aureus* isolates were stored in glycerol broth at -70°C and subcultured onto blood agar 48 hours prior to further study. Detection of *mecA*- Mediated Oxacillin Resistance in *S. aureus* strains: Disc diffusion test was performed on Mueller-Hinton agar (MAST Diagnostics, UK) and Cefoxitin (30-µg) disc was applied. The zone of inhibition was determined after 16-18 hrs of incubation at 33-35°C. Interpretations were according to Clinical Laboratory Standards Institute (CLSI) (2013) criteria: ≤ 21 mm is *mecA* positive and ≥ 22 mm is *mecA* negative. *S. aureus* isolates that were *mecA* positive were reported as oxacillin resistant.

Susceptibility of MRSA strains to antimicrobial agents: MIC of clinical isolates of MRSA to linezolid, vancomycin, imipenem and rifampicin was evaluated and interpreted using E test (AB Bio Disk Solna, Sweden) according to the manufacturer's instructions and CLSI (2013) guidelines: linezolid S ≤ 4 µg/ml and R ≥ 8 µg/ml; vancomycin S ≤ 2 µg/ml, I 4-8 µg/ml, R ≥ 16 µg/ml; imipenem S ≤ 4 µg/ml, I 8 µg/ml, R ≥ 16 µg/ml and rifampicin S ≤ 1 µg/ml, I 2 µg/ml, R ≥ 4 µg/ml.

Time kill curves were performed on the control strain and five MRSA strains representing samples of different origin and rifampicin susceptibility. All stock solutions were prepared in accordance with guidelines provided by the CLSI (2013). For each strain, linezolid was studied at the MIC (at 8x MIC) alone or in combination with vancomycin or rifampicin (at 8x MIC) or imipenem (at 32x MIC). Time-kill curve were done in Mueller-Hinton (MH) broth with an inoculum of 5×10^6 to 1×10^7 CFU/ml in the presence of a single antibiotic or in

combination with other antibiotics. A tube of inoculated MH broth with no antibiotic served as a control. The surviving bacteria were counted after 0, 3, 6, and 24 h of incubation at 37°C by subculturing 50 µl serial dilutions (in 0.9% sodium chloride) onto MH plates (Jacqueline *et al*, 2005). A bactericidal effect was defined as a ≥ 3 -log₁₀ CFU/ml decrease after 24 hours of incubation compared to the size of the initial inoculum. Synergy was defined as a 2 log₁₀ decrease in colony counts, when antibacterial activity of combinations was compared with that of the most active single agent. Indifference was defined as <2 log₁₀ increase in colony count at 24 hours by the combination compared by the most active single agent. Antagonism was defined as ≥ 2 log₁₀ increases in colony count at 24 hours by the combination compared with that by the most active single agent alone (Pankey and Ashcraft, 2005).

Scanning Electron Microscopy (SEM): MRSA isolates (overnight bacterial culture diluted to obtain 10⁷ CFU /ml) were cultured for 24 h in MH broth containing linezolid alone (at 8x MIC) or in combination with vancomycin or rifampicin (at 8x MIC) or Imipenem (at 32x MIC). The isolated bacteria in broth culture were centrifuged at 4,000 rpm for 15 minutes. The pellet was processed (Glauert, 1974). It was first immediately fixed for 2 hours in equal volumes of glutaraldehyde 4% and caccodylate 0.2 M, washed in equal volumes of sacchrose 0.4 M and caccodylate 0.2 M for 2 hours and then post fixed in osmium tetroxide 1% and caccodylate 0.3 M for 1 hour. The samples were then washed with distilled water and finally dehydrated in ascending grades of ethyl alcohol for 5 min each (30%.50%.70%.90%)

then absolute alcohol 100% for 10 min for 3 times. Specimens were examined with scanning electron microscope (Inspect S; FEI, Holland) operated at 10 - 30KV, at Electron Microscopy Unit of Theodor Bilharz Research Insitute (TBRI).

Transmission electron microscopy (TEM): Dehydrated pellet was processed for study. Substitution in a mixture of an epoxy resin and ethyl alcohol in equal volumes was the next step. Impregnation of the specimens in pure resins using Epon A and Epon B in equal volumes making three washes on three successive days. The fixed specimens were embedded in the epoxy resin to which was added an accelerator, DMP 30 (1.7%) in gelatin capsules which were left in an oven at 60°C for two days to polymerize and harden. Hardened material was then removed from the capsules and ultra-thin sections were cut using ultra-microtome. Thin sections were stained with uranyl acetate and lead citrate, and examined with a Philips FEI, EM 208 TEM (Silva and Sousa, 1973; Clement *et al*, 1986).

