Efficacies of Vancomycin, Arbekacin, and Gentamicin Alone or in Combination against Methicillin-Resistant *Staphylococcus aureus* in an In Vitro Infective Endocarditis Model

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We adopted an in vitro infective endocarditis model (IVIEM) to compare the efficacy of vancomycin (VAN), arbekacin (ABK), and gentamicin (GEN) alone or in combination. Using two strains of clinically isolated methicillin-resistant *Staphylococcus aureus*, one GEN susceptible (GS171) and one GEN resistant (GR153), fibrin clots were prepared and suspended in the IVIEM. Antibiotics were given as boluses every 6 h (q6h), q12h, or q24h or by continuous infusion with VAN, q12h or q24h with ABK, and q8h or q24h with GEN. For combination treatment, VAN q12h plus ABK q24h and VAN q12h plus GEN q24h were given. Fibrin clots were removed from each model at 0, 8, 24, 32, 48, and 72 h, and the bacterial densities were determined. The number of colonies within the fibrin clot was significantly decreased in all study groups compared with control groups ($P < 0.001$). When VAN and ABK were administered alone, the number of colonies was significantly lower in GS171 than in GR153 by 8 h after administration ($P = 0.02$) and was lowest in GS171 when ABK was administered q12h ($P = 0.01$). At 72 h, ABK or VAN alone produced equivalent bacterial reductions regardless of dosing frequency and GEN resistance. In GR153, VAN plus ABK showed an additive effect till 24 h, although VAN plus GEN showed indifference. Our data suggest that ABK could be used as an alternative to VAN in GEN-resistant staphylococcal endocarditis. An additive effect was seen when VAN and ABK were used together in GEN-resistant strains until 24 h; however, further studies are warranted for the clinical application of this combination.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important pathogen that could usually be treated with vancomycin. However, the response to vancomycin is not satisfactory in serious infection, and the number of vancomycin-resistant strains has increased, requiring measures to counter these problems (10, 18, 21, 24, 28). Arbekacin is an antibiotic with a high efficacy against gram-positive bacteria, and its MIC was shown to be effective in gentamicin-resistant *S. aureus* and MRSA so that it is known as one of the antibiotics that could replace vancomycin (4, 15, 16, 31, 32, 33). Various combinations of aminoglycosides had been tried in clinical strains which were not completely responsive to vancomycin, but the recent trend of increasing prevalence of aminoglycoside resistance makes treatment difficult (14, 22). Nonetheless, arbekacin is effective against MRSA, with a MIC range of 0.39 to 3.13 $\mu\text{g/ml}$. The MIC at which 90% of the isolates tested are inhibited was 1.56 $\mu\text{g/ml}$ (15), and some synergistic effect was demonstrated in in vitro studies if it was combined with vancomycin (17, 33). However, in vitro study results could not be applied directly to the clinical setting, so many studies with an in vitro infection model have been attempted in recent years (6, 13). We used the in vitro infective endocarditis model (IVIEM) to confirm the efficacies of each antibiotic, including vancomycin,

arbekacin, and gentamicin, against MRSA and to compare these efficacies to those of vancomycin and gentamicin in combination and vancomycin and arbekacin in combination.

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MATERIALS AND METHODS

Bacterial strains and test of antibiotic susceptibility. Two clinical strains of *S. aureus* without mucoid characteristics, GR153 and GS171, were used in the study. Both strains were methicillin resistant, GR153 was gentamicin resistant, and GS171 was gentamicin sensitive. These strains were chosen from 549 clinical strains of *S. aureus* (32).

Mueller-Hinton broth (CAMHB; Difco laboratories, Detroit, Mich.) containing 25 mg of calcium/liter and 12.5 mg of magnesium/liter was used in the test of antibiotic susceptibility and the infective endocarditis model. The number of colonies was measured by using tryptic soy agar (TSA; Difco). The liquid medium minimum dilution method was used based on the NCCLS method for MIC determination (25). The inoculation concentration was $\sim 5 \times 10^5$ CFU/ml. The standard strain used for quality control was *S. aureus* ATCC 29213. The strain was placed in tryptic soy broth (Difco) containing 10% glycerol, kept at -70°C , and subcultured in TSA for every week during the study period.

