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Original Article

Vancomycin Resistance of *Staphylococcus aureus* in Korean Primary Hospitals

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According to a United States study, 13 cases of vancomycin-resistant *Staphylococcus aureus* (VRSA) have been reported to date. In 2001, a survey conducted in Korea revealed that 0.5% of methicillin-resistant *S. aureus* (MRSA) isolates have a vancomycin minimum inhibitory concentration (MIC) of 4 μg/ml, and are thus referred to as vancomycin intermediate *S. aureus* (VISA). However there are no reports of VISA found in primary hospitals. We evaluated the MIC of vancomycin in MRSA samples obtained from primary hospitals to determine whether VISA was present in primary hospitals. The population analysis was performed to determine whether hetero-VISA was present in primary hospitals. As a result, twenty of the 103 isolates were *S. aureus* which were all MRSA and the vancomycin MIC was similar to that seen in tertiary hospitals. Population analysis confirmed that three strains were hetero-VISA, by showing that one strain grew in 8 μg/ml vancomycin and that two strains grew in 4 μg/ml vancomycin. In conclusion, hetero-VISA was detected in Korean primary hospitals, which may develop into VISA, however a larger sample size will be needed to confirm these results.

Key Words: *S. aureus*, Vancomycin intermediate *S. aureus* (VISA), Primary-hospital

INTRODUCTION

Although methicillin-resistant *Staphylococcus aureus* (MRSA) outbreaks have mainly been isolated to hospitals, MRSA has been also reported in community settings recently. The antibiotic vancomycin is considered the best course of treatment against MRSA. However, *S. aureus* infection with a minimum inhibitory concentration (MIC) to vancomycin of 8 μg/ml was reported in Japan in 1997 (1). In the United States, 12 cases of vancomycin-resistant *S. aureus* (VRSA) and more than 100 cases of vancomycin-intermediate *S. aureus* (VISA) have been reported to date. VRSA means the strain revealing vancomycin MIC more than 32 μg/ml, however The Clinical and Laboratory Standards Institute (CLSI) lowered the criteria from 32 μg/ml to 16 μg/ml in 2006. The CLSI lowered the VISA criteria from 8~16 μg/ml to 4~8 μg/ml also (2). Since Hiramatsu, *et al*. reported MRSA infections with a MIC of 8 μg/ml, it has been found that resistant subpopulations coexist with susceptible populations, which is termed heteroresistance (hetero-VISA) (3, 4). To date, there are no reported on vancomycin resistant strains in Korea, but frequency of strains with MIC of 4 μg/ml against vancomycin is 0.5% of MRSA in tertiary hospitals of Korea suggesting VISA (5). This frequency may be even lower in primary hospitals. To further account

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for the incidence of VISA in primary hospitals, we studied vancomycin-intermediate MRSA in the region of Bundang, Korea. We did observe the presence of heteroresistant subpopulations (hetero-VISA) of MRSA in the primary hospitals. In addition, we investigated whether these isolated strains acquired resistance when exposed to high concentrations of vancomycin solution.

MATERIALS AND METHODS

Bacterial isolates and antimicrobial susceptibility testing

The subject was 504 specimens from clinical samples, including closed and open pus, biopsy, wound, body fluid, and sputum from primary hospitals in the Bundang district from January to July, 2012. One hundred and three specimens revealed bacterial growth which were isolated. The identification of bacterial strains was performed with the Vitek System (bioMérieux, Inc., Marcy-l'Étoile, France). The antimicrobial test was performed with disk diffusion, the Vitek System (bioMérieux, Inc.) or Microscan (Dade Behring, Inc., West Sacramento, CA, USA). The control strain used was the *S. aureus* ATCC 29213. The antibiotic disk included penicillin, oxacillin, vancomycin, teicoplanin, erythromycin, tetracycline, clindamycin, trimethoprim/sulfamethoxazole, rifampin, linezolid, ciprofloxacin, chloramphenicol, and quinupristin/dalfopristin.

Determination of vancomycin MIC

The vancomycin MIC was determined in 10 isolates among 20 isolates of MRSA.

The vancomycin MIC was also determined after 24 hr incubation in TSB broth.

