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Glycopeptide Resistance in *S. aureus*

Hasan Cenk Mirza

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Abstract

The glycopeptides (particularly vancomycin) have been the recommended therapy for serious methicillin-resistant *Staphylococcus aureus* (MRSA) infections. The increased incidence of MRSA has led to the frequent use of vancomycin. Unfortunately, with the increased use of vancomycin, isolates of *S. aureus* have been discovered with reduced susceptibility to vancomycin. Several studies suggest that reduced vancomycin susceptibility is associated with vancomycin treatment failure. Various forms of glycopeptide resistance have appeared in MRSA strains, including high-level resistance, homogeneous and heterogeneous intermediate resistance. While vancomycin-resistant *S. aureus* (VRSA) strains are limited to a handful of reported cases and vancomycin-intermediate *S. aureus* (VISA) strains remain rare; heterogeneous VISA (hVISA) strains are more common. This article summarizes the current knowledge regarding the history, definition, mechanisms, detection methods, epidemiology and clinical significance of 'glycopeptide resistance in *S. aureus*' and discusses therapeutic options for the treatment of hVISA/VISA infections.

Keywords: *S. aureus*, glycopeptide resistance, vancomycin, teicoplanin, hVISA, VISA

1. Introduction

Glycopeptide group antibiotics—notably vancomycin—have traditionally been the mainstay of therapy for infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) [1]. However, the increased incidence of MRSA infections has led to increased use of vancomycin and has resulted in the emergence of *S. aureus* with reduced susceptibility to vancomycin. Both the terms vancomycin-intermediate *S. aureus* (VISA) and glycopeptide-intermediate *S. aureus* (GISA) have been used in the literature [2]. Since many VISA isolates also have been intermediate to glycopeptide teicoplanin, the term GISA may be more accurate. However, the

acronym VISA is more frequently used. Various studies have associated the presence of VISA and heterogeneous VISA (hVISA) with vancomycin treatment failure.

This article focuses on the history, definition, mechanisms, detection methods, epidemiology, and clinical significance of 'glycopeptide resistance in *S. aureus*' and the therapeutic options for the treatment of hVISA/VISA infections.

2. History and definition of glycopeptide resistance

The glycopeptide vancomycin was isolated from a Gram-positive filamentous actinomycete called *Amycolatopsis orientalis* and was approved for use by the U.S. Food and Drug Administration in 1958 [2, 3]. Vancomycin acts by inhibiting proper cell wall synthesis. It binds with high affinity to D-alanyl-D-alanine (D-Ala-D-Ala) terminal end of peptidoglycan precursors and prevents cross-linking of peptidoglycan by inhibiting the action of transglycosidase and transpeptidases. Vancomycin has been in clinical use for decades and there was no notable resistance to vancomycin reported in *S. aureus* until 1996. In 1996, a MRSA strain with vancomycin MIC of 8 μg/ml (Mu50, VISA) was isolated from the surgical wound infection from a 4-month-old male infant who had undergone cardiac surgery [4]. After this case, two patients from USA and one patient from France with infections due to *S. aureus* with intermediate resistance to vancomycin were reported [5, 6]. After the emergence of VISA; a new model of vancomycin resistance (hVISA) was defined by Hiramatsu *et al.* in 1997 [7]. The first hVISA strain Mu3 was isolated from the sputum of a 64-year-old patient with MRSA pneumonia who failed vancomycin therapy. hVISA strains are susceptible to vancomycin by the standard broth microdilution reference method (vancomycin MIC ≤2 μg/ml) but contain subpopulations of cells (one in every 10^5–10^6) for which the vancomycin MIC is in the intermediate range, currently defined as 4–8 μg/ml by the Clinical and Laboratory Standards Institute (CLSI) [8]. hVISA strains are more commonly found than VISA and different rates of hVISA are reported from different countries.

In 2002, the first *S. aureus* fully resistant to vancomycin [vancomycin-resistant *S. aureus* (VRSA), vancomycin MIC ≥16 μg/ml] was reported in Michigan, United States (U.S). Fortunately, VRSA is very rare as only 14 cases of VRSA have been reported in U.S. so far [9].

