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ORIGINAL ARTICLE

Phenotypic changes of methicillin-resistant *Staphylococcus aureus* during vancomycin therapy for persistent bacteraemia and related clinical outcome

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Abstract Persistent bacteraemia (PB) due to methicillin-resistant *Staphylococcus aureus* (MRSA) that fails to respond to glycopeptide therapy is a well-documented clinical problem. There are limited data on changes in *agr* functionality, vancomycin susceptibility and heteroresistance during MRSA PB. Thus, the frequency of these changes and their clinical significance remain unclear. Only patients with MRSA PB (≥7 days) from a prospective cohort of *S. aureus* bacteraemia were included. We collected isogenic paired strains and compared vancomycin MIC, vancomycin heteroresistance, and *agr* functionality between initial and final blood isolates. We also assessed the clinical outcome. A total of 49 patients had MRSA PB over 22 months. Bacteraemia persisted for a median of 13 days and most patients (98%) received glycopeptide as initial therapy. Among 49 isogenic pairs, only one pair showed a vancomycin MIC increase ≥2-fold by broth microdilution method, and only seven (14%) by E-test. Significant portions of initial isolates had vancomycin heteroresistance (49%) and *agr* dysfunction (76%). Development of vancomycin heteroresistance during PB occurred in four (16%) among 25 initial vancomycin-susceptible isolates, and acquisition of *agr* dysfunction occurred in two (16%) among 12 initial *agr*-functional isolates. Changes in the opposite direction occasionally occurred. These phenotypic changes during PB were not associated with mortality, whereas *agr* dysfunction of the initial isolates was significantly associated with mortality. During MRSA PB, phenotypic changes of MRSA isolates occurred occasionally under prolonged vancomycin exposure but were not significantly associated with clinical outcome. In contrast, initial *agr* dysfunction could be a predictor for mortality in MRSA PB.

Keywords MRSA · Persistent bacteraemia · Vancomycin · hVISA · *agr* dysfunction

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) infection still poses a substantial burden on healthcare systems. Among invasive MRSA infections, persistent MRSA bacteraemia that fails to respond to appropriate antibiotic therapy such as glycopeptide is a well-documented clinical problem often encountered in the management of *S. aureus* bacteraemia (SAB) [1, 2]. There are several reports examining clinical and microbiological factors associated with persistent MRSA bacteraemia [1–8]. Retention of infected devices, endovascular infection, methicillin resistance, and metastatic or multiple sites of infection are major clinical risk factors for persistent bacteraemia [1, 2, 4]. Vancomycin minimum inhibitory concentration (MIC) of 2 mg/L, vancomycin heteroresistance, *agr* dysfunction, and resistance to host defense cationic peptides have been suggested as

microbiological factors for persistent MRSA bacteraemia [3, 5–8]. Although these microbiological characteristics of MRSA isolates have been regarded as contributing factors for persistent bacteraemia, they inversely could be acquired during persistent MRSA bacteraemia, particularly under prolonged vancomycin therapy. These changes, in turn, may hinder the clearance of MRSA bacteraemia and lead to glycopeptide treatment failure. It has been suggested that prolonged vancomycin exposure can induce phenotypic changes such as an increase in vancomycin minimum inhibitory concentration (MIC) or acquisition of vancomycin heteroresistance [9, 10]. However, there is currently little data on the frequency of phenotypic changes during vancomycin therapy for persistent MRSA bacteraemia, such as increased vancomycin MIC or acquisition of vancomycin heteroresistance and *agr* dysfunction [10]. This sole study was constrained by small sample size and mainly focused on microbiological features, not on associated clinical impact. Hence, both the frequency of phenotypic changes during persistent MRSA bacteraemia and their clinical significance have not been well defined. We thus evaluated how frequently phenotypic changes in sequential isolates occur during vancomycin therapy for persistent MRSA bacteraemia and assessed their clinical significance in a prospectively enrolled SAB cohort.