Antibiotics. Oxacillin (Sigma Chemicals Co., St. Louis, Mo.), vancomycin (Sigma), gentamicin (Sigma), and arbekacin (Meiji Seika Kaisha, Tokyo, Japan) powders were prepared as solutions according to NCCLS or manufacture guidelines and kept at -70°C until use.

Fibrin clot preparation. Study strains were cultured for 18 h at 35°C in a shaking incubator, centrifuged at $3,500 \times g$ for 15 min, and diluted with CAMHB so the final concentration would be approximately 10^9 to 10^{10} CFU/0.1 ml (29). The fibrin clot suspension was prepared by adding 0.5 ml of cryoprecipitate

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(provided by the west blood bank of the Korean Red Cross), 0.035 ml of platelet concentrate (250,000 to 500,000 platelets/clot), 0.1 ml of suspension containing the strain, and 0.035 ml of aprotinin solution (2,000 U of inhibitory kallikrein/ml; Boehringer Mannheim, Heidelberg, Germany) into a 1.5-ml sterilized Eppendorf tube. A monofilament line was inserted into each sterilized Eppendorf tube, and 0.06 ml of bovine thrombin solution (5,000 U/ml; Sigma) dissolved with calcium (50 mmol; Sigma) was added. The prepared fibrin clot was taken from the Eppendorf tube with a 22-gauge needle, which was attached to the IIVIEM (8).

IIVIEM. The IIVIEM prepared was a one-compartment model and was equipped with a tube with a cap where the fibrin clot could be attached as previously described (13, 20). After 700 ml of CAMHB medium was placed inside the model by using 6 sets in 2 fibrin clots, the study was conducted by eliminating each set during 72 h. The temperature was maintained at 37°C for 72 h, and the medium within the model was stirred well with a stir bar. The medium was removed at a monoexponential elimination rate from the model containing the antibiotic with pharmacokinetics similar to those in humans, and new medium was supplied at the same rate as the removal rate. The half-life of vancomycin was 6 h, and those of arbekacin and gentamicin were 3 h (11).

The study for vancomycin was performed with two groups: the intermittent group, to which vancomycin was given every 6, 12, and 24 h as a bolus, and the continuous group, for which the loading dose would reach the maximum concentration of 20 µg/ml (11). Arbekacin was administered every 12 and 24 h, and gentamicin was administered every 8 and 24 h. Concentrations of human serum were simulated as follows. The target concentration of vancomycin in the continuous administration group was 20 µg/ml, whereas the target concentrations in the 6-, 12-, and 24-h groups were maximum concentrations of 25, 30, and 60 µg/ml, respectively (11). The minimum concentration was set at 5 µg/ml in all groups. The target maximum concentration for the arbekacin 12- and 24-h groups were 9 and 17 µg/ml, respectively, with a minimum target concentration of 0.5 µg/ml (15). The target maximum concentrations of the gentamicin 8- and 24-h administration groups were 5 and 15 µg/ml, respectively, with a minimum target concentration of 1 µg/ml (11). The removal rates of antibiotics in the vancomycin and arbekacin combination group and the vancomycin and gentamicin combination groups were set to those of arbekacin and gentamicin, respectively, which have faster half-lives. Vancomycin, having a relatively slower half-life, was supplemented through a supplemental chamber (3). The experiment was repeated twice with antibiotic-administered groups and control groups with no antibiotic administration.

Pharmacokinetic-pharmacodynamic analysis. To measure the concentrations of antibiotics, a 0.5-ml sample was taken from the medium at 0.25, 1, 2, 4, 6, 8, 24, 32, 48, and 72 h after administration and kept at -70°C until measurement. The pharmacokinetic parameters were from WinNonlin (version 3.0; Palo Alto, Calif.). All parameters were measured based on the one-compartment model according to the log-linear trapezoidal method. The pharmacodynamic parameters measured in vancomycin, arbekacin, and gentamicin were the ratio between the maximum concentration ($C_{\max}$) and the MIC ($C_{\max}/\text{MIC}$ ratio), the time period of maintaining the concentration higher than the MIC for 24 h ($T > \text{MIC}$), and the ratio of the area under the concentration-time curve from 0 to 24 h (AUC_{0-24}) to the MIC ($\text{AUC}_{0-24}/\text{MIC}$ ratio).