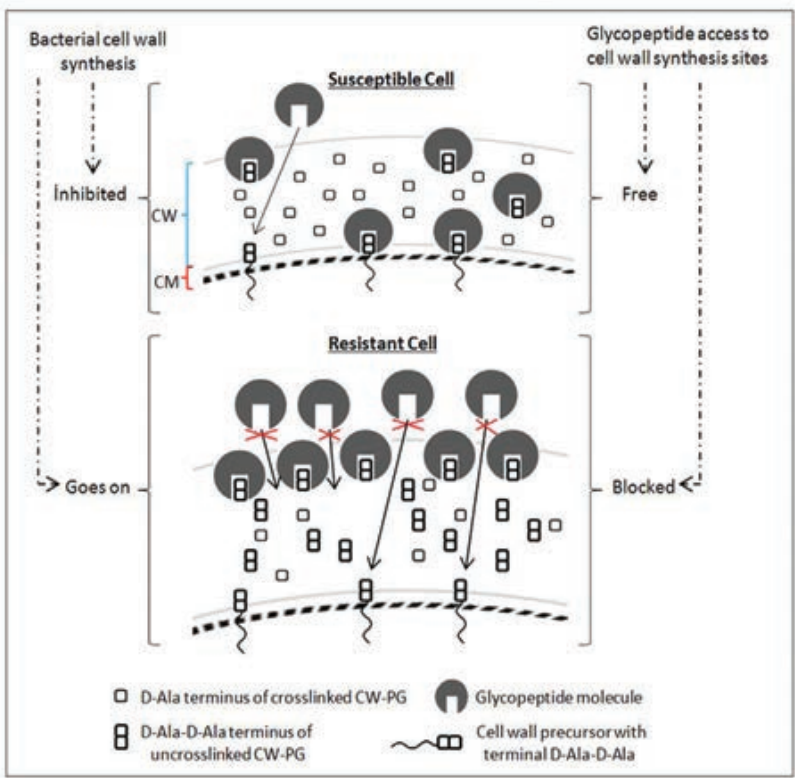## 3. Phenotypic changes and mechanisms of resistance

3.1. hVISA and VISA

Heterogeneous VISA appears to be the phase before the development of VISA. Vancomycin exhibits a selective pressure that leads to the growth of VISA subpopulations, eventually creating a uniform population of VISA [10].

One of the most common phenotypic changes observed in hVISA/VISA is the thickened cell wall with reduced peptidoglycan cross-linking (**Figure 1**) [2, 10, 11]. Reduced cross-linking of

peptidoglycan leads to an increase in free D-Ala-D-Ala residues (binding sites for vancomycin). It is supposed that vancomycin binds to these free D-Ala-D-Ala residues in the outer layers of the thickened cell wall and is unable to reach its site of action at the cell membrane [12]. The trapped vancomycin molecules within the cell wall clog the peptidoglycan meshwork and form a physical barrier towards further incoming vancomycin molecules. Thus, collaboration of the clogging and cell wall thickening leads to glycopeptide resistance (**Figure 2**) [13, 14].
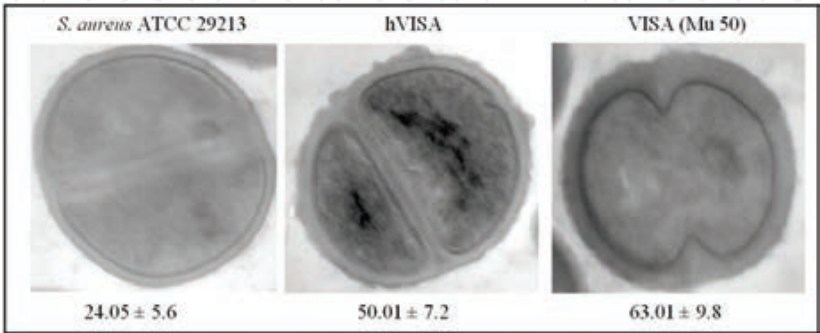
Figure 1. Comparison of the cell wall thickness of *S. aureus* ATCC 29213 [Vancomycin-susceptible *S. aureus* (VSSA)], hVISA and VISA strains by transmission electron microscopy. The cell wall thickness, in nanometers (mean ± SD), is given under each image. Magnification: × 60,000. (Adapted from Ref. [11] which was published under an open-access license agreement).