Materials and methods

Patients selection

The prospective observational cohort study of patients with SAB was performed at the Asan Medical Center, a 2700-bed teaching hospital in Seoul, Korea, from November 2008 to August 2010. All adult patients (aged ≥18 years) with SAB were enrolled and observed over a 12-week period. Patients were excluded if they had polymicrobial bacteraemia, or if they died or were discharged before positive blood culture results. In our hospital, almost all patients with SAB receive consultation from the Department of Infectious Diseases and are routinely recommended to undergo follow-up blood cultures at two- to three-day intervals until negative conversion and monitoring of vancomycin trough concentrations (just before the fourth dose and then at 3–4 day intervals). It is also recommended that vancomycin trough concentration be maintained at 15–20 mg/L in patients with MRSA bacteraemia. Among SAB patients, only those who had persistent MRSA bacteraemia, defined as bacteraemia for ≥7 days despite appropriate antibiotic therapy, were included in the analysis [1, 3]. This study was approved by the Asan Medical Center Institutional Review Board.

Data collection and study definition

Demographic characteristics, underlying diseases or conditions, severity of underlying disease, severity of bacteraemia, source of infection, and clinical outcome were recorded. The Charlson comorbidity index was used to provide a composite score of comorbid conditions [11]. The severity of bacteraemia at the time of the first positive blood culture was assessed using the Pitt bacteraemia score [12].

The duration of bacteraemia was calculated as the number of days between the first and last positive blood culture. The type of infection causing SAB was defined by the surveillance criteria of the Centers for Disease Control and Prevention except for catheter-related infection, infective endocarditis, and vascular graft infection [13]. Catheter-related infection [14], the presence of infective endocarditis [15], and vascular graft infection [16] were defined according to widely accepted criteria. Bacteraemia was classified as "community-onset" if a blood culture performed within the first 48 hours after admission was positive, and was further subclassified as healthcare-associated or community-acquired bacteraemia [17]. SAB was defined as "hospital-acquired" if an initial positive blood culture was obtained >48 hours after admission. The clinical outcome measure was death within 12 weeks after onset of MRSA bacteraemia.

Microbiologic data and genotypic assays

Isogenic paired strains (the initial and final blood isolates from each study patient) were used for microbiological and molecular assessments. All MRSA isolates were identified by standard methods. Antimicrobial susceptibilities were determined using the MicroScan system (Dade Behring, West Sacramento, CA) and standard criteria of the Clinical and Laboratory Standards Institute. Methicillin resistance was confirmed by polymerase chain reaction (PCR) detection of the *mec*A gene. Vancomycin MICs of all serial MRSA isolates were determined using both broth microdilution (BMD) and E-test (AB Biodisk, Piscataway, NJ) on Mueller-Hinton agar according to the manufacturer's instructions. All assays were performed in triplicate, and the most frequent concentration was chosen. Identification of heterogeneous vancomycin-intermediate *S. aureus* (hVISA) phenotype (vancomycin heteroresistance) was determined by population analysis profile (PAP) as previously described [18]. An isolate was identified as hVISA if the ratio of the area under the viable count–vancomycin curve (AUC) for the test isolate versus the AUC of the reference strain (Mu3; ATCC 700698) was ≥0.9.

The staphylococcal cassette chromosome *mec* (SCC*mec*) type, *agr* type, and *agr* functionality of isolates were determined using previously described methods [19–21]. We determined *agr* dysfunction by the level of δ-hemolysin production, measured by streaking the isolate adjacent to a β-hemolytic RN4220 strain (Remel, Lenexa, KS), because *agr* dysfunction results in deficient δ-hemolysin production [20, 22]. Multilocus sequence typing (MLST) was performed for all strains as described elsewhere [23], and sequence types were consistent in each isogenic pair.

Statistical analysis

Characteristics of patients and isolates are summarized using percentages or median and interquartile range (IQR). Categorical variables were compared using the $\chi 2$ test or the Fisher's exact test as appropriate, and continuous variables were compared using Student's *t* test or the Mann–Whitney *U* test. The time-to-event analyses were performed using Kaplan–Meier estimates and the log-rank test. To identify microbiological risk factors for 12-week mortality, significant variables in the univariate analysis were included in the multivariate analysis using penalized Cox regression model. All tests of significance were two-tailed, and *P* values of <0.05 were considered to indicate statistical significance. All statistical analyses were performed using SPSS version 21.0 (SPSS, Armonk, NY, USA) and R software version 3.1.2 (R Project for Statistical Computing, Vienna, Austria).

