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Original Article

Investigation of heteroresistant vancomycin intermediate *Staphylococcus aureus* among MRSA isolates

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Abstract

Introduction: Heteroresistant vancomycin intermediate *Staphylococcus aureus* (hVISA) testing is recommended when therapeutic failure is suspected in the clinics. In our research, we aimed to investigate the prevalence of hVISA among methicilline-resistant *S. aureus* (MRSA) isolates in our university hospital and compared three methods for detection of hVISA.

Methodology: One hundred MRSA clinical isolates were collected in our medical microbiology laboratory between 01.04.2018 and 01.10.2019. For screening of hVISA, we used two screening agar plates and used one commercial medium; brain heart infusion agar (BHI) plates containing 4 µg/mL vancomycin and 16 g/Lt casein (BHIA-VC; Satola's test), BHI agar plates containing 4 µg/mLvancomycin (BHIAV), and commercially obtained vancomycin resistant *Enterococci* (VRE) agar for detetection of hVISA. Colonies which could grow on plates were counted manually at 24th and 48th hours.

Results: Among 100 MRSA isolates, 43 (43%) were found as hVISA using Satola's test. BHIAV and VRE agar screening test results were found 70% and 4%, respectively. Finally, at the step, MIC values of 20 (47%) hVISA isolates reduced to 2 µg/mL after sub culturing for the gradient test.

Conclusions: We found higher rates of hVISA comparing other studies in Turkey. Both VRE agar and BHIAV screening test failed to detect hVISA properly. Meropenem in combination with vancomycin inhibited the growth of 90% hVISA isolates in our study.

Key words: *S. aureus;* MRSA; hVISA; screening; VRE agar.

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Introduction

Staphylococcus aureus is a Gram-positive microorganism which is responsible for many bacterial infections. The pathogen is characterized by a *coccus* morphology about 0.5 µ in diameter. The microorganisms can be observed singly, in pairs, in chain forms or in clusters [1]. *S. aureus* causes a broad spectrum of infections, which mainly involves skin, soft tissue, bone, and some biomedical devices such as catheters or prosthesis [2]. Methicillin resistant *S. aureus* (MRSA) is one of the most common nosocomial pathogens causing serious morbidity and high mortality rates [3]. Vancomycin is a glycopeptide antimicrobial, which has been one of the most effective therapeutic agents against MRSA for about thirty years [4]. Increased use of vancomycin to cover Gram-positive organisms (such as MRSA), has likely contributed to growing burden of less sensitive clones, and plenty of clinical research reports underlined the upward trend of vancomycin minimum inhibitory concentrations (MICs) for MRSA in the past two decades [5-7]. The first clinical vancomycin-intermediate *S. aureus* (VISA) strain (Mu50) with a vancomycin MIC of 8 µg/mL and the heteroresistant vancomycin-intermediate *S. aureus* (hVISA) isolate (Mu3) with an MIC of 2 µg/mL was reported in 1997 by Hiramatsu and co-workers [8]. hVISA is accepted to be a precursor of VISA and hVISA strain is composed of VISA subpopulations having different levels of vancomycin resistance [8]. Infections due to hVISA strains cause unique problems in the hospital settings. When routine antibiogram methods are used in the medical microbiology laboratory, hVISA strains are found susceptible to vancomycin (MIC < 4 µg/mL) and they are classified as susceptible even if these isolates contain subpopulations of 1 in 10^6 cells growing in the presence of ≥ 4 µg/mL of vancomycin [9,10]. The most reliable and reproducible approach for hVISA detection is the population analysis profile-area under the curve (PAP-AUC) method. However, this method is a labour-intensive and costly method which is not suitable for routine screening in the medical microbiology laboratories [11].

Satola *et al*. [12] proposed a practical method for screening of hVISA using brain heart infusion (BHI) agar containing vancomycin and casein. They reported that BHI screen agar plates containing 4 µg/mL vancomycin and 16 g/Lt casein (BHIA-VC) had 90% sensitivity and 95% specificity for detecting hVISA strains comparing with PAP-AUC method [12]. This study was cited by the EUCAST guideline for detection of resistance mechanisms Version 2.01 July 2017 with reference no.13 [12,13]. It is suggested that the MIC value of the isolate should be determined when using vancomycin to treat a patient with *S. aureus* infection. In some cases, especially when there is a therapeutic failure, testing for hVISA may also be warranted according to the EUCAST guideline for detection of resistance mechanisms in 2017. Since it is a complicated procedure to confirm hVISA, in the hospital settings, antimicrobial surveillance is performed by detection of VISA and vancomycin resistant *S. aureus* (VRSA) in practical. In our research, we investigated the prevalence of hVISA among MRSA strains which were isolated in our hospital and compared Satola's hVISA screening method with two other screening methods.