Figure 2. Model depicting the resistance mechanisms of hVISA/VISA. CW, cell wall; CM, cell membrane; PG, peptidoglycan. (Adapted with permission from Ref. [14]).

In addition to thickened cell wall, hVISA/VISA strains exhibit other phenotypic changes including reduced autolytic activity, reduced hemolytic activity and slow growth *in vitro* [10, 15].

3.1.1. Molecular mechanisms of resistance

The molecular mechanisms of glycopeptide resistance in hVISA/VISA are still not clearly understood. To date, no specific genetic determinants of hVISA/VISA have been defined. However, some of the genes whose expression has been found to be altered in VISA strains include *atl* (autolysin), *mprF* (phosphatidylglycerol lysyltransferase), *sceD* (transglycosylase), *sarA*, *sigB*, *tcaA*, and *ddh* [16]. Furthermore, mutations associated with the intermediate resistance phenotype have been identified in the two-component regulatory systems [*vraSR* (vancomycin resistance-associated sensor/regulator), *graSR* (glycopeptide resistance-associated sensor/regulator), *walKR*], and *rpoB* (RNA polymerase) gene [15].

Cellular physiology of hVISA/VISA is believed to be altered due to the cumulative effects of mutations and/or modulation of regulatory systems [17]. As a result, altered cell wall structure and metabolism resulting from multiple genetic changes appears to be responsible for intermediate resistance to glycopeptides.

3.2. VRSA

The mechanism of vancomycin resistance in VRSA strains is different from that of hVISA/VISA strains. The vanA gene complex, which confers high-level resistance to glycopeptides in enterococci, was detected in VRSA isolates.

To date, nine types of glycopeptide resistance have been described in enterococci. Eight of these types (VanA, VanB, VanD, VanE, VanG, VanL, VanM, and VanN) correspond to acquired resistance, whereas VanC is an intrinsic characteristic of *Enterococcus gallinarum* and *Enterococcus casseliflavus*. Glycopeptide resistance in enterococci results from the production of modified peptidoglycan precursors ending in D-alanyl-D-lactate (D-Ala-D-Lac) (VanA, VanB, VanD, and VanM) or D-alanyl-D-serine (D-Ala-D-Ser) (VanC, VanE, VanG, VanL, and VanN) to which vancomycin binds with low affinity and the elimination of high-affinity precursors ending in D-Ala-D-Ala [18].

Resistance in VRSA isolates is caused by the horizontal transfer of transposon Tn*1546* (carrying the *vanA* operon) from vancomycin-resistant *Enterococcus faecalis* [15, 19]. Vancomycin is known to act by binding to the terminal D-Ala-D-Ala of bacterial cell wall precursors. The *vanA gene* complex acquired by VRSA strains enables the bacteria to synthesize cell wall precursors terminating in D-Ala-D-Lac for which vancomycin has a greatly decreased affinity. In the presence of vancomycin, the novel cell wall precursors are synthesized, allowing continued peptidoglycan assembly [19, 20].

4. Laboratory detection of hVISA, VISA and VRSA

Laboratory detection of VISA and VRSA strains is easier than hVISA, as there are defined CLSI MIC criteria (MIC: 4–8 µg/ml for VISA and MIC ≥16 µg/ml for VRSA). These criteria have been defined using the reference broth microdilution (BMD) method. Results obtained by the use of other methods for determining the MIC should be confirmed with BMD [21]. Disk diffusion (Kirby-Bauer) is not an acceptable method for vancomycin susceptibility testing of *S.aureus* isolates.

Detection of hVISA is problematic for most clinical microbiology laboratories. The lack of a precise definition and standardized testing makes the detection of hVISA difficult [21]. hVISA strains appear susceptible to vancomycin (MIC ≤ 2 µg/ml) with conventional testing but contain subpopulations (1 per 10^5–10^6 organisms) that express reduced vancomycin suscepti-bility (MIC ≥4 µg/ml). Standardized methods for susceptibility testing [broth microdilution (BMD), agar dilution and standard Etest]—which use an inoculum of only 5×10^4 colony-forming unit (CFU)/well (BMD) or 1×10^4 CFU/spot (agar dilution)—fail to detect hVISA, in part due to the small inoculum, the relatively poor support of growth on Mueller-Hinton agar (MHA) plates, or a combination of both [2, 22].

