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Decreased colony spreading in *Staphylococcus aureus* with reduced vancomycin susceptibility

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ABSTRACT: *Staphylococcus aureus* has been a significant bacterial cause of both healthcare-associated and community-acquired infections. Currently, vancomycin is one of the most drugs of choice used for treatment of Gram-positive infection. Thus, the *S. aureus* strains with reduced vancomycin susceptibility including vancomycin-intermediate *S. aureus* (VISA) and heterogeneous vancomycin-intermediate *S. aureus* (hVISA) have been reported increasingly. In this study, we aimed to compare the colony spreading ability, a phenomenon that *S. aureus* forms a giant colony on a soft agar surface, between vancomycin-susceptible and non-susceptible *S. aureus*. A total of 79 *S. aureus* strains, 44 vancomycin-susceptible *S. aureus* (VSSA), 27 hVISA, and 8 VISA isolates, were cultured on soft agar, and the colony spreading was investigated after 24 h incubation. Reference strains of VSSA ATCC29213, VISA strain Mu50, and hVISA strain Mu3 were included as positive and negative controls. Each bacterial isolate was tested twice on soft agar. The results showed that 61.4% of VSSA and 22.2% of hVISA were positive for colony spreading on soft agar, whereas none of the VISA isolates showed spreading colony. There were statistical differences between VSSA and hVISA ($p = 0.0016$) and VISA ($p = 0.0014$), but no difference between hVISA and VISA was found. These results suggest that *S. aureus* with reduced vancomycin susceptibility has a defect in colony spreading ability.

KEYWORDS: *Staphylococcus aureus*, colony spreading, vancomycin susceptibility, virulence factor

INTRODUCTION

Bacterial migration plays an important role in the infectious process of the pathogenic bacteria [1]. There are various types of bacterial movement: the appendage-dependent such as swimming, swarming, and twitching; and the appendage-independent such as gliding and sliding. Some bacteria, though possessing flagella, could be motile without using flagella. For example, *Vibrio cholerae* and *Serratia marcescens* can spread on soft agar surfaces without using flagella [2]. Potassium ions and secreted surfactants are needed for *Bacillus subtilis* spreading on soft agar surfaces [3]. The sliding of *Mycobacterium smegmatis* colonies on the surface of soft agar requires cell surface glycopeptide lipids [4]. The flagellated bacteria move actively by the rotation of flagella via proton motive force, whereas the non-flagellated bacteria translocate by various factors [1, 5]. However, the molecular mechanisms of non-flagella translocation of bacteria are still unclear.

Staphylococcus aureus is a commensal pathogen that causes various infectious diseases [6, 7]. Methicillin-resistant *S. aureus* (MRSA) has been a global health-threatening organism in healthcare settings for over 60 years [8, 9]. Accordingly, vancomycin has been the first-line therapy for MRSA bacteremia and infective endocarditis for decades [10, 11]. Consequently, *S. aureus* with reduced vancomycin susceptibility have emerged in the last 20 years [12, 13]. Both vancomycin-intermediate *S. aureus* (VISA) and heterogeneous vancomycin-intermediate *S. aureus* (hVISA) have thickening cell wall and reduction of *agr* virulence regulation system [14]. Even without flagella, *S. aureus* can spread rapidly on the surface of soft agar, called "colony spreading", which is one of its plenty virulence factors [15]. The colony spreading of *S. aureus* requires cell wall teichoic acids and D-alanylation of teichoic acids [15]. Other factors involved such as the density of bacterial colony which triggered a quorum sensing response resulted in the synthesis of phenolic-soluble modulins (PSMs), a biosurfactant. This affected water surface tension, allowing the bacteria to spread rapidly [16]. Therefore, we investigated the colony spreading manner of VISA and hVISA isolates which have cell wall modification from the vancomycin-resistant phenotype compared to those of vancomycin-susceptible *S. aures* (VSSA).

MATERIALS AND METHODS

Bacterial strains

Seventy-nine MRSA isolates collected between 1997 and 2010 from a university hospital in Northeast of

Thailand were used in this study. They consisted of 44 VSSA, 27 hVISA, and 8 VISA. *S. aureus* ATCC 29213, ATCC 700698 (Mu3), and ATCC 700699 (Mu50) were included as the reference strains.