Results

Patients and isolates characteristics

A total of 235 patients were diagnosed with MRSA bacteraemia during the 22-month study period at our institution. Only the 49 patients with persistent MRSA bacteraemia and the isogenic paired strains isolated from these patients were included in the analysis. The median age of study patients was 65 years (IQR, 55–71 years) and the median duration of bacteraemia was 13 days (IQR, 8–18 days). Among these 49 cases, three (6%) were community-acquired, 13 (27%) were healthcare-associated, and 33 (67%) were hospital-acquired. Catheter-related infection was the most common site of infection (30 patients [61%]), followed by infective endocarditis (6 [12%]), skin and soft tissue infection (4 [8%]), vascular graft infection (4 [8%]), bone and joint infection (3 [6%]), and suppurative thrombophlebitis of varix (1 [2%]). All but one patient received glycopeptide (vancomycin in 92% and teicoplanin in 6% of patients) as initial therapy, whereas the remaining patient received linezolid. The median time from bacteraemia onset to initiation of appropriate antibiotic therapy was 1 day (IQR 0–2 days). Of 38 patients with an eradicable focus of MRSA bacteraemia, 32 (84%) underwent removal of the focus. Among 49 isogenic pairs, 35 (71%) were ST5-SCC*mec* II, and ten (20%) were ST72-SCC*mec* IV strains.

Change of vancomycin susceptibility during persistent bacteraemia

As shown in Fig. 1 and Table 1, only one isogenic pair showed a vancomycin MIC increase ≥2-fold by BMD and seven (14%) by E-test. Among the initial 49 isolates, 25 (51%) were vancomycin-susceptible *S. aureus* (VSSA) and 24 (49%) were hVISA. Acquisition of vancomycin heteroresistance was observed in four (16%) of 25 VSSA strains during persistent bacteraemia, whereas phenotypic change from hVISA to VSSA was observed in seven (29%) of 24 hVISA strains during persistent bacteraemia (Table 2). Change from VSSA to hVISA occurred only in ST5-MRSA.

Change of *agr* function during persistent bacteremia

Among 49 isogenic pairs, 14 (29%) were *agr* group I and 35 (71%) were *agr* group II. Of the initial 49 isolates, 37 (75%) showed *agr* dysfunction. Of the 12 initial isolates without *agr* dysfunction, two (17%) acquired *agr* dysfunction during persistent bacteraemia, whereas one of 37 initial isolates with *agr* dysfunction showed a phenotypic change to be *agr* functional during persistent bacteraemia (Table 2).

Outcome of patients with persistent bacteremia

When we compared the 12-week mortality, there were no significant differences between patients with and without changes in vancomycin MIC, development of hVISA, or acquisition of *agr* dysfunction (Table 3). In contrast, initial isolates with *agr* dysfunction were significantly associated with higher mortality than those with *agr* function (30% [11/37] vs. 0% [0/12], $P = 0.045$). Kaplan–Meier survival curves of 12-week mortality according to initial *agr* functionality are presented in Fig. 2 ($P = 0.04$). Underlying disease, the severity of infection, and duration of bacteraemia were comparable between groups with and without initial *agr* dysfunction (Table 4). However, hospital acquisition of bacteraemia, catheter-related infection, and receiving ICU care were significantly more frequent in patients with initial *agr* dysfunctional isolates than in patients with initial *agr* functional isolates. In addition, 34 (92%) of 37 initial isolates with *agr* dysfunction were ST5-SCC*mec* type II, whereas only one (8%) of 12 initial isolates with *agr* function were ST5-SCC*mec* type II. Initial *agr* dysfunction, ST5-MRSA and Charlson comorbidity index were included in a penalized Cox regression model to identify risk factors for 12-week mortality. Multivariate analysis revealed that initial *agr* dysfunction was the only significant prognostic factor for mortality (hazard ratio [HR], 14.3, 95% confidence interval [CI] 1.9–1820.5, P = 0.003).