The morphological features of hVISA/VISA isolates can be different from those of standard *S. aureus* cultures on agar plates. Careful observation may reveal smaller-sized colonies, "mixed" colony morphology (large and small colonies or colonies with different pigmenta-tions in a pure culture) and reduced pigmentation. However, these changes may be subjective and are not diagnostic [2, 21].

Methods for "hVISA detection" use higher inoculum, prolonged incubation or more nutritious agar to promote the growth of subpopulations with reduced susceptibility to vancomycin. Population analysis profile-area under the curve (PAP-AUC) is considered the gold standard method for hVISA detection. However, this method is labor-intensive, time-consuming and not suitable for routine use in clinical microbiology laboratories [10, 22]. As a consequence, several screening methods have been developed for the detection of hVISA.

4.1. Screening methods for hVISA

4.1.1. Screening plates

A number of screening plates containing various concentrations of vancomycin or teicopla-nin have been proposed for the detection of hVISA/VISA isolates [brain heart infusion agar (BHIA) with 3 µg/ml, 4 µg/ml or 6 µg/ml vancomycin, BHIA with 5 µg/ml teicoplanin, MHA with 5 µg/ml vancomycin or 5 µg/ml teicoplanin] [23–26]. **Figure 3** shows the growth of a hVISA strain on BHIA with 4 µg/ml vancomycin.
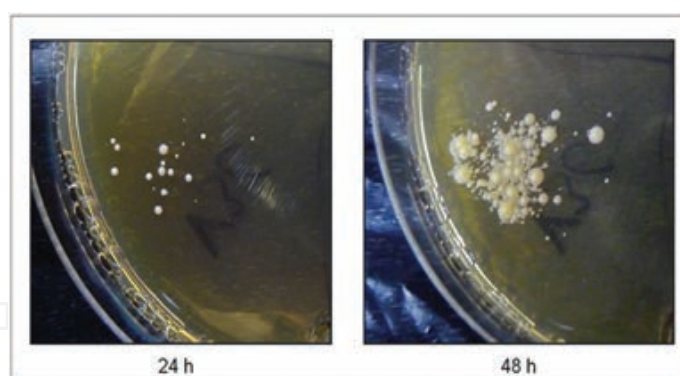
Figure 3. Photograph of a hVISA grown on BHIA with 4 µg/ml vancomycin for 24 and 48 h.

Antibiogram Committee of the French Society for Microbiology recommends the use of MHA with 5 µg/ml teicoplanin (MHA5T). This screening plate has been tested by various studies using an inoculum of 10 µl of a 2.0 McFarland standard suspension for the detection of VISA/hVISA isolates. Growth of one or more colonies is considered positive after 48 h of incubation. MHA5T has been shown to have sensitivity ranging from 65% to 79% and specificity ranging from 35 to 95% for the detection of hVISA [2].

In a study conducted by Satola *et al.*, BHIA containing 4 µg/ml vancomycin and 16 g/l pancreatic digest of casein has been shown to be 90% sensitive and 95% specific with a 0.5 McFarland inoculum and 100% sensitive and 68% specific with a 2.0 McFarland inoculum [22]. However, further studies are needed to determine the value of this screening plate.
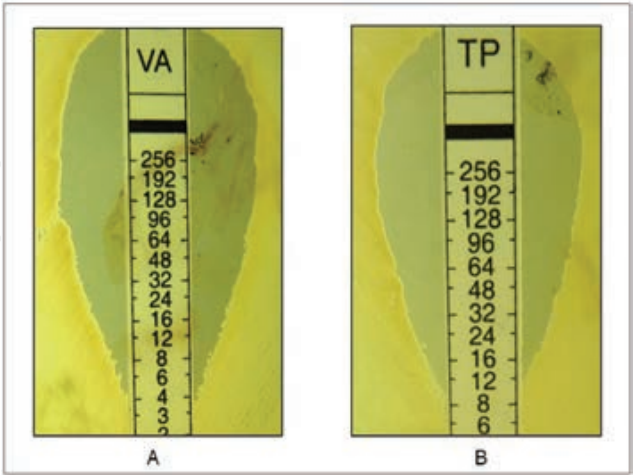
4.1.2. Etest macromethod