Fibrin clot killing curve. Two fibrin clots were taken from the model at 0, 8, 24, 32, 48, and 72 h. After measuring the weight of fibrin clots, the clots were finely homogenized with a homogenizer (clearance, 0.09 to 0.16 mm; Wheaton Science, Millville, N.J.), diluted in 0.9 ml of physiologic saline solution, and then plated on TSA plates. The media were cultured at 35°C for 24 h, and the number of colonies was measured and averaged as CFU per gram. To monitor the emergence of resistant organisms during the experiment, each antibiotic was divided in aliquots of media containing four and eight times the MIC. Potential antibiotic carryover samples (0.1 ml) were filtered through a 0.45-µm-pore-size filter (Millipore), placed on TSA plates, and then incubated for 24 h. The limit of detection in our laboratory has been determined to be 100 CFU/ml.

The synergic, additive, indifferent, and antagonistic effects were measured for the evaluation of antibiotic combinations. The synergic effect was defined as a ≥ 100 -fold increase in killing with the combination, in comparison with the most-active single drug. The additive effect was defined as a 10- to 100-fold decrease in the number of colonies when the combination was used compared with when an individual antibiotic was used at a given time. Indifference was defined as a decrease of less than 10-fold. The antagonistic effect was defined as a ≥ 100 -fold decrease in killing at a given time with the combination compared with the most-active single drug alone (7).

Measurement of antibiotic concentrations. The concentrations of antibiotics were measured by a fluorescence polarization immunoassay (Abbot Diagnostics

TABLE 1. Pharmacokinetic data in the IIVIEM^a

Drug and dosing regimen ^c	Concn (μg/ml) ^b			t _{1/2} (h) ^c
	C _{peak}	C _{trough}	C _{pss} ^d	
VAN				
CI			20.6 ± 0.7	5.8 ± 0.2
q6h	25.6 ± 3.2	8.9 ± 1.1		5.5 ± 0.5
q12h	32.4 ± 2.8	6.1 ± 1.7		7.0 ± 0.6
q24h	68.3 ± 2.1	8.2 ± 2.1		6.0 ± 0.4
ABK				
q12h	11.6 ± 0.7	0.76 ± 0.2		3.0 ± 0.2
q24h	18.0 ± 0.9	0.18 ± 0.1		3.6 ± 0.3
GEN				
q8h	7.3 ± 1.3	1.1 ± 0.3		2.9 ± 0.5
q24h	17.2 ± 1.9	0.2 ± 0.1		3.7 ± 0.3

^a All values are means ± standard deviations ($n = 4$).

^b Concentrations at steady state.

^c $t_{1/2}$, half-life.

^d C_{pss} , peak concentration at steady state.

^e Abbreviations: VAN, vancomycin; ABK, arbekacin; GEN, gentamicin; CI, continuous infusion; q6h, every 6 h; q8h, every 8 h; q12h, every 12 h; q24h, every 24 h.

TDx, Irving, Tex.), of which the interday and intraday coefficients of variation were less than 10% for all standards. The reagent for arbekacin was obtained from Meiji Seika Kaisha Co. The interday coefficients of variation for arbekacin could not be obtained because we measured all of the samples as a batch. The sensitivities of each antibiotic were 2.0, 0.4, and 0.27 µg/ml for vancomycin, arbekacin, and gentamicin, respectively. The control reagent was prepared within CAMHB, and the concentrations of control reagent and samples were measured twice.

Statistical analysis. The number of colonies ($\log_{10}$ CFU/gram) within the fibrin clot according to time (i.e., at 8, 24, and 72 h) was analyzed by one-way analysis of variance and Duncan's test in SPSS, version 10.0. Significance was determined at a P value of <0.05 .

RESULTS

MICs for strains. The MICs of oxacillin, vancomycin, arbekacin, and gentamicin were 16, 1, 1, and 32 µg/ml, respectively, for strain GR153, and 256, 0.5, 0.5, and 0.25, respectively, for strain GS171. Both GR153 and GS171 were MRSA, with a susceptibility for vancomycin and arbekacin. GR153 was a gentamicin-resistant strain, whereas GS171 was a gentamicin-susceptible strain.