Methodology

One hundred non-repetitive MRSA isolates were collected in our university hospital clinical microbiology laboratory between 01.04.2018 and 30.09.2019. The isolates were identified as *S. aureus* via Vitek2 automated identification system (Biomerieux, Marcy-l'Étoile, France) during routine workflow. Antimicrobial susceptibility testing of the isolates for vancomycin and other drugs were performed using the same Vitek2 automated system and vancomycin MICs were also confirmed by broth micro-dilution test [14]. The isolates were stored at -20 °C in trypticase soy broth (TSB; BD, Franklin Lakes, USA) containing 20% glycerol and were subcultured twice on 5% sheep blood agar prior to the study.

For hVISA investigation, we used a method recommended by Satola *et al*. and compared three hVISA detecting methods by preparing two different screening agar plates and using one commercial media:

1. BHI screening agar plates containing 4 µg/mL vancomycin (Sigma-Aldrich, St. Louis, USA) and 16 g/Lt casein (Sigma-Aldrich, St. Louis, USA) (BHIA-VC), which was previously recommended by Satola, *et al*. [12].

2. Manually prepared BHI screening agar plates containing 4 µg/mL vancomycin (BHIA-V)

3. Commercially obtained vancomycin resistant Enterococci screening agar (VRE agar, GBL, Istanbul, Turkey) containing 6 µg/mL vancomycin and 10 µg/mL meropenem.

In the first step, a standard of 0.5 McFarland from an overnight culture in TSB was prepared. Then, three different agar plates (BHIA-VC, BHIA-V and VRE agar plates) were inoculated with the bacterial suspensions in TSB.

Four 10 µl droplets (totally 40 *µL*) from each suspension were dropped onto the BHIA-VC plates by a pipette, and allowed to air dry for approximately 5 min. Then the plates were incubated at 35 °C. Plates were examined at 24th and 48th hours, and individual colonies in each droplet were counted. A droplet with confluent growth was scored as having confluent growth. An isolate was considered hVISA if at least one droplet had two or more colonies according to Satola's method (Figure 1) [12].

In the same time, BHIA-V plates were inoculated in accordance with Satola's test and VRE agar plates were inoculated with 100 *µL* amount of bacterial suspension from TSB. The drops from bacterial suspensions were spread to all surfaces of the agar media using loop. They were allowed to air dry for 5 minutes and incubated at 35 °C. Plates were examined at 24th and 48th hours after

Figure 1. hVISA screening method recommended by Satola et al. [12].

hVISA colonies growing on brain heart infusion agar plates (containing 4 µg/mL vancomycin and 16 g/Lt casein) are observed in cream color.

incubation. Any colony that can grow in the presence of vancomycin was accepted as hVISA.

In the second step, we determined vancomycin MICs for the colonies which could grow on BHIA-VC plates by using gradient test (Oxoid, Hampshire, UK). Here, we wanted to see whether the MIC values of VISA/hVISA colonies would reduce or not.

Results

Study isolates were collected from throat swab (23%), tracheal aspirate (21%), wound (%21), sputum (12%), blood culture (11%) and other specimens (12%). Specimens were sent to our laboratory from various departments including pediatric immunology (27%), intensive care (27%), orthopedics (9%), general pediatrics (9%), internal medicine (6%), and other departments (12%) in our university hospital. Average age of the patients with MRSA infection was 36. Fifty-four percent of patients were male and 46% of patients were female. Most common primary diagnosis of patients was cystic fibrosis (22%), cerebro-vascular accident (12%) and respiratory failure (8%).