Vancomycin MIC determination and PAP-AUC analysis

The minimum inhibitory concentration (MIC) of vancomycin for all isolates was determined using agar dilution method according to the CLSI 2023 [17].

A modified population analysis profile with area under the curve (PAP-AUC) for detection of hVISA phenotype was conducted according to a previous report [14]. Briefly, a serial 10-fold dilution (10^0 to 10^{-6}) of 0.5 McFarland bacterial suspension of each isolate was prepared. A 20 µl from each dilution was spread on brain heart infusion (BHI) (Oxoid, Hampshire, England) agar plate adding with vancomycin (Sigma-Aldrich, MO, USA) at the concentrations of 0, 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 µg/ml. After 48 h of incubation, the number of bacterial colonies grown on each plate was counted and calculated to the colony forming unit per milliliter (CFU/ml). Then, the log 10 of CFU/ml grown on each vancomycin concentration was plotted versus the vancomycin concentrations using the Graph Pad Prism software version 5.0.1 (GraphPad Software Inc., CA, USA). The PAP-AUC value was calculated from the ratio of areas under the curve of the tested isolate over that of the reference hVISA strain (Mu3). The isolates having PAP-AUC ratios less than 0.9, 0.9–1.3, and more than 1.3 were considered VSSA, hVISA, and VISA phenotypes, respectively [18, 19].

Colony spreading

The isolates from the frozen stocks were sub-cultured on blood agar (Oxoid) and incubated at 37 °C for 24 h. Bacterial colonies were then suspended in normal saline solution to obtain the turbidity of 0.5 McFarland Standard. Two µl of each bacterial suspension were dropped on the surface of soft agar plate, tryptone soya broth (Oxoid) added with 0.24% agar [15]. The plates were placed with the lid ajar in biosafety cabinet for 15 min. The spreading colony was observed after the petri dishes were incubated face up at 37 °C for 24 h. A large colony with a few branched arms or filaments was reported as a positive result. Each sample was tested in duplicate. The third test was performed when the duplicate test gave different results.

Statistical analysis

The Fisher Exact test was used for statistical analysis to evaluate any difference between the number of isolates that have spreading colony among each phenotype (VSSA vs. hVISA, VSSA vs. VISA, and hVISA vs. VISA). The *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Vancomycin MIC and PAP-AUC ratio

According to the PAP-AUC analysis, the 79 *S. aureus* isolates were 44 VSSA, 27 hVISA and 8 VISA isolates with their PAP-AUC ratios of 0.1–0.87, 0.95–1.17 and 1.35–1.91, respectively. The vancomycin MICs of the VSSA, hVISA, and VISA isolates were 0.5–2, 1–2, and 3–4 µg/ml, respectively.

Colony spreading

The colony spreading ability was seen in 27 (61.4%) from 44 VSSA and 6 (22.2%) from 27 hVISA, whereas no spreading colony was observed in any isolate of the 8 VISA isolates (Table 1). Most of the spreading colony had irregular filamentous shape, while the non-spreading colony showed entire smooth circular shape (Fig. 1).

Table 1 Comparison of colony spreading ability between VSSA, hVISA, and VISA.

	No. of isolate (%)		
	VSSA ($n = 44$)	hVISA ($n = 27$)	VISA ($n = 8$)
Colony spreading	27 (61.4)	6 (22.2)	0 (0)
p-value vs. VSSA		0.0016	0.0014
p-value vs. hVISA			0.2994

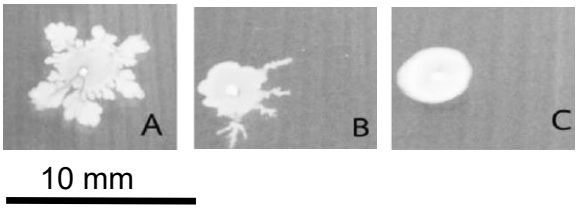

Fig. 1 Examples of the colony spreading of VSSA (A); hVISA (B); and VISA (C).

The Fisher exact test showed significant difference between the VSSA and hVISA groups ($p = 0.0016$) and between the VSSA and VISA groups ($p = 0.0014$). No significant difference was found between the hVISA and VISA groups ($p = 0.2994$).