Pharmacokinetic-pharmacodynamic analysis. The pharmacokinetic indices, including maximum and minimum concentrations and half-life, were as expected (Table 1). Vancomycin, for instance, maintained a concentration greater than the MIC throughout the study period in GR153 and GS171. The $\text{AUC}_{0-24}/\text{MIC}$ ratio was higher with vancomycin than with arbekacin in both GR153 and GS171 (Table 2). No difference was seen in the pharmacokinetics of antibiotics used in combination and alone.

Fibrin clot killing curve. The number of colonies within the fibrin clot was significantly decreased in all groups compared with control groups, except for gentamicin used in GR153 ($P < 0.001$). The numbers of colonies were significantly lower in GS171 by 8 h when vancomycin or arbekacin was used alone than those in GR153 ($P = 0.02$). The colony number was lowest when arbekacin was administered every 12 h in GS171 ($P = 0.01$). When either of two agents was given alone, no

TABLE 2. Pharmacodynamic data in the IVIEM^a

Drug and dosing regimen ^c	Result for strain:					
	GR153			GS171		
	<i>T</i> > MIC (%) ^b	AUC ₀₋₂₄ /MIC	<i>C</i> _{max} /MIC	<i>T</i> > MIC (%)	AUC ₀₋₂₄ /MIC	<i>C</i> _{max} /MIC
VAN						
CI	100	494.4 ± 9.5	ND ^d	100	988.8 ± 4.8	ND
q6h	100	562.8 ± 4.9	ND	100	1,124.7 ± 5.6	ND
q12h	100	465.9 ± 3.1	ND	100	931.8 ± 5.7	ND
q24h	100	798.7 ± 8.2	ND	100	1,597.4 ± 6.9	ND
ABK						
q12h	ND	102.4 ± 1.0	11.6 ± 0.7	ND	204.9 ± 1.8	22.9 ± 0.9
q24h	ND	94.0 ± 2.2	18.0 ± 0.9	ND	198.1 ± 1.5	37.2 ± 0.6
GEN						
q8h	ND	2.9 ± 0.9	0.2 ± 0.1	ND	375.6 ± 2.8	29.2 ± 1.8
q24h	ND	2.9 ± 0.5	0.5 ± 0.2	ND	372.8 ± 1.6	68.8 ± 2.1

^a All values are means ± standard deviations (*n* = 4).^b *T* > MIC, percentage of time that the drug concentration was above the MIC.^c Abbreviations: VAN, vancomycin; ABK, arbekacin; GEN, gentamicin; CI, continuous infusion; q6h, every 6 h; q8h, every 8 h; q12h, every 12 h; q24h, every 24 h.^d ND, not done.

difference was seen in the number of colonies (*P* = 0.11) and in the bacterial killing effect according to the administration time (*P* = 0.61).

Although no difference in bacterial killing effect was seen in GS171 when arbekacin or gentamicin was given individually every 8 and 12 h (*P* = 0.24), gentamicin showed a higher bacterial killing effect when administered every 24 h (*P* = 0.04) (Table 3).

The number of colonies was reduced with time in GR153 until the end of the study when vancomycin or arbekacin was administered alone. On the other hand, the number of colonies

was decreased significantly in GS171 when vancomycin, arbekacin, or gentamicin was administered individually until 8 h of administration and did not decrease significantly afterwards until the end of the study (Fig. 1).

An additive effect was seen when vancomycin and arbekacin were used in combination in GR153 until 24 h, and indifference was shown afterwards. On the other hand, indifference was seen throughout the study period both in GR153 with the combination of vancomycin and gentamicin and in GS171 with the combination of vancomycin and gentamicin and with the combination of vancomycin and arbekacin (Fig. 1).