Production of vancomycin stock solution

The weight of vancomycin powder was calculated according to below formula (6).

$$\text{Weight (mg)} = \frac{\text{Volume (ml)} \times \text{concentration (μg/ml)}}{\text{Vancomycin potency (900 μg/mg)}}$$

The vancomycin weight was calculated for a final concentration of 320 μg/ml, with consideration for vancomycin potency and the 10-fold dilution in agar medium. Weighed vancomycin powder was transferred to the sterilized tube, which was dissolved with 2 ml of sterile distilled water and filled with the rest with distilled water. Two-fold serial dilutions were then made, spanning the range of 32 μg/ml to 0.03 μg/ml. Each solution was stored at -70℃ refrigerator.

Determination of vancomycin MIC

Each bacterial specimen solution was adjusted to 10^5 CFU/spot, and 10 μl was added to each vancomycin concentrated Mueller hinton agar (CAMHA. Difco Laboratories BD, Detroit, MI, USA.) from 0.03 to 32 μg/ml. MIC was estimated following incubation at 35℃ for 24 hours. The control strain used was ATCC 29213 which is vancomycin susceptible.

Population analysis

The population analysis was performed to detect resistant subpopulations coexist with susceptible populations, which is termed hetero-VISA.

Production of vancomycin agar

Weighed Brain Heart Infusion Agar (BHIA Difco Laboratories BD, Detroit, MI, USA.) was added to 100 ml of distilled water and heated to 100℃, and then preserved at 50℃ in a water bath. The serial dilutions of vancomycin (2 ml of the 320 μg/ml to 0.3 μg/ml solutions) were each added to a separate sterile petri dish, and filled with 18 ml of warmed Brain Heart Infusion Agar (BHIA) to reach the final concentration of 32 to 0.03 μg/ml.

Population analysis

After10-fold dilution of bacterial solution from 10^{-1} to 10^{-7}, 100 μl of each serial dilution was inoculated on BHIA containing vancomycin with sterile tip, applied evenly with a sterile swab and incubated at 35℃ for 24 hours. The colony numbers were counted, and the results were charted. The control strains used were Mu50 which is strain with vancomycin MIC 16 and Mu3 which is strain of hetero VISA, obtained from the Korean Centers for Disease Control (CDC).

Acquired resistance after incubation in vancomycin solution

S. aureus colonies were incubated in solutions containing vancomycin of range from 0.03 mg/l to 1,280 mg/l. A thick bacterial suspension of 10 μl was added to each concentration of vancomycin BHIA, and incubated at 35℃ for 24 hours. We then observed if the strain acquired vancomycin resistance at higher concentrations, and if it acquired resistance proportionally. ATCC 29213 was used as control.

RESULTS

Twenty *S. aureus* strains were isolated from 103 specimens. Of these twenty, all were MRSA, with 100% methicillin resistance. Among 20 isolates of MRSA, vancomycin MIC against 10 isolates were determined; 1 strain at 0.5 μg/ml, 7 strains at 1 μg/ml, and 2 strains at 2 μg/ml. The control strain had a MIC of 1 μg/ml. Interestingly we found that the MIC increased after more 24 hr incubation in TSB broth (Table 1); 2 strains at 1 μg/ml, 5 strains at 2 μg/ml, 3 strains at 4 μg/ml.

Three strains that had a MIC 4 μg/ml were serially diluted from 10^{-1} to 10^{-7} CFU/ml and inoculated at each of the vancomycin concentrations and incubated. One strain (0501) grew up to 8 μg/ml, and the other 2 strains (1120, 1537) grew up to 4 μg/ml. The total colony count at each concentration was calculated by multiplying colony count by dilution factor. The colony number of 0501 strain was 2.7×10^5 at 1 μg/ml, 8.3×10^4 at 2 μg/ml, 7.0×10^4 at 4 μg/ml, and 1.1×10^3 at 8 μg/ml (Fig. 1). The resulting colony counts for the three strains at each concentration were then logarithmically graphed (Table 2, Fig. 2).