Etest macromethod is performed utilizing a higher inoculum of organism (2.0 McFarland vs. 0.5 McFarland utilized in standard Etest) streaked onto BHIA. Vancomycin and teicoplanin Etest strips are applied to the dry agar surface and read after 48 h of incubation (compared to 24 h for standard Etest) at 35°C [10]. Zones must be read at complete inhibition, with care, to visualize hazy growth or microcolonies. Heteroresistance is defined as MICs for vancomycin and teicoplanin of ≥8 µg/ml or a teicoplanin MIC of ≥12 µg/ml alone. It should be noted that the result of the Etest macromethod is just a cutoff level and is not a true MIC, because this method differs from the standard MIC calculation [2, 21].

Figure 4 shows a hVISA strain (confirmed by PAP-AUC) with positive Etest macromethod result. The presence of microcolonies inside the inhibition zones reflects the heterogeneous resistant character of the strain (**Figure 5**). Subculture from a single microcolony (**Figure 5B**) done on blood agar reveals heterogeneous colony morphology (a common feature of hVISA/VISA strains) (**Figure 6**).

Various studies have evaluated the performance of Etest macromethod, using PAP-AUC as the gold standard. Etest macromethod has been shown to have sensitivity ranging from 57 to 98.5% and specificity ranging from 55 to 96% for the detection of hVISA [22, 23, 25, 27–29]. The differences in sensitivity and specificity rates may be partially explained by the use of various

inoculum sizes for Etest macromethod (50, 100 or 200 μl) in different studies [27, 28, 30]. Currently, the manufacturer recommends the use of an inoculum of 100 μl [31].

Figure 4. Positive Etest macromethod result for a hVISA strain isolated from blood culture A: Vancomycin Etest, B: Teicoplanin Etest.
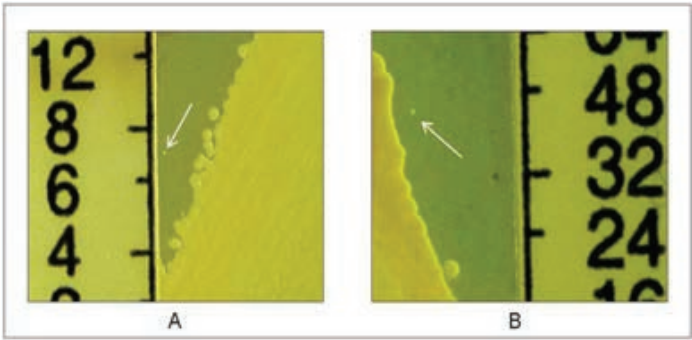
Figure 5. Magnified appearance of **Figure 4(A** and **B)**. The arrows indicate the presence of microcolonies growing within the zones of inhibition. A: Vancomycin Etest, B: Teicoplanin Etest.
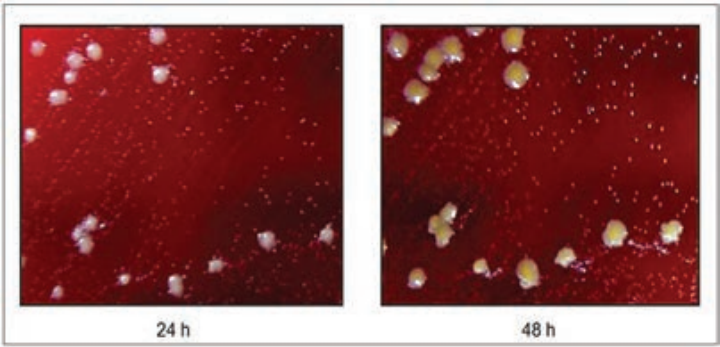
Figure 6. Subculture from a single microcolony (**Figure 5B**) on blood agar demonstrating different colony morphotypes.