Statistical analysis: Data were described in terms of frequencies (number of cases) and relative frequencies (percentages). All statistical calculations and graphs were done using computer programs Microsoft Excel 2007 (Microsoft Corporation, NY, U.S.A.).

Results

All MRSA isolates (100%) were sensitive to linezolid and vancomycin while 15 isolate (75%) and 14 isolates (70%) were sensitive to imipenem and rifamipcin respectively. Two MRSA isolates (10%) were intermediate to rifamipcin and one isolate (5%) to imipenem.

Table 1: Antimicrobial activity of antibiotics tested on clinical MRSA strains.

Antibiotic	Number of strains having MIC (µg/ml) equal to:														
	32	16	8	4	2	1	0.5	0.25	0.125	0.064	0.032	0.016	0.008	0.004	0.002
Linezolid	0	0	0	0	9	6	5	0	0	0	0	0	0	0	0
Vancomycin	0	0	0	0	13	5	1	1	0	0	0	0	0	0	0
Imipenem	4	0	1	1	0	0	0	2	4	4	3	0	0	1	0
Rifampicin	3	0	0	1	2	1	3	1	0	0	0	5	0	1	3

Table 2: MICs of the studied MRSA strains to different Antimicrobial Agents

Strain No.	Linezolid	Vancomycin	Imipenem	Rifampicin
MRSA ATCC 43300	2	1	0.064	0.004
1 (Pus)	0.5	1	0.125	0.002
8 (Sputum)	1	2	0.25	32
10 (Urine)	0.5	1	0.004	32
12 (Swab)	2	2	8	0.5
13 (Pleural fluid)	1	2	0.32	32