Discussion

We assessed the phenotypic changes in isogenic paired MRSA strains isolated from persistent bacteraemia patients and the associated clinical outcomes. During glycopeptide

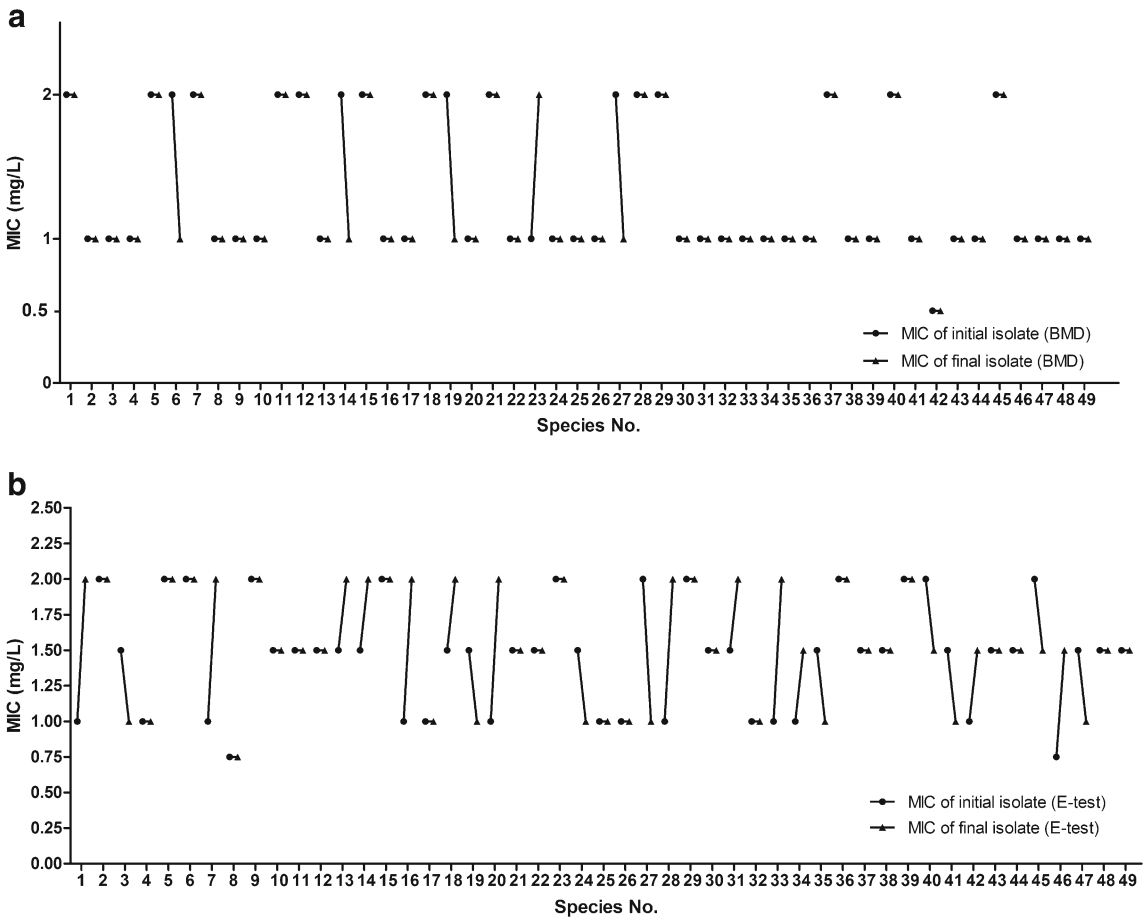

Fig. 1 Changes in vancomycin minimum inhibitory concentration (MIC) between initial and final isolates during persistent methicillin-resistant *Staphylococcus aureus* (MRSA) bacteraemia. **a** Broth microdilution (BMD). **b** E-test

therapy for persistent MRSA bacteraemia, phenotypic changes such as increased vancomycin MIC, development of vancomycin heteroresistance, and loss of *agr* function occurred occasionally, even in the opposite direction, but did not affect mortality. In contrast, *agr* dysfunction of initial isolates was significantly associated with adverse outcome.

In the present study, vancomycin MIC increased in only one pair (2%) as determined by BMD and seven pairs (14%) by E-test during vancomycin therapy for persistent MRSA bacteraemia. In addition, four pairs (16%) developed hVISA phenotype from initial VSSA strains. There are limited data on phenotypic changes such as increased vancomycin MIC and

Table 1 Changes in vancomycin minimum inhibitory concentration (MIC) between initial and final isolates during persistent methicillin-resistant *Staphylococcus aureus* (MRSA) bacteraemia

