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Effects of Linezolid on Suppressing Heteroresistant Vancomycin Subpopulations among Heteroresistant Vancomycin Intermediate *Staphylococcus aureus* (hVISA)

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Infections due to heterogeneous Vancomycin Intermediate *Staphylococcus aureus* (hVISA) are associated with vancomycin treatment failure, in which they raise a great concern for treatment options for hVISA infections. Earlier studies indicate that the combination of vancomycin and linezolid should be avoided. The aim of this study was to compare the *in vitro* activities of vancomycin and linezolid against hVISA in a setup, to help formulate a better treatment and reduce the emergence of hVISA. In this particular study, 8 hVISA were studied. Different methods were applied to all the isolates in order to assess their tolerance to vancomycin and to detect the presence of heterogeneous subpopulations within strains, in the presence of different concentrations of linezolid. The methods included minimum inhibitory concentration, Time-Kill experiments and Population Analysis Profiling. When linezolid was combined with vancomycin, slight or no antagonism was observed. Despite this, we find that the combination with linezolid reduces the emergence of heteroresistant vancomycin resistant among hVISA. Despite previous studies indicating that vancomycin and linezolid in combination should be avoided, Sub MIC of Linezolid could be used to reduce vancomycin treatment failures among hVISA infections.

Key words: Linezolid, Vancomycin MRSA, VISA, hVISA.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial and community acquired infections that continue to cause a variety of clinical syndromes all over the world. There are few numbers of new antibacterial agents available to treat these lethal infections (Thati *et al.*, 2011). Glycopeptides, such as vancomycin have been widely used for the treatment of MRSA, which created a selection pressure resulting in the development of new strains with a reduced susceptibility to vancomycin (Tarai *et al.*, 2013).

Sequential mutations in vancomycin-Susceptible *Staphylococcus aureus*(VSSA) lead to the emergence of vancomycin-intermediate *Staphylococcus aureus* (VISA) and hVISA. Increases in the prevalence of strains with higher level vancomycin resistant are a challenging and serious public health concern (Sharma, 2012). Site-Specific Mutation, frequently occurring in genes important for cell wall metabolism such

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as vraRS and graRS, have been associated with the cell wall modifications. Although it is accepted that hVISA strains are phenotypically susceptible to vancomycin by using Minimum Inhibitory concentration method (MIC); subpopulation analysis profile, however reveals subpopulations of bacterial cells with increase resistant to vancomycin.. The proportion of *S. aureus* isolates that are hVISA increase as the vancomycin MIC increases with consequent adverse clinical outcome (Howden *et al.*,2011).

In 2006, the Clinical and Laboratory Standards Institute (CLSI) decrease the vancomycin susceptibility break point for *S. aureus* from 4 to 2 µg/ml. Currently, the CLSI defined vancomycin breakpoints as follows: susceptible at ≤2 ug/ml, intermediate at 4- 8 ug /ml, and resistant at ≥16 µg/ml (CLSI, 2012). In the other hand, both the British Society for Antimicrobial Chemotherapy (BSAC) define *S. aureus* strains as vancomycin susceptible (MIC ≤ 2 µg/ml) or resistant (MIC > 2 µg/ml) (BSAC, 2011).

MRSA infections such as bacteraemia, endocarditis and osteomyelitis have been associated with clinical strains with aheteroresistance to vancomycin, and this may increase the rates of vancomycin treatment failure. The oxazolidinone antibiotics, linezolid, is one of the recent treatment options for Gram Positive Bacteria and has exhibited a good activity against most multidrugs resistant bacteria, including MRSA (Rubinstein *et al.*, 2001). Oxazolidinone antibiotics act by inhibiting ribosomal protein synthesis at an early stage of bacterial replication which results to the absence of cross-resistance with other antimicrobials agents (Lentino *et al.*, 2008).