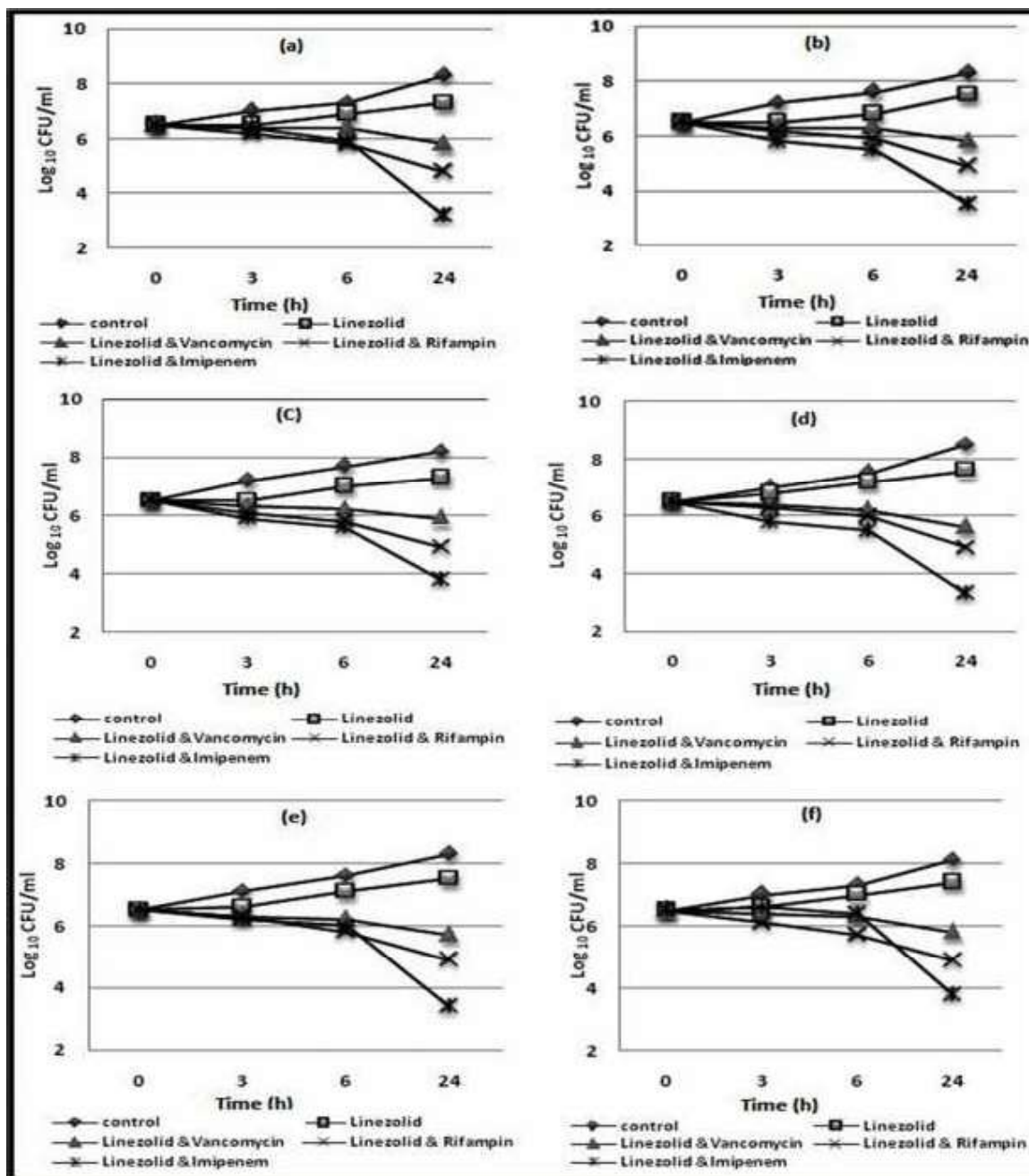


Fig.1: Time kill curve showed antibacterial activity of linezolid alone and in combination with vancomycin or rifampin (at $8 \times$ MIC) and imipenem (at $32 \times$ MIC) against (a) MRSA ATCC 43300 strain (b) strain1, (c) strain 8, (d) strain10, (e) strain 12, (f) strain13

Time kill curve were done on selected five MRSA strains and the MRSA ATCC 43300

control strain. Linezolid (at 8x MIC) combined with imipenem low concentrations (at 32x MIC) showed synergy against all the tested MRSA strains. When the linezolid and vancomycin (at 8x MIC) were combined, they were less active than either agent alone. No synergistic activity was seen in any of the six strains of MRSA and they exhibited indifference activity. The combination linezolid-rifampin (at 8x MIC) did not display any synergism or antagonism against the strains tested. Regrowth of rifampicin-resistant mutants occurred for all strains incubated with rifampicin alone (at 8x MIC). The addition of linezolid prevented the selection of resistant mutants at 24 hrs. MRSA strains with linezolid alone or combined with imipenem, vancomycin or rifampicin were examined by scanning electron microscopy to compare morphological alterations (Fig. 2). SEM of untreated MRSA (Fig. 2a) showed that bacteria were roughly spherical and smooth. Linezolid alone (at 8 × MIC) exhibited moderate alterations in shape and abnormal forms were visible. No cell destruction was observed (Fig. 2b). Linezolid plus vancomycin combination showed abnormal forms without separation of the central septum, and no bacterial lysis was observed (Fig. 2c). Rifampicin in combination with linezolid showed morphological alterations, some showed distortion of cell wall denoting beginning of lysis (Fig. 2d). Linezolid and imipenem combination showed abnormal forms with shape changes (Fig. 2e).

TEM showed rounded bacteria with transverse septa, regular outer membrane and dense core in the control (Fig. 3a). Linezolid alone showed longitudinal forms with relatively faint core and shrunken DNA with condensation of chromatin along the bacterial membrane (Fig. 3-b). Linezolid plus vancomycin showed elongated forms with corrugated walls, faint cores and thick septa with small vacuoles (Fig. 3c). Linezolid and rifampicin showed elongated forms with faint cores in addition to abnormal shapes

with vacuolated changes starting bacterial lysis and rarefaction of DNA content (Fig. 3d). Linezolid and imipenem showed longitudinal forms with corrugated outer membrane and faint cores (Fig. 3e)

Discussion

New anti-staphylococcal drugs as linezolid, which is the first oxazolidinone antibiotic to be approved by FDA, have developed and shown efficacy comparable to that of vancomycin for the treatment of pneumonia and soft tissue infections (Basri *et al*, 2014). Linezolid has unique mechanism of action by inhibiting ribosomal protein synthesis at an early stage of bacterial replication which leads to the absence of cross resistance with other antimicrobials (Rubinstein *et al*, 2010). Although linezolid unsusceptible strains are unusual, long courses of oxazolidinone therapy could select resistant mutants (Wilson *et al*, 2003) hence, the use of a combined strategy might be considered in clinical practice (Ribes *et al*, 2010). Combination therapy, with the goal to enhance the antibacterial activity of known and effective antibiotics, cannot only enhance the activity of known antibiotics but can possibly support the clinical development of agents previously found to be very effective but too toxic for the host. Another advantage is that this approach might lead to shorter and/or lower dosing regimens, which has the potential to reduce the rate of acquirement of resistance in pathogens (Melakea and Zakariab, 2012). Very few studies have reported the effects of combinations of linezolid with other antimicrobial agents.

