Preferential Emergence of Reduced Vancomycin Susceptibility in Health Care-Associated Methicillin-Resistant *Staphylococcus aureus* Isolates during Continuous-Infusion Vancomycin Therapy in an *In Vitro* Dynamic Model[▽]

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Bacterial killing and the development of reduced vancomycin susceptibility during continuous-infusion vancomycin (CIV) therapy were dependent on the area under the concentration-time curve over 24 h divided by the MIC (fAUC$_{24}$/MIC), with values of $\geq$240 (equivalent total serum AUC$_{24}$/MICs of $\geq$480) being bactericidal and suppressing emerging resistance in methicillin-resistant *Staphylococcus aureus* (MRSA). Also, vancomycin therapy was less likely to be bactericidal and 4.4 times more likely to lead to reduced vancomycin susceptibility in health care-associated MRSA than in community-associated MRSA.

Infections associated with methicillin-resistant *Staphylococcus aureus* (MRSA) with higher vancomycin MICs (e.g., >1 mg/liter) have been linked to poor clinical outcomes (9, 16, 28). Furthermore, exposure to low vancomycin concentrations for prolonged durations has been associated with increasing or selecting higher MICs, especially when the bacterial burden is large or a foreign device is involved (11, 13). Concerns that traditional vancomycin dosing may be inadequate for treating MRSA infections have led to notable changes in practice. In 2006, the Clinical and Laboratory Standards Institute (CLSI) lowered the vancomycin susceptibility breakpoint from 4 to 2 mg/liter (29). Furthermore, a 2009 consensus review of vancomycin therapeutic monitoring by the American Society of Health-System Pharmacists, the Infectious Diseases Society of America, and the Society of Infectious Diseases Pharmacists recommended more aggressive dosing to achieve serum troughs of $\geq$10 or 15 to 20 mg/liter for serious and complicated infections (22). These levels were proposed as practical surrogates for vancomycin AUC$_{24}$/MICs (area under the concentration-time curve over 24 h divided by the MIC) of $\geq$400, which is the significant pharmacodynamic threshold based on available clinical evidence (14). The consensus review also cautioned that "therapeutic" AUC$_{24}$/MICs would most likely be achieved with the "recommended" troughs when pathogen MICs are $\leq$1 mg/liter.

New approaches to vancomycin dosing in the clinical setting are required to achieve the higher targets. In response, the administration of vancomycin by continuous infusion has been proposed. Although limited study to date has not shown significant differences in clinical outcome compared with standard intermittent vancomycin dosing (3, 33), advantages in more effectively achieving and maintaining target concentrations in populations such as the critically ill have been demonstrated (17, 18). Our goal was to study continuous-infusion vancomycin (CIV) against both health care-associated (HCA) and community-associated (CA) MRSA strains in an *in vitro* pharmacodynamic model. Although such models have been used to study intermittent vancomycin dosing, ours is the first, to our knowledge, to investigate CIV and emerging resistance in MRSA.

Six clinical MRSA isolates collected from patients in 2008 were obtained from the CANWARD surveillance program conducted by The Canadian Antimicrobial Resistance Alliance (http://www.can-r.com/index.php). Three genotypically defined HCA and CA strains were selected as detailed in Table 1. Vancomycin MICs were measured using broth microdilution according to CLSI methods in addition to the Etest (AB Biodisk, bioMérieux, Solna, Sweden). Our MIC data were consistent with observations that vancomycin MICs may vary with the method used. Several studies have shown consistently higher MICs with Etest than with broth microdilution, with one investigation reporting values which were 0.5 to 1.5 log$_2$ dilution steps higher (23). Our study used the standard clinical test, broth microdilution, for vancomycin pharmacodynamic (AUC$_{24}$/MIC) analyses and the more discriminating Etest to detect MIC changes during CIV therapy. Broth microdilution MIC breakpoints of the CSLI were used to define vancomycin-susceptible ($\leq$2 mg/liter), -intermediate (4 to 8 mg/liter), and -resistant ($\geq$16 mg/liter) isolates. One isolate (77900) was an hVISA (heteroresistant vancomycin-intermediate *S. aureus*) strain as determined by the Etest macromethod (32). A one-compartment dilutional *in vitro* dynamic model containing Mueller-Hinton broth (Becton Dickinson & Company, Sparks, MD) supplemented with calcium (25 mg/liter) and magnesium (12.5 mg/liter) and maintained at 37°C in a heated water bath was used. A bacterial suspension was injected into the infection flasks (250 ml), yielding initial inocula of $\sim$1 $\times$ 10^6 CFU/ml. A

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TABLE 1. Characteristics of MRSA clinical isolates used in this study