4.1.3. Etest GRD

Etest glycopeptide resistance detection (GRD) is a newer Etest method for the detection of hVISA. This method involves the use of a double-ended Etest strip that contains vancomycin, teicoplanin, and a nutritional supplement to enhance the growth of hVISA. A 0.5 McFarland standard inoculum, rather than the 2 McFarland standard used for Etest macromethod, is used and inoculated onto MHA + 5% blood [2]. Etest GRD strip is applied to the agar surface and the zone of inhibition is read after 48 h of incubation at 35°C. The strain is considered positive for hVISA if the Etest GRD result is ≥8 µg/ml for either vancomycin or teicoplanin [21, 22]. **Figure 7** shows a hVISA strain with positive Etest GRD result.

Figure 7. A hVISA strain with positive Etest GRD result. (Photograph courtesy of Dr M. Wootton).

Etest GRD has been reported to have sensitivity and specificity of 57–94% and 82–97%, respectively [22, 28, 29, 32]. Some of these differences may reflect the instability of hVISA phenotype. hVISA strains are known to be unstable, with the ability to revert to vancomycin-susceptible *S. aureus* (VSSA) under various conditions, including passage of the isolate on vancomycin-free media [29].

A possible barrier to large-scale usage of modified Etest methods is the potentially high cost to the clinical laboratory. This can be considered a disadvantage [10].

4.2. Confirmatory methods for hVISA

4.2.1. PAP-AUC

Population analysis profile-area under the curve (PAP-AUC) remains the gold standard method for detection of hVISA [10]. PAP-AUC method is performed as follows:

The isolate is incubated in tryptone soya broth (TSB) for 24 h. An undiluted culture and dilutions of $1/10^8$ and $1/10^5$ are spiral plated onto BHIA plates containing 0.5, 1, 2, 2.5, 4, and 8 µg of vancomycin per ml. After 48 h of incubation at 35°C, the colonies are counted. Log_{10} of the colony numbers (log_{10} CFU/ml) are plotted against the vancomycin concentrations. The "area under the curve (AUC)" of the isolate is calculated. The VSSA strain ATCC 29213 is used as negative control. Reference strains of hVISA (Mu3, ATCC 700698) and VISA (Mu50, ATCC 700699) are used as positive controls. A ratio is calculated by dividing the AUC of the test strain by the AUC of reference hVISA strain (Mu3). The ratios of <0.90, 0.90–1.30, and >1.30 are interpreted as VSSA, hVISA, and VISA, respectively [28, 33]. **Figure 8** shows an example

of population analysis for *S. aureus* ATCC 29213 (VSSA), Mu3 (hVISA), Mu50 (VISA), a clinical hVISA isolate and a clinical VSSA isolate [11].
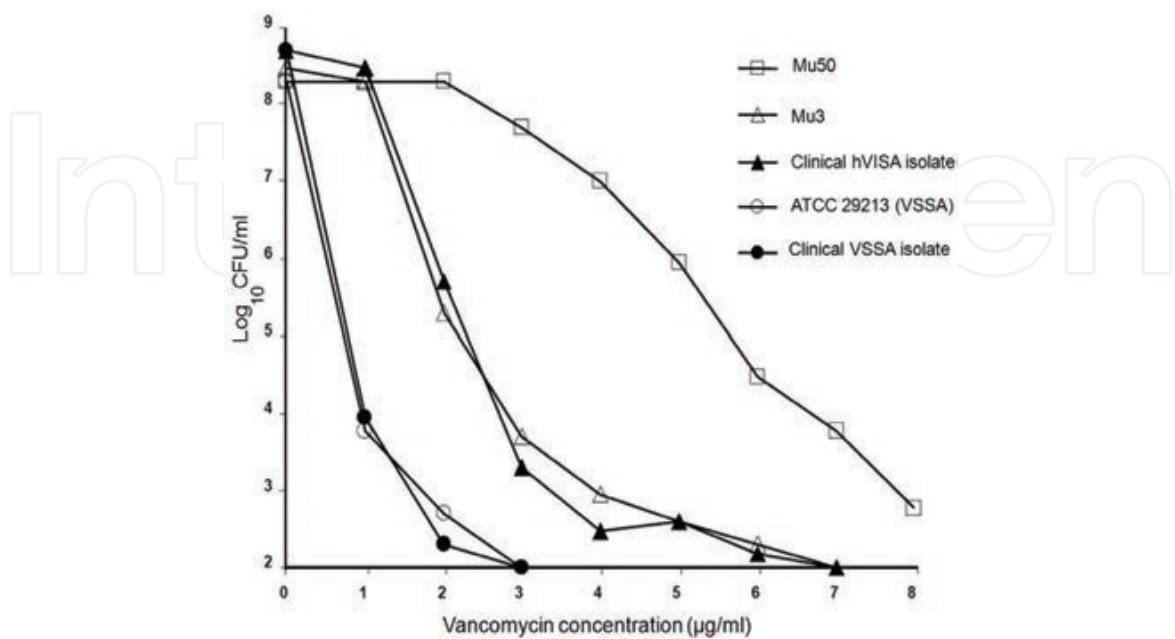
Figure 8. Example of population analysis profile curves for *S. aureus* ATCC 29213 (VSSA), Mu3 (hVISA), Mu50 (VISA), a clinical hVISA isolate and a clinical VSSA isolate. (Adapted from Ref. [11] which was published under an open-access licence agreement).