To compare the colony spreading activity of these *S. aureus* isolates with the genetic variations, part of the data from our previous study were analyzed [18, 20]. The SCC*mec* and *spa* types together with the mutations in *psm-mec* region of 54 from the 79 isolates are shown in Table 2. Most of them were SCC*mec* III - *spa* type t037, followed by SCC*mec* II - *spa* types t045. The spreading and non-spreading isolates had both intact and mutation in *psm-mec* region.

Table 2 Genetic characteristics of 54 from the 79 *S. aureus* isolates.

Bacterial group (n)	Colony spreading (n)	SCC*mec* type (n)*			*spa* type (n)*	Mutation in *psm-mec* region*
		II	III	IX		
VSSA (34)	+(25)	6			t045 (3)	−7T>C
					t463 (2)	−7T>C
					t2460 (1)	absence
			19		t037 (16)	intact
					t037 (1)	1.3 kb insertion
					t463 (1)	−7T>C
					t1921 (1)	+101T>C
	−(9)		6		t037 (5)	intact
					t1921 (1)	+101T>C
				3	t337 (3)	absence
hVISA (12)	+(8)	3			t463 (2)	−7T>C
					t045 (1)	−7T>C
				5	t037 (5)	intact
	−(4)			3	t037 (2)	intact
					t037 (1)	1.3 kb insertion
				1	t337 (1)	absence
VISA (8)	−(8)	3			t045 (1)	intact
					t045 (1)	absence
					t2460 (1)	absence
			5		t037 (3)	intact
					t037 (1)	absence
					t1504 (1)	intact

* Part of results were from Tabuchi et al [20] and Lulitanond et al [18].

DISCUSSION

S. aureus generally produces spreading colony on a soft agar surface. However, this study found approximately 40% of VSSA strains did not have spreading colony ability. The colony spreading of *S. aureus* has been reported to relate with several factors. In 2007, Kaito and Sekimizu showed that the colony spreading ability of *S. aureus* decreased by the disruption of *dltABCD* operon functioning at the step of adding D-alanine to teichoic acids and by the disruption of the *tagO* gene responsible for teichoic acids wall synthesis [15]. These results indicated that the teichoic acids wall and D-alanylation of teichoic acids are required for colony spreading. In 2012, Omae et al [21] reported that *hld*-disrupted *S. aureus* mutants had higher ability to spread than the parent strains, suggesting a negative regulation of colony spreading by the secretion of δ-hemolysin. In 2016, Lin et al [16] found that a quorum sensing response triggered the biosurfactants and phenolic-soluble modulin (PSM) synthesis. The PSMs play a role in weakening the surface tension of water, causing it to flood on the agar surface and allowing the bacteria to spread.

The present study investigated the colony spreading among *S. aureus* with different vancomycin susceptibility. More colony spreading was found in vancomycin-susceptible strains (VSSA) than in reduced vancomycin-susceptible strains (hVISA and VISA). It has been known that an important mechanism of reduced vancomycin susceptibility in hVISA and VISA is the production of excess free D-alanyl-D-alanine residues and a thickening cell wall [12, 22]. In addition, several genes of VISA and hVISA have been found to change their regulation such as a reduction of *atl* and *lyt*M expression leading to a low autolysis activity, a reduced *agr*-functionality [23–26] resulting in a reduction of D-alanylation of teichoic acids, and the *dlt*A overexpression resulting in a reduction of the net negative cell-envelope charge [23, 24]. The increasing positive charge of the bacteria may result in sticking the cell to the agar surface.

The *S. aureus* strains with *agr* dysfunction tended to have low metabolic pathway. Therefore, it has been hypothesized that in the high bacterial density with stationary phase growth, the quorum sensing may facilitate the defective autolysis, the biofilm formation, and the production of thicker cell walls [27]. The cell wall teichoic acid synthesis is initiated by the TarO enzyme, and lipoteichoic acid polymerization involved the enzymes encoded by the *ltaA*, *ltaS*, and *ypfP* genes [28, 29]. The mutations of these genes may affect the