In the present study, linezolid, a new drug with potent activity on Gram-positive pathogens such as MRSA, was highly effective with 100% susceptibility. The results agreed with Matynia *et al*. (2005) and Kohno *et al*. (2007) to be fully susceptible. Although imipenem did not exhibit bactericidal activity against MRSA strains, many studies reported on its efficacy when used in combination with the other antimicrobial agents (Jacqueline *et al*, 2005). In this study, sub-

inhibitory concentrations (at 32x MIC) of imipenem in combination with linezolid showed the maximal bactericidal activity. These results agreed with Jacqueline *et al.* (2005) who reported that linezolid (at the MIC) combined with low concentrations of imipenem (1/8 to 1/512 the MIC) showed synergy against all the MRSA strains tested. Sweeney and Zurenko (2003) stated that linezolid plus imipenem was synergistic and no antagonism was determined. Jang *et al.* (2009) found that the combined use of linezolid and carbapenem has a synergistic bactericidal effect on *S. aureus* in vitro and in an animal endocarditis model. Matsuda *et al.* (1995) showed that in MRSA strains imipenem preferentially binds to penicillin binding protein 4 (PBP) & PBP 1 and then to PBP 3 & PBP 2. Subinhibitory concentrations of imipenem could interfere only with one PBP suggested that classical PBP binding-mediated activity of imipenem is probably not involved in the synergy between the two antimicrobial agents. The use of higher imipenem concentrations decreased the antibacterial activity of the combination suggesting a slight antagonism between the drugs under these conditions (Jacqueline *et al.*, 2005).

Numerous regimens were tested in the era of multidrug-resistant organisms. It was observed that, an increase in the use of linezolid and vancomycin together, with little evidence to support this practice (Singh *et al.*, 2009). In this study, no synergistic activity was seen when the linezolid and vancomycin were combined. These results agreed with Sweeney and Zurenko (2003), Chiang and Climo (2003) and Sahuquillo *et al.* (2006) who found that linezolid in combination with vancomycin showed indifference against MRSA strains. Mulazimoglu *et al.* (1996), linezolid was tested at one-quarter its MIC with vancomycin and the combination had no synergistic or antagonistic effect. However, Singh *et al.* (2009) stated that three of the five MRSA strains exhibited antagonistic activity when linezolid was added

to vancomycin and the other two strains were equivocal. Although in vitro synergy testing in Chiang and Climo (2003) revealed indifferent activity between the two drugs and in vivo antagonism was demonstrated by using rabbit model. It was hypothesized that this antagonism may be due to a reduced ability on the part of vancomycin to bind to cells exposed to linezolid (Singh *et al.*, 2009). Vancomycin is a cell wall synthesis inhibitor, which means that bacteria must be in growth phase to be subject to its bactericidal activity. Linezolid is a bacteriostatic agent, and its action on the ribosome inhibits bacterial growth. Consequently, the bactericidal activity of vancomycin could be partially inhibited by linezolid in a concentration-dependent manner (Jacqueline *et al.*, 2003). This data indicates that combination therapy is of no benefit and that vancomycin and linezolid should not be used together for MRSA infections. High-level resistant mutant selection limits the use of rifampicin as a single drug and regrowth of rifampicin resistant subpopulations always when this antibiotic was used alone. Thus, the use of antimicrobial combinations to prevent the development of rifampicin resistance during treatment has been investigated (Baldoni *et al.*, 2009). In the present study, combined linezolid-rifampin did not display any synergism or antagonism against MRSA strains tested, addition of linezolid prevented the selection of resistant mutants. These results agreed with Sahuquillo *et al.* (2006) who observed the indifference activity against the strains tested when linezolid combined with rifampicin. In another study (Mulazimoglu *et al.*, 1996), linezolid was tested at one-quarter its MIC with rifampin and the combination had no synergistic or antagonistic effect. Grohs *et al.* (2003) reported that, different combinations using the four or eight times MICs of rifampin and linezolid against five methicillin-sensitive *S. aureus* and five MRSA strains showed no synergistic or antagonistic effect and the presence of linezolid prevented the selection of mutants re-

sistant. Jacqueline *et al.* (2003) found that the combination of linezolid with rifampicin inhibited the regrowth of rifampicin resistant mutants. Moreover, an improvement of the antibacterial activity was observed with the combination compared with the most active single agent (i.e. linezolid). In a study for the detection of the efficacy of linezolid in the eradication of primary and secondary infective foci in experimental endocarditis by MRSA and the significance of the addition of rifampicin, it was found that rifampicin did not enhance the overall efficacy of linezolid either in survival or in the reduction of bacteria in valves. The co-administration of rifampicin and linezolid favored the suppression of bacterial growth in the lung (Tsaganos *et al.*, 2008). Linezolid targets the formation of the initiation complex composed of 30S and 50S ribosome units, mRNA and N-formylmethionyl-tRNA. Rifampicin, by binding to DNA directed RNA polymerase, prevents elongation of the RNA chain and stops bacterial growth (Stratton, 1996). When the two drugs are combined, rifampicin normally acts before linezolid in the ribosome cycle and might prevent linezolid action. Consequently, the antibacterial activity observed in time-kill curves during the first 6 hours was probably the result of rifampicin alone. Owing to the appearance of in vitro resistant variants, the antibacterial activity of the combination over the 6–24 hours period is probably due to the action of linezolid alone. Linezolid takes over from rifampicin on RNA polymerase-mutated bacteria by acting later in the ribosome cycle (Jacqueline *et al.*, 2003).