Change in vancomycin MIC	All isogenic pairs (N = 49)	ST5-MRSA pairs (N = 35)	Non-ST5-MRSA pairs[a] (N = 14)
By broth microdilution			
No changes	45 (92)	34 (97)	11 (79)
≥2-fold increase	1 (2)	0	1 (7)
≥2-fold decrease	3 (6)	1 (3)	2 (14)
By E-test			
No changes	41 (84)	30 (86)	11 (79)
≥2-fold increase	7 (14)	5 (14)	2 (14)
≥2-fold decrease	1 (2)	0	1 (7)

Data are presented as the number of isogenic pairs (% among initial isolates with a specific phenotype)

[a] ST72 (n = 10), ST239 (n = 3), and ST8 (n = 1)

Table 2 Changes in vancomycin heteroresistance and *agr* functionality between initial and final isolates during persistent methicillin-resistant *Staphylococcus aureus* (MRSA) bacteraemia

Phenotypic change from initial to final isolate	All isogenic pairs (N = 49)	ST5-MRSA pairs (N = 35)	Non-ST5-MRSA pairs (N = 14)
Change in vancomycin heteroresistance			
From VSSA to VSSA	21 (84)	13 (76)	8 (100)
From VSSA to hVISA	4 (16)	4 (24)	0
From hVISA to hVISA	17 (71)	14 (78)	3 (50)
From hVISA to VSSA	7 (29)	4 (22)	3 (50)
Change in *agr* functionality			
From *agr* function to *agr* function	10 (83)	1 (100)	9 (82)
From *agr* to *agr* dysfunction	2 (17)	0	2 (18)
From *agr* dysfunction to *agr* dysfunction	36 (97)	34 (100)	2 (67)
From *agr* dysfunction to *agr* function	1 (3)	0	1 (33)

VSSA vancomycin-susceptible *S. aureus*, *hVISA* heterogeneous vancomycin-intermediate *S. aureus*

Data are presented as the number of isogenic pairs (% among initial isolates with a specific phenotype)

development of hVISA phenotype during persistent MRSA bacteraemia with glycopeptide therapy [10]. In a retrospective study, Lin et al. [24] reported that the frequency of vancomycin MIC increase (≥2-fold by agar dilution method) during persistent MRSA bacteraemia was 25% for subsequent isolates and 20% for the final isolates in 199 patients. Infrequent phenotypic changes of our data are consistent with another previous study, which found that only two pairs (9%) of isolates exhibited a slight increase of vancomycin MIC by E-test in brain-heart infusion agar during persistent bacteraemia and three isolates (14%) acquired heteroresistance out of 22 pairs during persistent or recurrent MRSA bacteraemia [10]. This discrepancy in the frequency of vancomycin MIC increase among studies might arise from the different vancomycin susceptibility testing methods. Given that duration of glycopeptide therapy in cases with phenotypic changes seemed to be longer than in cases without those changes (Table 3), the length of glycopeptide exposure also could contribute to inconsistent results among studies.

Because several reports implicated *agr* dysfunction as a cause of persistent MRSA bacteraemia [3, 5, 22], we assumed that *agr* dysfunction would be acquired in many cases of persistent MRSA bacteraemia. However, the acquisition of *agr* dysfunction was not common (17%, [2/12]), and change from *agr* dysfunction to *agr* function was observed in one isogenic pair (3% [1/34]). Initial infection caused by mixed MRSA populations with both traits could provide some explanation for unexpected changes from *agr* dysfunction to *agr* function or from hVISA to VSSA during glycopeptide therapy [20]. Although it has been suggested that *agr* dysfunction is associated with hVISA [22, 25], neither of the two isolates acquiring *agr* dysfunction during persistent bacteraemia developed hVISA phenotype in the present study. Interestingly, all four isolates that developed hVISA phenotype during persistent bacteraemia were initially *agr* dysfunctional. Therefore, we can postulate that *agr* dysfunctional isolates can more easily adapt to glycopeptide selection pressure during persistent bacteraemia than *agr* functional isolates [26].