The higher incidence of nosocomial infections due to MRSA and the report of therapeutic failures associated with standard therapy highlight the significant of identifying new synergistic drug combinations to avoid this problem. The value of linezolid as part of a drug combination has been investigated against MRSA strains, but very few data has been reported against hVISA or VISA strains (Sacar *et al.*, 2007).

This study is purposed to re-evaluate the *in vitro* activities of vancomycin and linezolid against hVISA in a set up to help formulate a better treatment and reduce the emergence of hVISA.

MATERIALS AND METHODS

Bacterial strains

Eight different clinical isolates of hVISA with different degrees of resistance to vancomycin were included. The MRSA isolates obtained from different clinical infection sites at Ohad Hospital, Madinah, Saudi Arabia. *S. aureus* Mu3 strain (*S. aureus* ATCC 700698) was used as a control throughout the test. PCR method used for confirmation of methicillin resistance by amplification of the methicillin resistance gene mecA. Forward primer AAA ATC GAT GGT AAA GGT TGG C and reverse primer AGT TCT GCA GTA CCG GAT TTG C were used in this work (Murakami & Minamide, 1991).

Determination of antimicrobial activity

MIC method was used on all the isolates in order to assess the strains susceptibility to the different antimicrobial agents. The MICs were determined according to CLSI guidelines. All susceptibility testing were performed using cation-adjusted Mueller-Hinton broth (CAMHB) and stock solutions were prepared in accordance with CLSI guidelines [CLSI, 2012)

Time–kill studies

For time-kill assays, bacteria were grown in CAMHB until exponential phase then diluted to approximately $5x10^5$ CFU/ml in media containing antimicrobial agents alone or in combination, and exposed for 24 h at 37°C . Synergy was defined as a 2 log10 decrease in CFU/ml between the combination and its most active constituent after 24 h (at least one of the drugs must be present at a concentration that does not affect the growth curve of the test organism), Antagonism was defined as an increase in the colony count of ≤ 2 log10 CFU/ml with the combination in comparison with the count obtained with the most active single agent. An indifferent effect was defined as <1 log (increase or decrease) in killing (Singh *et al.*, 2009).

All experiments were repeated at least three times, and the results of a representative experiment are presented; data points are averages from duplicate CFU/ml determinations within an experiment.

Population Analysis Profiles

Population Analysis Profiles (PAPs) were constructed as previously described with the following modification: Overnight cultures of bacteria (≥10^8cfu/ml) were plated at a series of

dilutions on Muller-Hinton plates containing vancomycin-free medium or twofold dilutions of vancomycin ranging from 0 –32 ug/ml. All the plates have the same sub-MIC concentration of linezolid. The Plates were incubated at 37°C for 48 h, and the number of bacterial colonies was counted. Plotting cfu (in log 10) against vancomycin concentrations provides a graphic display. hVISA is defined as a strain that is susceptible to vancomycin using the MIC method but contains a resistant subpopulation usually at a frequency of 1 in 10^6 cells that can grow in the presence of ≥ 2 µg/ml of vancomycin (Hiramatsu *et al.,* 1997, Wootton *et al.,* 2011).

RESULTS

The PCR test Confirmed that all the strains are containing the mecA gen and confirmed as a true MRSA strains. (Fig. 1 show the results for hVISA-6). The antimicrobial activity was evaluated on eight different hVISA. Significant antimicrobial effects, expressed as various concentrations of Vancomycin and Linzolid, were observed against all the isolates and the control strains. All the strains were susceptible to vancomycin and Linezolid using a standard MIC method. The results are summarized in Table 1.

When linezolid was combined with vancomycin, slight or no antagonism was observed using Time-kill tests, despite that, by using PAPs test, we found that the presence of sub-MIC of linezolid reduced the emergence of heteroresistant to vancomycin for all the isolates (Figs. 2 and 3 show the results for hVISA-6).