In this study, parallel scanning and transmission electron microscopy experiments were performed to confirm the interactions observed with time-killing curves. Electron microscopy observations confirmed the interactions observed in time kill experiments. Jacqueline *et al.* (2003) found that vancomycin has an effect on the morphological structure of the cell wall, and inhibits cell division reported. These effects did not oc-

cur with linezolid plus vancomycin combination, and the bacterial cells observed were similar to those with linezolid alone which confirms that linezolid partially inhibits the antibacterial activity of vancomycin.

In the present study, two types of cells were observed when bacteria were treated by rifampicin in combination with linezolid. Some was showing elongated forms with faint cores due to decreased protein synthesis in addition to abnormal shapes and others had undergone bacterial lysis. The elongated deformity with the irregular extended cellular membrane seen by transmission electron microscopy explains and confirms the presence of the abnormal forms seen by scanning electron microscopy. Jacqueline *et al.* (2003) reported that there was no difference in morphological alteration was observed when bacteria were treated by rifampicin alone or in combination with linezolid.

Conclusion

Linezolid proved specific to treat staphylococcal infections mainly those caused by MRSA. Linezolid in combination with sub-inhibitory concentrations of imipenem have bactericidal activity against MRSA strains specially for severe MRSA infections. However, one would not expect a synergistic its effect with vancomycin or rifampin, but its least of linezolid could prevent the emergence of mutants resistant to very efficient antistaphylococcal compounds such as rifampin. EM well correlated with the interactions observed in time-kill experiments. Because of the lack of a correlation between different methods for in vitro assessment of the antibiotic combinations activities, in vivo experimental models are required to confirm the interactions observed in vitro.

References

- Alicem, T, Tuba, D, Özcan, D, Recep, T, Nida, Ö, *et al.*, 2014: In vitro susceptibility to methicillin, vancomycin and linezolid of staphylococci isolated from bloodstream infections in eastern Turkey. *Brazil. J. Microbiol.* 45, 3:829-33.
- Almeida, LM, Araújo, MR, Sacramento, AG, Pavez, M, Souza, AG, 2013: Linezolid resis-