Unfortunately, PAP-AUC method is time-consuming, labor-intensive and costly which limits its use in routine clinical laboratories.

5. Epidemiology

While VRSA strains are limited to a handful of reported cases (14 total cases of VRSA in the U.S.) and VISA strains remain rare; hVISA strains are more common [9, 12, 34]. The true prevalence of hVISA is unknown, and estimates vary widely due to nonstandardized detection methods or absence of routine hVISA screening, variation in interpretation, clinical setting, geographical region, and differing patient populations [35]. Global hVISA rates among MRSA isolates have been reported to range from 0 to 73.7% [36].

In many studies, only the isolates suspected of being hVISA by screening methods (screening plates, Etest macromethod, Etest GRD) have been subjected to PAP-AUC. Some hVISA isolates may have been missed by screening methods in these studies. This may lead to an underestimation of the true prevalence [2, 27].

Although reported predominantly for MRSA; hVISA/VISA can be detected among methicillin-susceptible *S. aureus* (MSSA) strains [2, 37]. However, routine testing of MSSA isolates for the presence of hVISA/VISA for clinical purposes is not necessary and not recommended [10].

High-level resistance to vancomycin in *S. aureus* is very rare and all VRSA strains reported to date have been MRSA [9].

The proportion of *S. aureus* isolates demonstrating heteroresistance increases with increasing vancomycin MICs within the susceptible range, but heteroresistance has been reported in strains with MICs as low as 0.5 µg/ml [21, 38].

The main risk factors for hVISA and VISA infection appear to be prior MRSA colonization or infection and exposure to vancomycin. Most of hVISA/VISA infections occur in patients with serious underlying diseases such as malignancy, renal failure and diabetes, or in patients who have undergone major surgery [2, 12]. Nosocomial spread and rare outbreaks caused by VISA or hVISA have also been reported [34].

6. Clinical significance of hVISA/VISA and VRSA

The clinical significance of hVISA/VISA is difficult to determine due to differences in definitions and laboratory detection as well as the lack of well-controlled prospective studies [2, 21]. Commonly reported associations with hVISA/VISA infections include vancomycin treatment failure and high-inoculum infections such as bacteremia, endocarditis, deep abscesses, osteomyelitis, and prosthetic device infections [2, 21, 35, 36]. Some authors consider that hVISA/VISA could be responsible for treatment failure, whereas others have suggested that it has arisen as a consequence of treatment failure and prolonged vancomycin exposures [39]. It is difficult to determine, especially if it is not clear when the VISA or hVISA isolate was detected in the course of infection [21].

Interestingly, attenuated virulence of *S. aureus* with reduced susceptibility to vancomycin has been reported in some animal infection models [40, 41]. Pooled data from a meta-analysis showed similar mortality rates for hVISA and VSSA infections [36]. However, two recent studies have found a link between hVISA and higher mortality rate. Claeys *et al*. reported that patients with hVISA pneumonia experienced significantly higher inpatient mortality than those with VSSA pneumonia [42]. Hu *et al*. demonstrated that patients with hVISA bacteremia had a significantly higher in-hospital mortality than those with VSSA bacteremia [43].

The clinical significance of VRSA is unclear as only a few cases have been reported to date.