Parameter	Result obtained with isolate with stock no.:					
	77900	81655	80424	77564	79002	77339
nuc (nuclease) gene	Positive	Positive	Positive	Positive	Positive	Positive
mecA gene	Positive	Positive	Positive	Positive	Positive	Positive
Genotype (PFGE)[a]	USA100	USA100	USA100	USA400	USA300	USA300
Panton-Valentine leukocidin	Negative	Negative	Negative	Positive	Positive	Positive
agr (accessory gene regulator) type	II	II	II	I	I	I
Source	Blood	Blood	Blood	Wound	Wound	Blood
Broth microdilution MIC (mg/liter) of:						
Vancomycin (Etest MIC)	2 (2)	1 (1.5)	1 (2)	1 (1.5)	1 (1.5)	0.5 (1)
Ceftobiprole	2	1	1	1	1	1
Clarithromycin	>32	>32	>32	0.25	>32	>32
Clindamycin	>8	>8	>8	≤0.12	≤0.12	≤0.12
Daptomycin	0.25	0.25	0.12	0.25	0.25	0.5
Levofloxacin	>32	>32	>32	0.12	8	8
Linezolid	2	1	2	2	2	2
Moxifloxacin	>16	>16	8	≤0.06	2	2
Tigecycline	0.25	0.25	0.5	0.12	0.25	0.25
Trimethoprim-sulfamethoxazole[b]	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12

[a] PFGE, pulsed-field gel electrophoresis.
[b] Trimethoprim MIC.

computerized pump (Masterflex; Cole-Parmer, Chicago, IL) with flexible tubing delivered sterile broth through the infection flasks to produce a vancomycin half-life of 7 h. Chemical grade vancomycin was obtained from Sigma-Aldrich Canada (Oakville, ON) and prepared prior to each use according to the manufacturer's instructions. Active vancomycin concentrations representing the free (f) fraction present in serum or interstitial/extracellular fluid were studied. The equivalent total serum concentration was predicted to be twice the free value based on an estimated 50% protein binding. A bolus dose was injected into the infection flasks, and a reservoir flask supplied CIV at a steady-state concentration of 2.5 mg/liter (fAUC$_{24}$ = 60 mg/liter × h), 5 mg/liter (fAUC$_{24}$ = 120 mg/liter × h), 10 mg/liter (fAUC$_{24}$ = 240 mg/liter × h), or 20 mg/liter (fAUC$_{24}$ = 480 mg/liter × h) over 48 h. Samples were collected at 0, 24, and 48 h; serially diluted in normal saline; plated on solid Mueller-Hinton agar; and incubated at 35°C for 24 h. Bacterial colony counts were performed, and MICs were determined using the Etest. An immunoassay (TDx) was used to ensure vancomycin levels within 10% of the target concentration. Experiments were conducted at least in triplicate on separate occasions and with growth controls. Data on bacterial killing at 24 h (BK$_{24}$) and at 48 h (BK$_{48}$) were subjected to analysis of variance with Tukey's *post hoc* tests, and the occurrence of reduced vancomycin susceptibility during therapy was compared using Fisher's exact test (α = 0.05).

Vancomycin showed fAUC$_{24}$/MIC-dependent activity, as detailed in Table 2 and Fig. 1. CIV producing fAUC$_{24}$/MICs of ≤120 was bacteriostatic, with a median (interquartile range) BK$_{48}$ of 2.3 (1.0 to 3.2) log$_{10}$ CFU/ml, whereas CIV achieving higher fAUC$_{24}$/MICs of ≥240 was bactericidal, with a BK$_{48}$ of 3.7 (2.9 to 4.0) log$_{10}$ CFU/ml ($P < 0.0001$). As shown in Table 2 and Fig. 2, the occurrence of reduced vancomycin susceptibility during CIV therapy was also associated with the fAUC$_{24}$/MIC. Reduced vancomycin susceptibility characterized by increasing MICs to ≥3 mg/liter was detected in 31% (16/51) of the experiments with fAUC$_{24}$/MICs of ≤120 and in 5% (2/39) of those with values of ≥240 ($P = 0.003$). Furthermore, only 11% (2/18) of the cases of reduced vancomycin susceptibility occurred with fAUC$_{24}$/MICs of ≥240 or equivalent total serum AUC$_{24}$/MICs of ≥480, and no cases were detected with fAUC$_{24}$/MICs of ≥480.