- tance in Brazilian *Staphylococcus hominis* strains is associated with L3 and 23S rRNA ribosomal mutations. *Antimicrob. Agents Chemother.* 57:4082-3.
- Baldoni, D, Haschke, M, Rajacic, Z, Zimmerli, W, Trampuz, A, 2009:** Linezolid alone or combined with rifampin against methicillin-resistant *Staphylococcus aureus* in experimental foreign-body infection. *Antimicrob. Agents Chemother.* 53, 3:1142-8.
- Basri, DF, Xian, LW, Abdul Shukor, NI, Latip, J, 2014:** Bacteriostatic antimicrobial combination: antagonistic interaction between epsilon-viniferin and vancomycin against Methicillin-Resistant *Staphylococcus aureus*. *BioMed Res. Int. ID* 461756-64
- Chiang, FY, Climo, M, 2003:** Efficacy of linezolid alone or in combination with vancomycin for treatment of experimental endocarditis due to methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 47, 9:3002-4.
- Clement, B, Rissel, M, Peyrol, S, Mazurier, Y, Grimand, JA, et al, 1986:** A procedure from light and electron microscopic intracellular immunolocalization of collagen and fibronectin in rat liver. *Journal of Histochemistry and cytochemistry*, 33(5): 407-9.
- Clinical and Laboratory Standards Institute, 2010:** Performance standards for antimicrobial susceptibility testing; 20th informational supplement M100-S20. Wayne, PA.
- Cunha, BA, 2005:** Methicillin-resistance *Staphylococcus aureus*: Clinical manifestations and anti-microbial therapy. *Clin. Microbiol. Infect.* 11, 1:33-42.
- Daniela, E, Alejandra, C, Pedro, R, Claudia, M, et al, 2014:** Antibacterial activity of *Mulinum spinosum* extracts against slime-producing *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* isolated from nasal carriers. *Sci. World J.* 2014: 342-4.
- DeLeo, FR, Chambers, HF, 2009:** Reemergence of antibiotic-resistant *Staphylococcus aureus* in the genomics era. *J. Clin. Invest.* 119: 2464-74.
- Glauert, AM, 1974:** Fixation, dehydration and embedding of biological specimens. In: *Practical Methods in Electron Microscopy*. Glauert AM (ed.) Amsterdam Oxford.
- Grohs, P, Kitzis, MD, Gutmann, L, 2003:** In Vitro bactericidal activities of linezolid in combination with vancomycin, gentamicin, ciprofloxacin, fusidic acid, and rifampin against *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 47, 1:418-20.
- Jacqueline C, Caillon J, Mabecquel V, et al, 2003:** In vitro activity of linezolid alone and in combination with gentamicin, vancomycin and rifampicin against methicillin-resistant *Staphylococcus aureus* by time-kill curve methods. *J. Antimicrob. Chemother.* 51:857-64.
- Jacqueline, C, Navas, D, Batard, E, et al, 2005:** In vitro and in vivo synergistic activities of Linezolid combined with sub inhibitory concentrations of Imipenem against methicillin-resistant. *St. aureus* *Antimicrob. Agents chemother.* 49, 1:45-51
- Jang, HC, Kim, SH, Kim, KH, Kim, CJ, Lee, S, et al, 2009:** Salvage treatment for persistent methicillin-resistant *Staphylococcus aureus* bacteremia: Efficacy of linezolid with or without carbapenem. *Clin. Infect. Dis.* 49, 3:395-401.
- Joel, J, Graham, SM, Cooper, A, Korres, N, Tsouchnica, H, et al, 2014:** Clinical results of linezolid in arthroplasty and trauma MRSA related infections. *World J. Orthop.* 18, 2:151-7
- Kishore, S, Verma, D, Siddique, ME, 2014:** Comparison of *in-vitro* activities of linezolid and vancomycin against *Staphylococcus aureus* Isolated from A Tertiary Care Hospital. *J. Clin. Diag. Res.* 8, 5:DC12-5.
- Kohno, S, Yamaguchi, K, Aikawa, N, Sumiyama, Y, Odagiri, S, et al, 2007:** Linezolid versus vancomycin for the treatment of infections caused by methicillin resistant *Staphylococcus aureus* in Japan. *J. Antimicrob. Chemother.* 60:1361-9.
- Lincopan, N, de Almeida, LM, de Araújo, M R, Mamizuka, EM, 2009:** Linezolid resistance in *Staphylococcus epidermidis* associated with a G2603T mutation in the 23S rRNA gene. *Int. J. Antimicrob. Agents* 34:281-2.
- Livermore, DM, Mushtaq, S, Warner, M, Woodford, N, 2009:** Activity of oxazolidinone TR-700 against linezolid -susceptible and -resistant staphylococci and enterococci. *J. Antimicrobial Chemother.* 63:713-5.
- Matsuda, K, Nakamura, K, Adachi, Y, Inoue, M, Kawakami, M, 1995:** Autolysis of methicillin-resistant *Staphylococcus aureus* is involved in synergism between imipenem and cefotiam. *Antimicrob. Agents Chemother.* 39:2631-4.
- Matynia, B, Młodzinska, E, Hryniewicz, W, 2005:** Antimicrobial susceptibility patterns of *S. aureus* in Poland obtained by the national quality assurance program. *Clin. Microbiol. Infect.*

11:379-85.

Melakea, NA, Zakariab, AS, 2012: In vitro activity of linezolid alone and in combination with glycopeptides against methicillin-resistant *Staphylococcus aureus*. *Int. Res. J. Microbiol.* 3, 4: 127-35.

Mulazimoglu, L, Drenning, SD, Yu, VL, 1996: In vitro activities of two novel oxazolidinones (U100592 & U100766), a new fluoroquinolone (trovafloxacin) and dalfopristinquinupristin against *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Antimicrob. Agents Chemother.* 40:2428-30.

Pankey, GA, Ashcraft, DS, 2005: In vitro synergy of ciprofloxacin and gatifloxacin against ciprofloxacin-resistant *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 49:2959-64.

Ribes, S, Pachón, ME, Domínguez, M A, Fernández, R, Tubau, F, et al, 2010: In vitro and in vivo activities of linezolid alone and combined with vancomycin and imipenem against *Staphylococcus aureus* with reduced susceptibility to glycopeptides. *Eur. J. Clin. Microbiol. Infect. Dis.* 29:1361-7.