TABLE 3. Residual organisms at 8 and 72 h in bacterial inocula in the IVIEM^a

Antibiotic regimen ^b	Log ₁₀ CFU/g			
	GR153		GS171	
	8 h	72 h	8 h	72 h
Growth control	11.90 ± 0.11	12.40 ± 0.30	11.89 ± 0.10	12.42 ± 0.31
VAN				
CI	8.36 ± 0.07 ^c	6.25 ± 0.50 ^c	5.94 ± 0.01 ^{c,d}	6.28 ± 0.01 ^c
q6h	7.01 ± 0.25 ^c	6.12 ± 0.32 ^c	7.37 ± 0.07 ^{c,d}	6.20 ± 0.07 ^c
q12h	8.22 ± 0.22 ^c	6.00 ± 0.77 ^c	6.20 ± 0.30 ^{c,d}	6.30 ± 0.21 ^c
q24h	7.76 ± 0.13 ^c	5.90 ± 0.40 ^c	5.81 ± 0.10 ^{c,d}	5.92 ± 0.10 ^c
ABK				
q12h	7.64 ± 0.22 ^c	6.31 ± 0.10 ^c	4.56 ± 0.053 ^{c,d,e}	6.20 ± 0.77 ^c
q24h	8.45 ± 0.17 ^c	6.19 ± 0.30 ^c	6.47 ± 0.16 ^{c,d}	6.00 ± 0.04 ^c
GEN				
q8h	10.90 ± 0.50	12.14 ± 0.21	5.17 ± 0.16 ^{c,d}	6.18 ± 0.27 ^c
q24h	11.60 ± 0.80	12.10 ± 0.59	5.93 ± 0.13 ^{c,d}	4.75 ± 0.83 ^{c,f}
VAN q12h + ABK q24h	7.10 ± 0.19 ^c	5.72 ± 0.85 ^c	6.03 ± 0.92 ^{c,d}	5.76 ± 0.27 ^c
VAN q12h + GEN q24h	7.80 ± 0.50 ^c	5.48 ± 0.18 ^c	5.54 ± 0.09 ^{c,d}	4.69 ± 0.18 ^c

^a All values are means ± standard deviations (*n* = 4).^b CI, continuous infusion; VAN, vancomycin; ABK, arbekacin; GEN, gentamicin; q6h, every 6 h; q8h, every 8 h; q12h, every 12 h; q24h, every 24 h.^c Statistically significantly different from growth control (*P* < 0.0001).^d Statistically significantly different from colony count of GR153 at 8 h (*P* = 0.02).^e Statistically significantly different from colony count of GR153 and GS171 at 8 h (*P* = 0.01).^f Statistically significantly different from arbekacin q24h in GS171 at 72 h (*P* = 0.04).

FIG. 1. Fibrin clot killing curves for GRI153 (A, C, E, and G) and GSI71 (B, D, F, and H) in the IVIEM. The results are presented as means $\pm$ standard deviations of colony counts (Log_{10} CFU/gram) for the results from quadruplicate measurements. Abbreviations: VAN, vancomycin; ABK, arbekacin; GEN, gentamicin; q6h, every 6 h; q12h, every 12 h; q24h, every 24 h.

The development of resistance was not detected throughout the entire study period in either strain.

DISCUSSION

MRSA poses a serious threat, since it frequently shows resistance to various antibiotics, including aminoglycosides, macrolides, and fluoroquinolones as well as β -lactams (1, 23, 27). Though vancomycin is the drug of choice for MRSA infection, it sometimes shows delayed response or even clinical failure in serious infection; there is an urgent need to develop alternative antimicrobials (10, 18).

Arbekacin is a derivative of dibekacin, it shows no cross-resistance with other aminoglycosides, and it has a low modification rate for the several aminoglycoside-modifying enzymes, so this antibiotic is expected to be used to treat MRSA infection (12, 14, 16). However, most of the studies done on the effect of arbekacin were performed on the basis of an in vitro method (4, 17, 30, 33). An in vitro infection model offers the benefits of effectiveness in evaluation of the efficacy of an antibiotic (since the half-life, dose, and interval could be regulated to fit human pharmacokinetics), no difference compared with animal models, and the fact that the study environment could be regulated (8, 9, 19).

When vancomycin, arbekacin, or gentamicin was administered alone, although the early bactericidal effect of these antibiotics was delayed in the gentamicin-resistant MRSA compared with the susceptible strain, the effect became similar with time. The possibilities of a relationship between gentamicin resistance and a delayed initial bactericidal effect might be considered.