As also shown in Table 2, vancomycin activity was strain dependent, with significantly less overall BK$_{48}$ in HCA MRSA (2.7 [1.4 to 3.9] log$_{10}$ CFU/ml) than in CA MRSA (3.2 [2.3 to 4.0] log$_{10}$ CFU/ml) ($P = 0.011$). Most notably, reduced vanco-

TABLE 2. Bacterial killing at 48 h and occurrence of reduced vancomycin susceptibility during CIV

fAUC$_{24}$/MIC[a]	Total AUC$_{24}$/MIC equivalent[a,b]	HCA and CA MRSA		HCA MRSA		CA MRSA	
		BK$_{48}$ (log$_{10}$ CFU/ml)[c]	RVS[d] at 48 h	BK$_{48}$ (log$_{10}$ CFU/ml)	RVS at 48 h	BK$_{48}$ (log$_{10}$ CFU/ml)	RVS at 48 h
30–60	60–120	2.2 (−1.4, 3.4)	10/24 (42)	1.8 (−1.7, 3.9)	10/18 (56)	2.7 (2.1, 2.9)	0/6 (0)
120	240	2.3 (1.3, 3.0)	6/27 (22)	2.2 (1.4, 2.7)	4/14 (29)	2.7 (1.3, 4.0)	2/13 (15)
240	480	3.7 (3.1, 4.0)	2/21 (10)	3.7 (3.2, 4.0)	1/10 (10)	3.8 (2.9, 4.0)	1/11 (9)
480–960	960–1,920	3.8 (2.9, 4.0)	0/18 (0)	3.8 (3.1, 4.0)	0/6 (0)	3.8 (2.9, 4.0)	0/12 (0)

[a] Based on broth microdilution MIC.
[b] Equivalent total AUC$_{24}$/MIC based on 50% protein binding in serum.
[c] BK$_{48}$ data are presented as median (interquartile range) values.
[d] The number of isolates with reduced vancomycin susceptibility (RVS)/total (%) is shown.
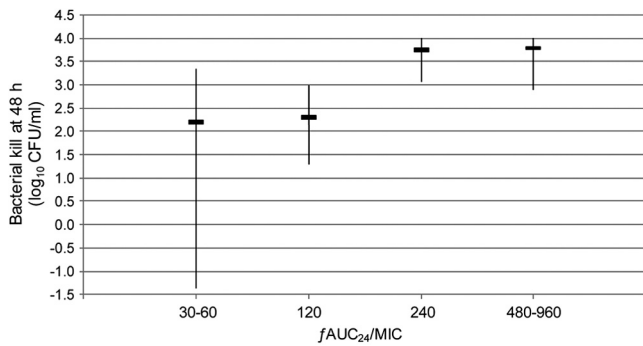
FIG. 1. Bacterial killing at 48 h stratified according to the vancomycin fAUC$_{24}$/MIC for all isolates. Data are presented as median (interquartile range) values, and negative values indicate net bacterial growth at 48 h.
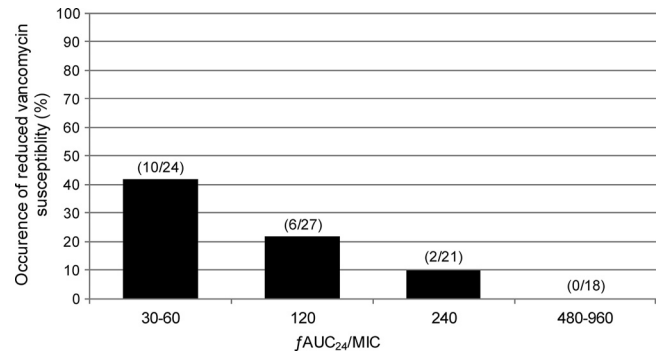

FIG. 2. Reduced vancomycin susceptibility during therapy stratified according to the vancomycin fAUC$_{24}$/MIC for all isolates.

mycin activity was associated with preferential emerging resistance in HCA MRSA strains, which were involved in 83% (15/18) of the cases of reduced vancomycin susceptibility (P = 0.007). Increasing MICs during therapy were significantly more common in HCA than in CA MRSA, even when accounting for differences in the initial MICs. In our study of HCA MRSA isolates, reduced vancomycin susceptibility was often documented with the lowest fAUC$_{24}$/MICs of 30 to 60 (56% of experiments), less frequently with fAUC$_{24}$/MICs of 120 and 240 (29% and 10% of experiments, respectively), and not at all with fAUC$_{24}$/MICs of ≥480. Although our study did not address the cause, the higher occurrence of reduced vancomycin susceptibility in HCA MRSA may be related to accessory gene regulator (agr) operons, which are global regulons controlling numerous critical virulence pathways in S. aureus (6). DNA sequence polymorphisms have identified four agr types, including groups I and II, which are most commonly found in HCA MRSA, and groups III and IV, which are most commonly found in CA MRSA. Some have suggested agr group II involvement in reduced vancomycin susceptibility in the hVISA and VISA phenotypes (24–26), although group I has also been identified (6). Notably, agr dysfunction or downregulation has been associated with reduced vancomycin susceptibility and increasing MICs during vancomycin exposure (21, 31). In addition, the identification of agr group II versus other genotypes in emerging resistance during therapy has raised concerns of potential agr group II-related risk for persistent infection and treatment failure (15, 27). Although only observational data, our findings of preferential increasing MICs in HCA MRSA agr group II genotypes during CIV therapy are consistent with such theories.