In a recent retrospective study, elevated vancomycin MIC during persistent MRSA bacteraemia (in 25% of subsequent isolates) was associated with increased mortality [24]. However, we found that vancomycin MIC increase and development of hVISA phenotype during persistent MRSA bacteraemia were not significantly associated with mortality. Instead, *agr* dysfunction of initial isolates was significantly associated with mortality in our data. This result is partly consistent with that of Schweizer et al. [27], who demonstrated an independent association between *agr* dysfunction and mortality among severely ill patients with SAB (adjusted HR, 1.82; 95% confidence interval, 1.03–3.21). Although they did not quantify the duration of bacteraemia due to the retrospective nature of their study, they suspected that higher mortality by *agr* dysfunctional isolates may result from a longer duration of bacteraemia. However, in our study that all patients had persistent bacteraemia, there was no significant difference in the duration of bacteraemia between patients infected by *agr* dysfunctional and *agr* functional isolates. Interestingly, there were significant differences in several clinical characteristics (i.e., site of acquisition, site of infection, and ICU care during bacteremia) between these patient groups, which could affect clinical outcome. Sakoulas et al. [26] and Vuong et al. [28] suggested that the loss of *agr* function may confer survival advantages to MRSA, such as promoting biofilm formation and physiological changes that support colonization and survival in the nosocomial setting under glycopeptide selection

Table 3 Comparison of clinical and genotypic characteristics between cases with and without phenotypic changes in the pathogen during persistent bacteraemia

Phenotypic change from initial to final isolate	From VSSA to hVISA, N = 4	From VSSA to VSSA, N = 21	From *agr* function to *agr* dysfunction, N = 2	From *agr* function to *agr* function, N = 10
Prior glycopeptide use	0	3 (14)	0	0
Duration of glycopeptide therapy (days), median (range)	27 (10–38)	17 (4–91)	57 (11–103)	21 (4–91)
Site of infection				
Catheter-related infection	4 (100)	13 (62)		2 (20)
Infective endocarditis		3 (14)		3 (30)
Skin and soft tissue infection		2 (10)		3 (30)
Vascular graft infection			1 (50)	1 (10)
Bone and joint infection		1 (5)	1 (50)	1 (10)
Others		1 (5)		
Eradicable focus	4 (100)	15 (72)	1 (50)	6 (60)
Removal of eradicable focus	4/4 (100)	15/15 (100)	0/1	5/6 (83)
Time to removal (days), median (range)	1 (0–3)	3 (0–17)	3 (N/A)	13 (0–17)
Genotype				
ST5-SCC*mec* II-*agr* II	4 (100)	13 (62)		1 (10)
ST72-SCC*mec* IV-*agr* I		7 (33)	1 (50)	8 (80)
ST239-SCC*mec* III-*agr* I				1 (10)
Other [b]		1 (5)	1 (50)	
12-week mortality	1 (25)	5 (24)	0	0

IQR interquartile range

Data are presented as the number of patients (%) unless otherwise specified

[a] One strain was ST8-SCC*mec* IV-*agr* I

pressure. Recently, Laabei et al. found that *agr* dysfunctional isolates are more fit in human serum, and as a result, are more associated with severe, invasive disease than *agr* function isolates [29]. These characteristics may explain the clinical features and increased mortality of our patients infected with *agr* dysfunctional isolates. Additional studies are needed to verify the impact of *agr* dysfunction on mortality in persistent MRSA bacteraemia.

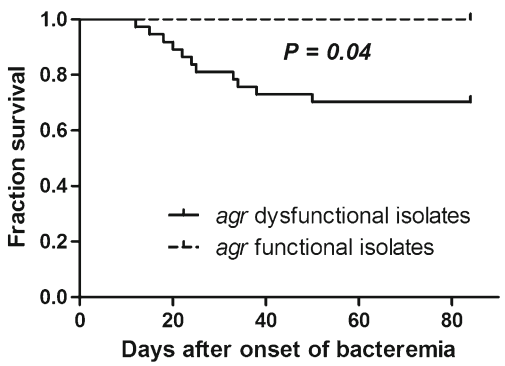

Fig. 2 Kaplan–Meier survival curves of patients with persistent MRSA bacteraemia caused by initially *agr* dysfunctional isolates or initially *agr* functional isolates

Our study has some limitations. First, the majority of initial isolates already had *agr* dysfunction, which may have contributed to the lower acquisition rate (17%) of *agr* dysfunction than anticipated during persistent bacteraemia. A study performed in patients with a higher proportion of *agr* functional isolates may provide a better estimate of the acquisition rate of *agr* dysfunction during persistent MRSA bacteraemia. Second, this study was performed at a single tertiary care center, so a multicenter prospective study is warranted to generalize our findings. Despite these limitations, our findings provide some important implications for understanding the nature of persistent MRSA bacteraemia. To the best of our knowledge, this is the first relatively large-scale study to evaluate not only serial phenotypic changes during persistent MRSA bacteraemia but also their clinical significance on the outcome.