Next, we tested whether the strains could acquire vancomycin resistance. Five strains with MIC of 1 μg/ml were incubated in vancomycin solutions ranging from 0.03 to 1,280 mg/l. After this initial incubation step, all grew at l μg/ml. Additionally, the strains incubated at higher concentrations of vancomycin, of 640 and 1,280 mg/l, acquired increased resistance and grew at 2 μg/ml. The control strain grew at l μg/ml but didn't grow at 2 μg/ml.

Table 1. Minimal inhibitory concentraion of MRSA isolates against vancomycin

No	Specimen	MIC (μg/ml)	MIC 24 hr
1	0501	2	4
2	1049	1	2
3	1054	1	2
4	1055	2	2
5	1056	1	1
6	1064	0.5	2
7	1120	1	4
8	1537	1	4
9	2041	1	2
10	2518	1	1

Each bacterial specimen solution was adjusted to 10^5 CFU/spot, and 10 μl was added to each vancomycin concentrated Mueller hinton agar from 0,03 to 32 μg/ml. MIC was estimated following incubation at 35℃ for 24 hours.

Table 2. Growth rate of hetero-VISA strains at each vancomycin concentration

	Colony count at each vancomycin concentration (μg/ml)					
	0.25**	0.5	1	2	4	8
0501*	Confluent†	Confluent	2.7×10^5	8.3×10^4	7.0×10^4	1.1×10^4
1120	Confluent	2.6×10^5	8.1×10^4	1.6×10^4	3.2×10^3	0
1537	Confluent	Confluent	1.6×10^5	5.5×10^4	3.0×10^3	0

*; specimen number
**; vancomycin concentration, μg/ml
†; colony count

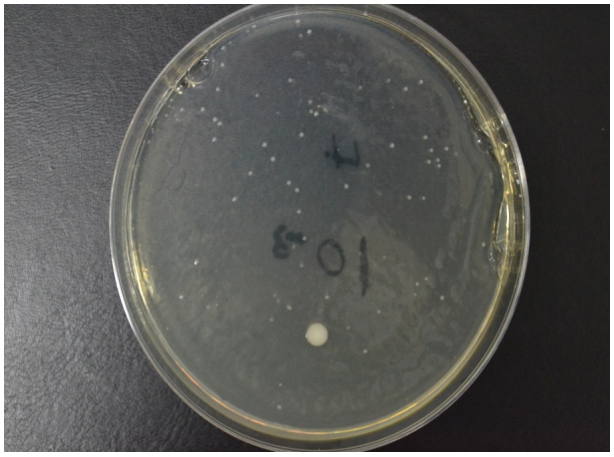

Figure 1. The count of CFU at each vancomycin concentration. After 10-fold dilution of bacterial solution (strain 0501) from 10^{-1} to 10^{-7}, 100 μl inoculated to each serial dilution of vancomycin BHIA with sterile tip, applied evenly with a sterile swab and incubated at 35℃ for 24 hours. We counted colony number and found the growth of 70 colonies of strain 0501 with 10^{-3} dilution at vancomycin 4 μg/ml.

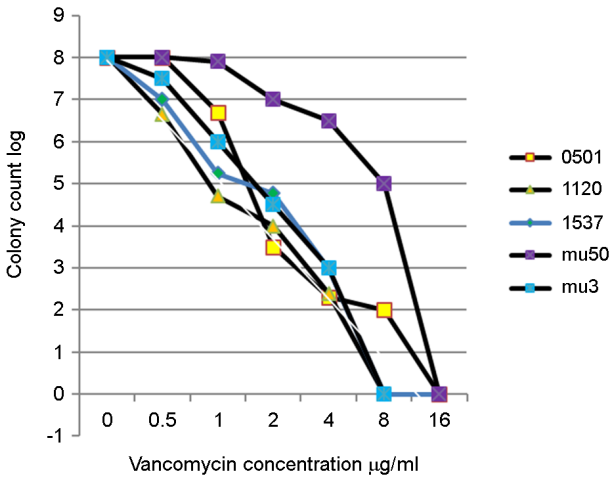

Figure 2. Logarithmically charted CFU at each vancomycin concentration. After 10-fold dilution of bacterial suspension from 10^{-1} to 10^{-7}, 100 μl of each serial dilution was inoculated on BHIA containing vancomycin, After incubation the colony numbers were counted and the colony number at each vancomycin concentration was charted logarithmically. (axis X, vancomycin concentration range; 0~16 μg/ml, axis Y, colony count at each dilution from 10^{-1} to 10^{-7}, range; 0~8)
mu50, mu3; control strain, 0501,1120,1537; test strain