The effectiveness of arbekacin was comparable to that of vancomycin after 72 h of administration, which suggested that arbekacin could also be effective in the treatment of infective endocarditis by MRSA. Comparing the effectiveness of arbekacin and gentamicin alone, the effect of gentamicin administered every 24 h was highest against GS171, possibly because the $C_{\max}/\text{MIC}$ ratio was the highest.

As a time-dependent antibiotic, it was reported that vancomycin would theoretically show the most efficacy when administered continuously (26). Nonetheless, no difference in killing was present in the present study, as shown in Fig. 1, probably because the concentration was higher than the MIC during the entire period of study in the intermittent and continuous treatment groups. While the magnitude of the $\text{AUC}_{0-24}/\text{MIC}$ ratio varied according to the dosing interval, no difference was noted in the fibrin clot killing curve in this study. It would be partially due to the fact that all the regimens achieved the threshold of maximum killing, which was yet unknown. Since at least 50% of vancomycin is protein bound, the level of functioning free vancomycin was actually half the concentration we simulated. So the results in this study could be overestimated. But, as seen in the results, the trough level of vancomycin exceeded the MIC in both strains throughout this experiment, even considering the protein binding portion. It could not be concluded whether using the level of serum rather than the level of free vancomycin resulted in overestimation of bactericidal effect or not. But as a time-dependent antibiotic, the efficacy of vancomycin may not be overestimated at least.

Arbekacin also did not show any difference in bactericidal

ability according to the dosing interval, but it was most effective at a 12-h interval in GR153 and GS171 both until 24 h and showed no difference afterwards, suggesting that once-daily administration of arbekacin would be effective in both GR153 and GS171.

Some studies reported that combining vancomycin and gentamicin might show a partial synergic effect; however, this synergic effect has not been established and differs according to MIC of gentamicin (11, 14, 22, 31). Furthermore, considering the high gentamicin resistance rate of MRSA in Korea (MIC at which 90% of isolates tested are inhibited, 128 $\mu\text{g}/\text{ml}$; susceptibility, 6.8%) (32), the possibility of a synergic effect of vancomycin plus gentamicin is very low. However, an in vitro study reported that a synergic effect was present in most cases of arbekacin plus vancomycin (17, 33). Preliminary in vitro time-kill data showed indifferent effects when vancomycin was combined with gentamicin or arbekacin, irrespective of gentamicin resistance (data not shown).

According to the study by Kim et al. (14), no synergic effect was present in the regimen of vancomycin plus gentamicin on the strain for which the MIC of gentamicin was 32 $\mu\text{g}/\text{ml}$, as in the case of GR153 used in the present study. In our study, no synergic effect was demonstrated when vancomycin was combined with gentamicin, but in case of vancomycin plus arbekacin in GR153, an additive effect was seen until 24 h. Moreover, indifference was seen when vancomycin plus arbekacin or vancomycin plus gentamicin was used in GS171. This result was seen probably because the synergic effect was affected not only by the degree of gentamicin resistance but also by the inoculum size, the activity of bacteria, the ability to infiltrate into the fibrin clot according to time, and the degree of protein binding.

Although not seen in the results, in the case of vancomycin, the concentration of this antibiotic within the fibrin clot was maintained at a similar level within the medium during 8 h so that the concentration remained similar throughout the study period. On the other hand, the concentrations of arbekacin and gentamicin by 24 h were about half the initial doses, showing significant concentration changes according to time. The degree of invasion into the fibrin clot was different according to the types of antibiotics, and the antibiotic concentration difference was present between serum and the fibrin clot and between the center and edge of the fibrin clot (2, 5). This suggests that the concentration difference between the medium and the fibrin clot and the concentration difference according to the types of antibiotics and time affected the antibiotic ability against MRSA.

In conclusion, the antibiotic effect of arbekacin against MRSA was comparable to that of vancomycin in the IVIEM; thus, this drug could be used as an alternative when vancomycin is no longer effective in patients with MRSA infective endocarditis. Furthermore, an additive effect until 24 h of administration when vancomycin is used in combination with arbekacin for gentamicin-resistant MRSA suggests the usefulness of this combination at the early stage in patients with infective endocarditis. Additional studies would be needed in the future to evaluate the clinical significance of the present study results and to search for other antibiotic therapies, such as the administration of high-dose arbekacin and combinations of other antibiotics.

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