Table 1. Antimicrobial susceptibility pattern of the MRSA strains for Vancomycin and Linzolid

Strains	MIC (µg/ml)	
	Vancomycin	Linezolid
MRSA-1	0.5	1
MRSA-2	0.125	0.5
MRSA-3	0.5	2
MRSA-4	0.25	1
MRSA-5	0.125	1
MRSA-6	0.5	2
MRSA-7	0.5	0.5
MRSA-8	0.5	1

DISCUSSION

Detection of *S. aureus* with VISA and hVISA remains problematic. In this study MIC of vancomycin was 0.125- 0.5ug/ml against the tested hVISA isolates. This agreed with Leonard *et al.* who reported that by using Population analysis profile as a standard method, hVISA can be detected for strains of *S. aureus* with vancomycin MICs as low as 0.5 to 1 µg/ ml. In contrast, other studies concluded that rates of hVISA detection increase as the vancomycin MIC increases. (Rybak *et al.,* 2008)

Also on the clinical basis that increased mortality and treatment failure has been reported in infections with VSSA isolates with elevated vancomycin MIC. Typically these isolates have MICs near the susceptibility breakpoint 1.5 or 2 µg/mL using different MIC methodologies (Holmes *et al.,* 2011).

A significant heterogeneity in clinical features and infection types, different MIC testing methods, and different MIC values linked with poorer outcomes and treatment failure has been reported (Van Hal and Paterson, 2011).

The difference in the level of MIC of vancomycin for hVISA between the different studies could be due to the use of higher or lower

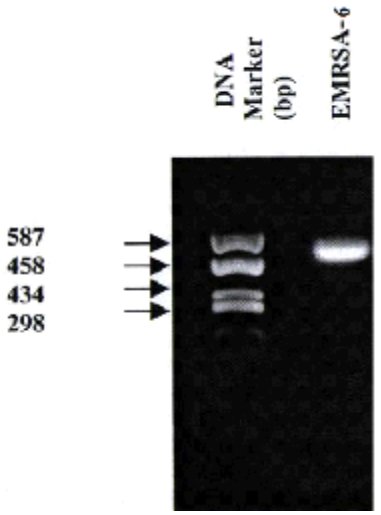

Fig. 1. Agarose gel electrophoresis of PCR products for detection of the mecA. The molecular weight marker used is pUC 18 HeaIII Digest. The PCR product sizes 560 bp.

inoculum size as reported by Warren *et al*., 2009 who showed that the activity of vancomycin against hVISA and even non hVISA isolates, using *in vitro* tests was reduced in the presence of a high inoculum amount. A possibility of a further reduction in vancomycin susceptibility breakpoints may be needed to avoid vancomycin associated treatment failure due to emerging hVISA strains (Warren *et al.*, 2009).

Linezolid showed high activity against hVISA isolates in comparison to vancomycin (0.125- 0.25 ug/ml for linezolid. versus 0.125- 0.5ug/ml) . This is consistent with other researchers who reported that all isolates of MRSA in their study were susceptible to ≤ 1 ug/ml of linezolid and the MIC of linezolid for MRSA is 0.023-0.75 ug/ml whereas the MIC of vancomycin is 0.5-3 ug/ml. (Ranjan *et al*.,2010).

Also on clinical basis, the antibiotic linezolid may be more effective than vancomycin with higher cure rate against methicillin MRSA (Aisling *et al.*, 2010)

Our study indicates that linezolid combinations preserve the activity of linezolid alone and might be considered as therapeutic options in the management of infections caused by *S. aureus* strains with reduced susceptibility to vancomycin. Indeed, an indifferent effect (<1 log increase or decrease) in killing was found when subMIC of linezolid concentrations was combined with vancomycin against hVISA. However an antagonistic effect was noticed with the use of MIC and double MIC of linezolid. This coincides with indifferent effect reported against MSSA. (Sahuquillo *et al.*, 2006). Shveta *et al*, recommend not to use vancomycin in combination with linezolid in the treatment of MRSA infections and found no synergistic activity for this combination. However this combination may be of value in delaying the emergence of hVISA. (Shveta *et al*.,2009). Others study found synergistic killing with sub-MIC concentrations of both antibiotics in combination against the vancomycin intermediate *S. aureus* (VISA) strain. (Ribes *et al.*, 2010).