Persistent signs of infection and positive cultures for MRSA despite the administration of glycopeptide therapy, or relapse of infection after glycopeptide therapy can suggest an infection with hVISA or VISA [12]. Well-designed, large-scale prospective studies are needed to evaluate the clinical significance of these strains.

7. Therapeutic options

7.1. Role of surgery

Many patients with infections due to hVISA/VISA have high-inoculum infections (endocarditis, deep abscesses, osteomyelitis/septic arthritis and prosthetic device infections) [2, 12, 36].

Surgery is a useful adjunct to antimicrobial therapy for these patients. Main types of surgery include the drainage of abscesses or infected joints and removal of the infected prostheses [12].

7.2. Antimicrobial therapy

The emergence of hVISA/VISA clinical isolates has prompted the search for new antibiotics. While there are no guidelines regarding alternative antimicrobial therapy, there are a number of antimicrobial agents that have potential to be used in treatment of hVISA/VISA infections.

7.2.1. Daptomycin

Daptomycin is a lipopeptide antibiotic with activity against Gram-positive bacteria. In a study conducted by Wootton *et al.*, despite slightly raised MICs seen for strains with reduced susceptibility to vancomycin; daptomycin showed greater bactericidal activity than vancomycin for hVISA and VISA [44]. This indicates that, while there is a potential for cross-resistance between daptomycin and vancomycin, susceptibility to daptomycin is minimally affected by the presence of hVISA or VISA [10]. In several studies, daptomycin has been shown to have a good antimicrobial activity against the majority of hVISA isolates [27, 44, 45]. The highest rate of daptomycin nonsusceptibility was reported in a study evaluating 47 isolates of hVISA/VISA. In this study, the percentage of daptomycin nonsusceptible isolates was 15% for hVISA and 38% for VISA [35, 46]. Additionally, daptomycin has been shown to have *in vitro* activity against VRSA isolates. In a study conducted by Sievert *et al.*, one daptomycin nonsusceptible isolate was observed among 7 VRSA [47].

7.2.2. Linezolid

Linezolid is a synthetic antibacterial agent of the oxazolidinone class. Although resistance to linezolid has been reported in *S. aureus* isolates, rates of resistance remain very low. Linezolid was found to be useful for the treatment of hVISA/VISA infections [2, 48]. Also, data from a study demonstrated potent *in vitro* activity for linezolid against VRSA strains [47]. However, treatment with linezolid may be limited by toxicity. High rates of adverse reactions have been found for complex patients (seriously ill patients with multiple comorbidities) who received prolonged linezolid therapy [49]. Therefore, prolonged therapy should be used with caution in this patient group.

7.2.3. Tigecycline

Tigecycline is a glycylcycline antibiotic for intravenous infusion. In a study conducted by Sun *et al.*, 26 hVISA isolates and 1 VISA isolate were tested for tigecycline susceptibility. All isolates were found to be susceptible to tigecycline [50]. In another study, *in vitro* activity of tigecycline was evaluated against 33 VISA and 13 VRSA isolates. Tigecycline susceptibility rates were 97 and 92% for VISA and VRSA, respectively [51]. *In vitro* data have shown that tigecycline is active against hVISA/VISA as well as VRSA. Clinical studies are needed to determine the role of tigecycline in infections caused by *S. aureus* with reduced susceptibility to vancomycin.

7.2.4. New cephalosporins

New cephalosporins like ceftaroline and ceftobiprole have been shown to be active against hVISA and VISA *in vitro* and in animal studies. In rabbit models of endocarditis, these agents were superior to vancomycin against hVISA and VISA [2]. While results of *in vitro* and *in vivo* testing of these cephalosporins have been positive against hVISA/VISA, their clinical utility for infections caused by hVISA or VISA remains unknown [10].

7.2.5. Other antimicrobial agents

Other potentially active antimicrobials include lipoglycopeptides (dalbavancin, oritavancin, telavancin), quinupristin-dalfopristin, rifampin and fusidic acid. However, resistance develops rapidly with monotherapy with rifampin or fusidic acid. Therefore, these agents should be used in combination with another antistaphylococcal agent. The combination of rifampin and fusidic acid is an effective option [2]. Studies also suggest the potential for synergistic activity between vancomycin and various antimicrobials including beta-lactams and gentamicin against *S. aureus* with reduced vancomycin susceptibility [10].

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