In summary, phenotypic changes such as increased vancomycin MIC, development of heteroresistance, and acquisition of *agr* dysfunction occurred occasionally, and changes in the opposite direction also occurred, during persistent MRSA bacteraemia, particularly under prolonged glycopeptide therapy. However, these changes did not significantly affect mortality. In contrast, initial *agr* dysfunction might be a predictor of poor outcome. Furthermore, as some clinical and microbiological characteristics (i.e., hospital-acquired bacteraemia,

Table 4 Clinical and microbiological characteristics of patients with persistent bacteraemia caused by initially *agr* dysfunctional isolates and initially *agr* functional isolates

Characteristic	*agr* dysfunctional isolates, N = 37	*agr* functional isolates, N = 12	All isolates, N = 49	*P*-value
Age (years), median (IQR)	65 (56–71)	65 (44–71)	65 (55–71)	0.49
Male	23 (62)	8 (67)	31 (63)	>0.99
Site of acquisition				0.001
Community-onset Community-acquired	0	3 (25)	3 (6)	
Healthcare-associated	8 (22)	5 (42)	13 (27)	
Hospital-acquired	29 (79)	4 (33)	33 (67)	
Underlying disease/condition				
Solid tumor	15 (41)	1 (8)	16 (32)	0.07
Haematological malignancy	5 (14)	1 (8)	6 (12)	>0.99
Diabetes mellitus	11 (30)	5 (42)	16 (33)	0.49
End-stage renal disease	6 (16)	1 (8)	7 (14)	0.67
Liver cirrhosis	4 (11)	0	4 (8)	0.56
Neutropenia	3 (8)	0	3 (6)	0.57
Immunosuppressant use	5 (14)	1 (8)	6 (12)	>0.99
Recent surgery	14 (38)	3 (25)	17 (35)	0.50
Recent chemotherapy	4 (11)	1 (8)	5 (10)	>0.99
Charlson comorbidity index, median (IQR)	3 (2–5)	2 (1–3)	3 (1–4)	0.05
Pitt bacteraemia score, median (IQR)	1 (0–2)	1 (0–4)	1 (0–2)	0.82
Septic shock	8 (22)	3 (25)	11 (22)	>0.99
Duration of bacteraemia (days), median (IQR)	13 (8–18)	13 (8–19)	13 (8–18)	0.86
Site of infection				0.01
Catheter-related infection	28 (76)	2 (17)	30 (61)	
Infective endocarditis	3 (8)	3 (25)	6 (12)	
Skin and soft tissue infection	1 (3)	3 (25)	4 (8)	
Vascular graft infection	2 (5)	2 (17)	4 (8)	
Bone and joint infection	1 (3)	2 (17)	3 (6)	
ICU care	14 (38)	0	14 (29)	0.01
Removal of eradicable focus	27/31 (87)	5/7 (71)	32/38 (84)	0.30
Time to removal (days), median (IQR)	1 (0–2)	1 (0–16)	1 (0–3)	0.55
Genotype				<0.001
ST5-SCC*mec* II-*agr* II	34 (92)	1 (8)	35 (71)	
ST72-SCC*mec* IV-*agr* I	1 (3)	9 (75)	10 (20)	
ST239-SCC*mec* III-*agr* I	2 (5)	1 (8)	3 (6)	
Other[a]	0	1 (8)	1 (2)	

IQR interquartile range

Data are presented as the number of patients (%) unless otherwise specified

[a] One strain was ST8-SCC*mec* IV-*agr* I

catheter-related infection, ICU care, and ST5-MRSA strain) were closely related to initial *agr* dysfunction, early aggressive treatment for patients with these characteristics may help improve outcome.

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Compliance with ethical standards

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Disclosure of potential conflicts of interest We have no conflicts of interest to declare.

Ethical approval This study was approved by the Institutional Review Board of Asan Medical Center (No. 2008-0274).

Informed consent The IRB waived the requirement for informed consent in view of the observational nature of the study, and the patient records were anonymized and deidentified.

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