Among 100 MRSA isolates, bacterial growth was observed in 70 (70%) isolates with BHI-VA method and in 4 (4%) isolates with VRE agar screening method. We detected growing of hVISA colonies in 43 (43%) isolates using BHIA-VC method which was the reference method for our study (Satola *et al*., 2010). Comparing Satola's method, the sensitivities of the BHI-VA method and VRE agar screening method were 58.1% and 6.9%, respectively. Also, the specifities of the two tests were 21% and 98.2%, respectively.

In the second step, vancomycin gradient test results for 43 hVISA strains growing on BHIA-VC were; 8 µg/mL for two strains (4.6%), 4 µg/mL for 21 strains (48.8%), and 2 µg/mL for 20 strains (46.5%). MIC50 and MIC90 values of the 43 hVISA colonies were found 4 and 4 µg/mL, respectively, using gradient test method. Two strains whose vancomycin MICs were found 8 µg/mL by gradient test were also tested using broth micro-dilution method to confirm the vancomycin resistance, but MIC values reduced to susceptible ranges 0.5 and 2 µg/mL using broth micro-dilution method.

Discussion

Sancak, *et al*. [15] investigated 256 clinical MRSA isolates from 256 patients in a study in Turkey. After screening with BHI agar containing vancomycin, they confirmed positive results using PAP-AUC method. They reported that 46 (17.97%) isolates were hetero-VISA in their study . In a study in 2017, Tunç, *et al*. [16] investigated 52 MRSA clinical isolates and concluded that nine bacterial strains (17.3%) were hVISA according to the population analysis profile results in Malatya, Turkey . A great deal of researches was performed for detection of hVISA isolates. Hiramatsu *et al*. [8] found the prevalence of hVISA 9.3% in a study including 20 university hospitals, this rate was 20% in another university hospital, and 1.3% in non-university hospitals and clinics . Recently , Amberpet, *et al*. [17] investigated 500 non-repetitive MRSA isolates obtained from various clinical samples in India and they reported a rate of 12.4 % for isolation of hVISA (Amberpet *et al*., 2019). Comparing those studies, we have found higher rates of hVISA. The reason why we found higher rates may be the fact that there is an increasing trend of VISA and hVISA in the world and most of the samples sent to our laboratory were obtained from intensive care patients who were repeatedly exposed to vancomycin and other antibiotics. Significant amount of our study isolates were from cystic fibrosis patients (22%). Since the cystic fibrosis clinic in our university hospital is the only unit in our region and the third largest clinic in our country, we isolated high rate of MRSA from this clinic.

Walsh *et al*. [18] investigated 284 MRSA isolates and 45 staphylococcal strains with reduced susceptibility vancomycin using seven different methods including agar dilution, broth micro-dilution, gradient test (0.5 and 2.0 McFarland), vancomycin agar screening, modified vancomycin agar screening method and simplified population analysis. They compared the sensitivity and specificity of these methods with reference PAP-AUC method. In the study, they reported that BHI agar screening method (with 6 µg/mL vancomycin) had 22% sensitivity and 97% specificity. The sensitivity of the Mueller Hinton agar screening method (with 5 µg/mL vancomycin) was 20% and the specificity was 99%; the sensitivity of the simplified population analysis method was 71% and its specificity was 88%; the sensitivity of the gradient test (0.5 McFarland) was 82% and the specificity was 93%; the sensitivity of the other gradient test (2.0 McFarland) was 96% and the specificity was 97%.

In 2010, Satola *et al*. [12] investigated 140 MRSA clinical isolates with vancomycin MICs of 2 µg/mL by using reference broth micro-dilution method and they screened these isolates for hVISA using PAP-AUC and additional methods including E-test macro-method (with vancomycin and teicoplanin strips), E-test with vancomycin-teicoplanin double-sided and BHI screen agar plates containing 4 µg/mL vancomycin and 16 g/Lt casein (BHIA-VC). Each method was compared with

PAP-AUC as the reference test and the sensitivity of each method for detecting hVISA was found higher when the results were read at 48 h. In the study, BHIA-VC screen agar method was found 90% sensitive and 95% specific with a 0.5 McFarland inoculum having the best score of all tests [12].