Rubinstein, E, Cammarata, S, Oliphant, T, Wunderink, R, 2001: Linezolid (PNU-100766) versus vancomycin in the treatment of hospitalized patients with nosocomial pneumonia: a randomized, double-blind, multicenter study. *Clin. Infect. Dis.* 32:402-12

Sahuquillo, JM, Colombo, GE, Gil, A, Ortiz, ER, Cantón, E, et al, 2006: In vitro activity of linezolid in combination with doxycycline, fosfomicin, levofloxacin, rifampicin and vancomycin against methicillin-susceptible *Staphylococcus aureus*. *Rev. Esp. Quimioterap-Septiem.* 19, 3:252-7

Shi, J, Mao, NF, Wang, Li, Zhang, HB, Chen, Q, et al, 2014: Efficacy of combined vancomycin and fosfomicin against methicillin-resistant *Staphylococcus aureus* in biofilms *in-vivo*. *PLOS ONE*, DOI:10.1371/journal.pone.0113133

Silva, MT, Sousa, JCF, 1973: Ultrastructure of the cell wall and cytoplasmic membrane of Gram negative bacteria with different fixation techniques. *J. Bacteriol.* 113:953-62.

Singh, SR, Bacon, AE, Young, DC, Couch, K A, 2009: In Vitro 24 hour time-kill studies of vancomycin and linezolid in combination versus Methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 53, 10: 4495-7.

Stratton CW (1996). Mechanisms of action for antimicrobial agents: general principles and mechanisms for selected classes of antibiotics. In *Antibiotics in Laboratory Medicine*, 4th ed. (Lorian, V.,Ed.). Williams & Wilkins Co., Baltimore, MD, USA

Sweeney, MT, Zurenko, GE, 2003: In vitro activities of linezolid combined with other antimicrobial agents against Staphylococci, Enterococci, Pneumococci, and selected Gram-negative organisms. *Antimicrobial. Agents Chemother.* 47, 6:1902-6.

Tian, Y, Li, T, Zhu, Y, Wang, B, Zou, X, et al, 2014: Mechanisms of linezolid resistance in staphylococci and enterococci isolated from two teaching hospitals in Shanghai, China. *BMC Microbiol.* 14:292-9.

Tsaganos, T, Skiadas, I, Koutoukas, P, Adamis, T, Baxevano,s N, et al, 2008: Efficacy and pharmacodynamics of linezolid, alone and in combination with rifampicin, in an experimental model of methicillin-resistant *Staphylococcus aureus* endocarditis *J. Antimicrobial Chemother.* 62: 381-3.

Tsiodras, S, Gold, HS, Sakoulas, G, Eliopoulos, GM, Wennersten, C, 2001: Linezolid resistance in a clinical isolate of *Staphylococcus aureus*. *Lancet*, 358:207-8.

Wilson, P, Andrews, JA, Charlesworth, R, Walesby, R, Singer, M, et al, 2003: Linezolid resistance in clinical isolates of *Staphylococcus aureus*. *J. Antimicrob. Chemother.* 51:186-8