DISCUSSION

There have been reports of vancomycin-resistant *Staphylococci* since the 1990s (1). The VISA criteria was lowered in 2006 from 8~16 μg/ml to 4~8 μg/ml to raise awareness about vancomycin resistance (2), along with concerns that there could be treatment failure even at 4 μg/ml. In Korea, VISA was first reported by Bae in 2000 (7). Kim, *et al*. reported 218 (0.5%) of 3,756 MRSA strains had a MIC of 4 μg/ml in 2001 (5). An additional 2 cases of MIC of 4 μg/ml were added in 2008 (8). Hiramatsu, *et al*. reported occurrence of "heteroresistance," where there is presence of a vancomycin-resistant subpopulation within the susceptible population (3, 9). It is not thought that VRSA is derived from vancomycin-resistant *Enterococcus* (VRE) because there are no vanA or vanB resistant genes present and the MIC is low (8). The more likely resistance mechanism is due to thickened cell walls or alterations in the cell wall peptidoglycan after selective pressure from vancomycin exposure, which then prevents permeability to vancomycin (10). Other possible explanations would be new genetic alterations that allow for vancomycin resistance (11, 12). For the effective treatment of vancomycin-resistant infections, positive results have been found with a combination of quinupristin-dalfopristin, linezolid, and arbekacin, or vancomycin and β-lactam (13).

In this study, 20 *S. aureus* were isolated from 103 specimens. Although the study number was small, the results were found to be significant. All 20 isolates of *S. aureus* were found to be MRSA with 100% resistance. To our knowledge, this is the first study to report 100% methicillin-resistant strains of *S. aureus* in Korean clinical isolates. Additional studies with a larger sample size are necessary.

These strains were then examined for vancomycin resistance. Ten strains which was estimated the MIC were found to have vancomycin-susceptible, ranging of a MIC from 0.5 μg/ml to 2 μg/ml. The control strain *S. aureus* ATCC 29213 which is susceptible to vancomycin showed 1 μg/ml. The MIC have been reported at less than 0.5 μg/ml 9.2%, 1 μg/ml 79.9%, 2 μg/ml 10.5%, 4 μg/ml 0.3%

in tertiary hospitals (14). Our study reveals that VRSA occurrence is not lower in primary hospitals than that in tertiary hospitals. We expected lower MIC in primary hospitals, but it did not turn out to be lower than that in tertiary or university hospitals.

Interestingly, we found that the MIC increased after more 24 hr incubation in TSB broth (Table 1); 2 strains at 1 μg/ml, 5 strains at 2 μg/ml, 3 strains at 4 μg/ml. Three strains that had a MIC 4 μg/ml were serially diluted and population analyzed.

In this study, 3 strains (30%) from primary hospitals were found to be heteroresistant. This percentage is compared to findings of 9.3% in Japan (3), 12% in the USA (4). These studies were of tertiary or university hospitals, and the results were similar between that of tertiary hospitals and that of primary hospitals.

We also examined the ability of these strains to acquire resistance. Five strains were randomly selected and all were grew at 1 μg/ml. Bacteria incubated at the higher concentrations of vancomycin of 640 and 1,280 mg/l acquired resistance and grew at 2 μg/ml. Only the strains incubated at these higher concentrations effectively grew at 2 μg/ml, suggesting increased vancomycin resistance. These findings support the possibility that cell wall alterations convey resistance to high concentrations of vancomycin (15).

In conclusion, clinical samples from Korean primary hospitals showed 100% MRSA. The calculated vancomycin MIC occurrence was not lower in primary hospitals than that in tertiary hospitals. Population analysis revealed that heteroresistant-VISA is present in Korean primary hospitals.

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