Screening for hVISA by PAP-AUC method is the most reliable and reproducible approach but this method is labor-intensive, costly, and unsuitable for routine use in clinical laboratories [11]. The new method which was recommended by Satola, *et al*. [12] is easy to perform, inexpensive and suitable for routine use. This method was also cited as a promising method in the EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance Version 2.01 July 2017 with reference no.13 [12]. When we compared VRE agar and BHIA-V method with Satola's BHIA-VC method, we concluded that we both two tests failed to detect hVISA properly. Since VRE agar screening method had 6.9% sensitivity and BHI-VA method had 21% specicifity, it is not possible to use these tests as screening methods.

In our study, we included commercial VRE agar plates to see if these commercial and ready-to-use plates could also be used for screening hVISA isolates. In this stage, only 4 hVISA isolates could survive on VRE agar most probably to due to the inhibitory effect of meropenem (plus vancomycin) inside the commercial medium, so it is understood that this medium cannot be used for screening hVISA in the clinical microbiology laboratory settings. Since VRE agar containing 6 µg/mL vancomycin and 10 µg/mL meropenem inhibited the growth of 90% of the hVISA isolates, we thought that this combination might be used for eradication of hVISA. It was reported that infections with MRSA that have elevated vancomycin MICs within the range considered susceptible and infections with MRSA isolates which are found to be hVISA may be risk factors for the failure of vancomycin chemotherapy [19-21]. Combined antimicrobial therapy with other antibiotics having MRSA activity could potentially provide broader coverage to include these more-recalcitrant strains [22]. In our study, vancomycin plus meropenem inhibited the emergence of VISA sub-populations in the hVISA isolates. We think that further studies should be performed to see the synergistic effect of vancomycin plus meropenem on hVISA and investigate whether this combination could reduce growing of hVISA and emergence of VISA isolates in the clinics or not. As a limitation of our research, the concentration of meropenem used in the study was higher than the dose recommended for antimicrobial therapy. Novel studies investigating the inhibitory effects of new antimicrobial drug combinations on hVISA may be useful to find a way to overcome emergence of VISA and therapeutic failure. Accordingly, Lai and co-workers reported that cephalosporins in combination with vancomycin showed synergistic activity on hVISA clinical isolates [23]. They also concluded that the combination was effective irrespective of the MIC level of the cephalosporin used. In another study in 2017, Tran and co-workers reported that vancomycin in combination with beta-lactam antibiotics was synergistic against hVISA and showed higher antimicrobial activity than the vancomycin therapy alone [24]. If the culture result (MRSA) is accepted as a pathogen by the clinician, vancomycin mono-therapy is administered to the patient by the confirmation of infectious diseases consultant in our university hospital. When we searched the literature, we could not find any study investigating the effect of vancomycin plus meropenem on emergence of hVISA. We think that there is a lack of such studies in the field and our research may give a clue about the potential effects of carbapenem plus vancomycin combinations on hVISA strains.

As another point, Hanaki *et al*. [25] stated that the MICs of the strains established from the agar plates tend to be lower than those expected from the nominal vancomycin concentrations of the agar plates on which the colonies were formed. As an example, the strain established from the colonies formed on the agar plate containing 4 µg/mL may record an MIC of 2–4 µg/mL instead of expected MIC of > 4 µg/mL. We also investigated this phenomenon, and accordingly, MIC values of 20 (46.5%) hVISA isolates reduced to 2 µg/mL after subculturing for the test, however, MIC values of the 23 (53%) hVISA isolates did not reduce after subculturing during susceptibility test for vancomycin.

Conclusions

We detected higher rates of hVISA comparing other studies in Turkey. We found that BHIA-V and VRE agar screening methods failed to detect hVISA properly. So we concluded taht it is not possible to use these methods for screening of hVISA. In accordance with the previous studies, MICs of most hVISA isolates did reduce after subculturing. As another result, using meropenem in combination with vancomycin in the clinics may reduce the emergence of hVISA isolates. Futher studies investigating the effects of different doses of carbapenems in combination with vancomycin

are needed in order to see the inhibitory effects of these combinations on the emergence of hVISA strains.

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Authors' Contributions

DG was responsible for supervision, hypothesis, conceptualization of manuscript, analysis and interpretation of research. YZ was responsible for supervision, conceptualization of the paper and the analysis and interpretation of the data. ME and ABM were responsible for the conceptualization of the paper, analysis and interpretation of the data, and all authors; DG, YZ, ME and ABM contributed to the drafting and reviewing of the manuscript.

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