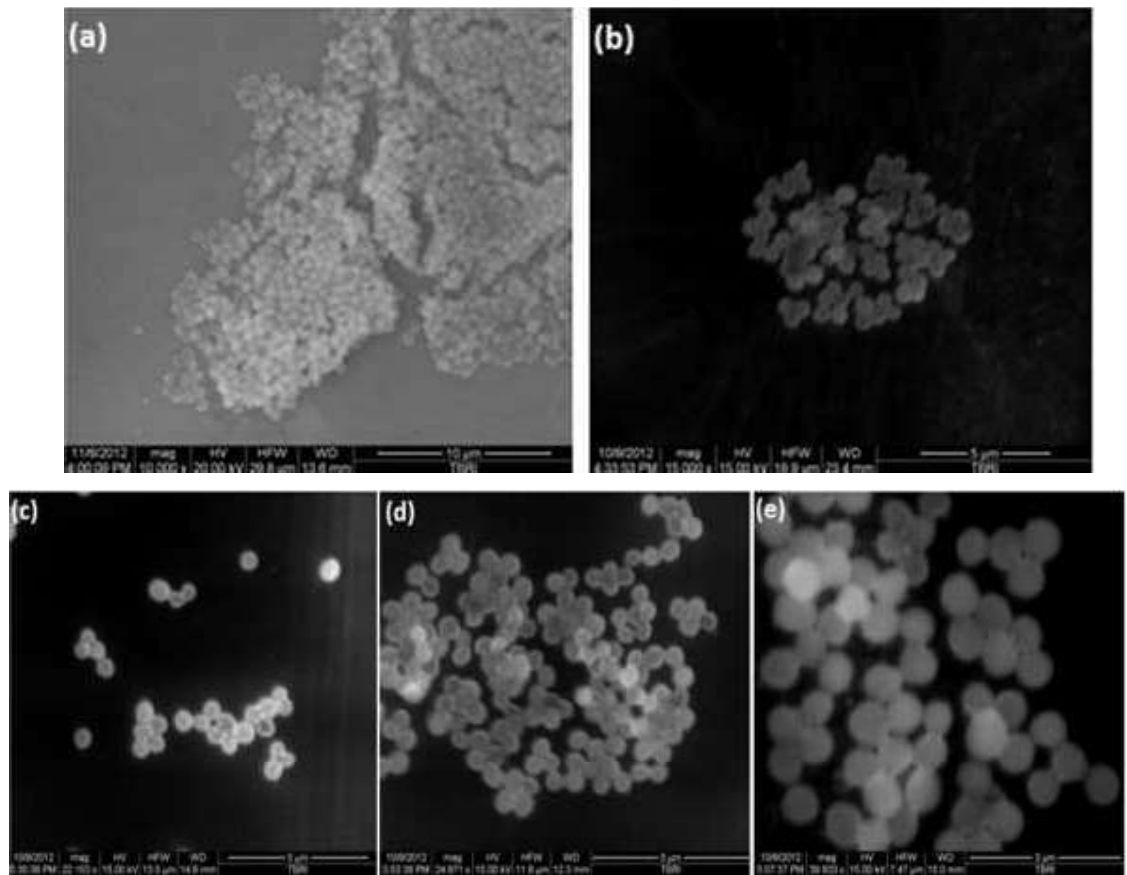


Fig. 2: SEM of MRSA ATCC 43300 strain exposed to linezolid alone and in combination with vancomycin or rifampicin, at $8 \times$ MIC and imipenem at $32 \times$ MIC (a) Control, (b) linezolid, (c) linezolid and vancomycin, (d) linezolid and rifampicin, (e) linezolid and imipenem.

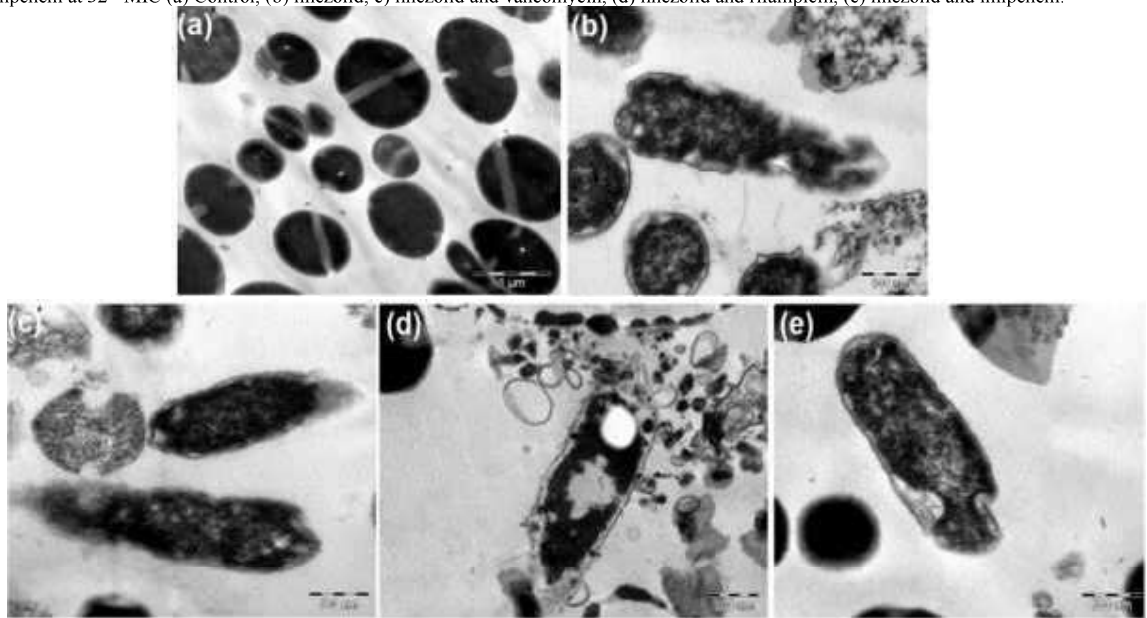


Fig. 3: TEM of MRSA ATCC 43300 strain exposed to linezolid alone and in combination with vancomycin or rifampicin, at $8 \times$ MIC and imipenem at $32 \times$ MIC (a) control (b) linezolid alone (c) linezolid and vancomycin (d) linezolid and rifampicin (e) linezolid and imipenem.