Other in vitro model studies have investigated vancomycin pharmacodynamics and emerging resistance in MRSA during intermittent dosing. Harigaya et al. found suppression of regrowth and resistance in MRSA when the fAUC$_{24}$/MIC was ≥280 (4). Studies by Rose et al. and Tsuji and colleagues found that agr dysfunctional or knockout strains were associated with less vancomycin activity and increasing MICs during therapy and required higher fAUC$_{24}$/MICs to suppress resistance than did wild-type strains (21, 31). Rose et al. were able to reduce resistance with fAUC$_{24}$/MICs of ≥165.6, whereas Tsuji et al. required values of 112 to 169 to suppress resistance. Two

subsequent in vitro dynamic studies by Rose et al. showed that hVISA strains had higher propensities to become resistant which were difficult to suppress with more aggressive vancomycin therapy alone (19, 20). Our findings were similar, with most cases of reduced vancomycin susceptibility occurring with fAUC$_{24}$/MICs of ≤120 and few with values of ≥240.

In practical terms, our study found that CIV achieving equivalent total serum AUC$_{24}$/MICs of ≤240 was bacteriostatic, with increasing MICs during therapy in almost one-third of cases. On the other hand, CIV attaining total AUC$_{24}$/MICs of ≥480 was bactericidal and only occasionally associated with emerging resistance. These effective pharmacodynamic targets would be achieved with CIV at a steady-state concentration of 20 mg/liter when pathogen MICs are ≤1 mg/liter (i.e., total AUC$_{24}$/MIC of ≥480). However, the pharmacodynamics of CIV at 20 mg/liter would predict significantly less activity with emerging resistance when the pathogen has a MIC of 2 mg/liter (i.e., a total AUC$_{24}$/MIC of 240) and is still susceptible according to CLSI breakpoints.

The foremost clinical evidence for the AUC$_{24}$/MIC target of ≥400 comes from an observational study of 63 patients with S. aureus pneumonia conducted by Moise-Broder and colleagues (14). Importantly, their study included infections associated with either methicillin-sensitive S. aureus or MRSA and therapies which frequently involved antibiotic combinations. Furthermore, unlike in vitro models which represent free antibiotic concentrations, their clinical target was based on total serum vancomycin concentrations, including the protein-bound portion. Despite these confounding factors, our significant fAUC$_{24}$/MIC threshold of ≥240 or the equivalent total serum AUC$_{24}$/MIC of ≥480 for bactericidal activity and suppression of emerging resistance in MRSA in vitro is very consistent with the clinically derived target of ≥400.

Although our findings are limited to the study of six clinical isolates, the dosing range and MIC distribution allowed relatively robust pharmacodynamic analyses of the vancomycin fAUC$_{24}$/MIC. An important limitation of our in vitro results, however, is the lack of host immunity factors which may influence the pharmacodynamic targets in patients. Furthermore, the presence of fully active antibiotic concentrations in in vitro models also differs from the in vivo situation. We used an estimated 50% protein binding in our discussions; however, the free fraction of vancomycin in serum is quite variable, as con-

firmed by recent investigations (1). Our experiments were conducted over 48 h using standard initial inocula and did not examine the effects of longer durations of vancomycin exposure or larger inocula (i.e., 1×10^8 CFU/ml), which have been shown to reduce vancomycin activity and increase resistance, particularly in hVISA (2, 20, 30). Finally, our pharmacodynamic study of CIV did not consider suggestions that more aggressive vancomycin dosing may be nephrotoxic (10, 12). Notably, the clinical evidence to date is limited to retrospective or observational findings of higher doses, or more commonly higher troughs, in association with declining renal function. These data are significantly influenced and potentially obscured by the presence of confounding variables for worsening renal function such as critical illness and other nephrotoxic agents (5). Higher serum drug concentrations are as, or more, likely to be the result of declining renal function as the cause of it. Interestingly, some have suggested that CIV may reduce the incidence of adverse effects compared with intermittent vancomycin dosing (7, 8).

In conclusion, our study showed that bacterial killing and the development of reduced vancomycin susceptibility during CIV therapy were $f\text{AUC}_{24}/\text{MIC}$ dependent, with values of ≥ 240 (equivalent total serum $\text{AUC}_{24}/\text{MICs}$ of ≥ 480) being bactericidal and suppressing emerging resistance in MRSA. These effective pharmacodynamic targets would be achieved with CIV at a steady-state concentration of 20 mg/liter when pathogen MICs are ≤ 1 mg/liter (total $\text{AUC}_{24}/\text{MIC}$ of ≥ 480). Also, vancomycin therapy was less likely to be bactericidal and 4.4 times more likely to lead to reduced vancomycin susceptibility in HCA than in CA MRSA.

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