Also, Booker *et al*, showed the ability of subinhibitory concentrations of linezolid to diminish production of several toxins by *S. aureus* which led to their use in combination with vancomycin so that further studies are required to clarify this issue *in vivo* also. (Booker *et al.*, 2005)

A related study found that the addition

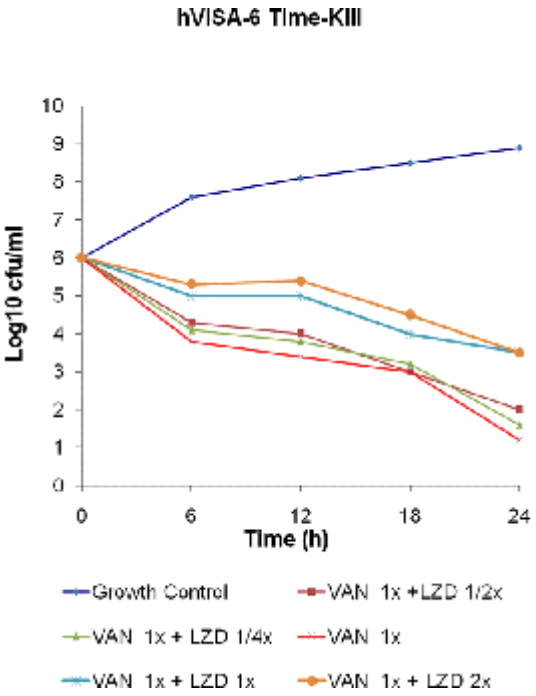

Fig. 2. Time–kill curves of the combinations of linezolid plus vancomycin for hVISA-6. LZD, linezolid; VAN, vancomycin

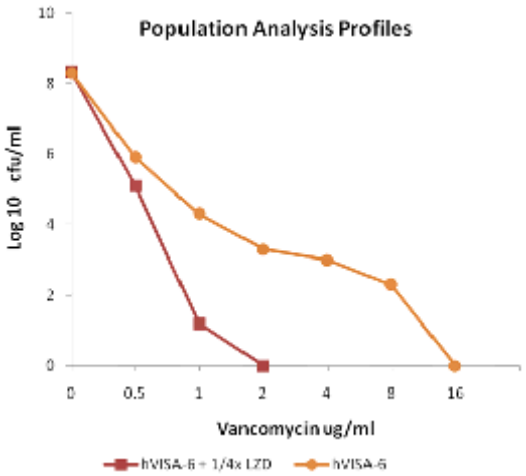

Fig. 3. Distribution profiles of vancomycin-resistant subpopulations against various vancomycin concentrations in the present of Sub MIC concentration of linezolid (1/4x MIC) for hVISA-6

of linezolid decreased the rate of vancomycin killing of MRSA by 100–1000-fold. Antagonism between these two antibiotics was also found by another group of investigators using time-kill analysis (Grohs*et al*, 2003).

This difference is due to the use 1xMIC and >1xMIC for linezolid in combination with vancomycin (Deresinski , 2009).

Population analytic profiles for hVISA strains to study the effect of linezolid on the emergence of hVISA under the effect of different vancomycin concentrations (0 –32 ug/ml) showed decrease in the emergence of these subpopulations when linezolid was combined to vancomycin . This might indicate the possible use of this combination to avoid treatment failure with vancomycin alone especially in life threating infections with MRSA.

We should emphasise that *in vitro* drug combination may not translate into clinical efficacy, mainly because of the different mechanisms involved in *in vivo*. So we recommend studying the *in vivo* efficacy of this combination in order for use in the clinical field.

CONCLUSION

Despite previous studies indicating that vancomycin and linezolid in combination should be avoided, Sub MIC of linezolid could be used to reduce vancomycin treatment failures among hVISA.

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