cell wall synthesis and the colony spreading ability of *S. aureus*. However, these factors may take a complex role in a negative regulation for colony spreading in the VISA and the hVISA isolates. Unexpectedly, we found a non-spreading colony in 17 VSSA isolates as well as spreading colony in 6 hVISA isolates. This may be due to other factors such as PSM protein production. The *psm-mec* gene, which is located on Type II or Type III SCC*mec*, inhibits toxin production and colony spreading [30]. Most of the isolates in this study were Type III, followed by Type II SCC*mec*. Among the Type II SCC*mec* isolates, both VSSA and hVISA groups (9 isolates) exhibited spreading colony, while the 3 non-spreading isolates were seen in the VISA group. For the Type III SCC*mec* isolates, they showed both spreading and non-spreading colony except for the VISA isolates giving non-spreading colony only. Most vancomycin non-susceptible isolates were reported as Type II SCC*mec* [31], like this study. In addition, all the VISA isolates with either Type II or Type III SCC*mec* showed non-spreading colony. However, further investigation with larger samples is necessary.

It was reported that the transcription of *psm-mec* ORF inhibits the expression of *psm*α, contributing to the decreased colony spreading in MRSA strain. In addition, a mutation in the *psm-mec* promoter (–7T>C) resulted in the decrease of the amount of *psm-mec* mRNA and no inhibition of colony spreading [32]. The *psm-mec* ORF of 54 isolates from the 79 MRSA strains used in this study has previously been characterized by Tabuchi et al [20]. Among 34 of the 44 VSSA isolates, 5 non-spreading VSSA isolates had intact *psm-mec*, whereas –7T>C mutation was found in other 6 spreading VSSA isolates, corresponding to the role of *psm-mec* ORF and the –7T>C mutation of the *psm-mec* promoter. In contrast, among the 8 VISA isolates giving non-spreading colony, 5 and 3 isolates had intact and absence of *psm-mec*, respectively. The non-spreading colony of these isolates may be affected by the change in the cell wall and reduced regulation of related genes. Similarly, among 4 non-spreading hVISA isolates, 2 isolates had intact *psm-mec*, whereas the 2 remaining isolates each contained either 1.3 kb insertion or no *psm-mec*, suggesting factors other than *psm-mec*.

Overall, this study showed that vancomycin non-susceptibility in MRSA may be another factor that affects the expression of its colony spreading ability on the surface of soft agar. This may result in the pathogenic characteristic of the strains. However, our study has a limitation of the small number of samples in each group and lack of information of cell wall analysis and *psm-mec* ORF for all the samples. Further study of the *psm-mec* ORF, *dltABCD* operon, and cell wall analysis should be investigated in larger samples.

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REFERENCES

1. Zegadło K, Gieroń M, Żarnowiec P, Durlik-Popińska K, Kręcisz B, Kaca W, Czerwonka G (2023) Bacterial motility and its role in skin and wound infections. *Int J Mol Sci* **24**, 1707–1718.
2. Brown II, Häse CC (2001) Flagellum-independent surface migration of *Vibrio cholerae* and *Escherichia coli*. *J Bacteriol* **183**, 3784–3790.
3. Kinsinger RF, Kearns DB, Hale M, Fall R (2005) Genetic requirements for potassium ion-dependent colony spreading in *Bacillus subtilis*. *J Bacteriol* **187**, 8462–8469.
4. Recht J, Kolter R (2001) Glycopeptidolipid acetylation affects sliding motility and biofilm formation in *Mycobacterium smegmatis*. *J Bacteriol* **183**, 5718–5724.
5. Wadhwa N, Berg HC (2022) Bacterial motility: machinery and mechanisms. *Nat Rev Microbiol* **20**, 161–173.
6. Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG Jr (2015) *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin Microbiol Rev* **28**, 603–661.
7. Tribuddharat C, Pummangura C, Srifuengfung M, Pipobwatthana P, Thuncharoon H, Rodjun V, Chongtrakool P, Srifuengfung S (2022) Prevalence and antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* clones: A study at Taksin Hospital, Bangkok, Thailand. *ScienceAsia* **48**, 159–164.
8. Harkins CP, Pichon B, Doumith M, Parkhill J, Westh H, Tomasz A, de Lencastre H, Bentley SD, et al (2017) Methicillin-resistant *Staphylococcus aureus* emerged long before the introduction of methicillin into clinical practice. *Genome Biol* **18**, 130–140.
9. Turner NA, Sharma-Kuinkel BK, Maskarinec SA, Eichenberger EM, Shah PP, Carugati M, Holland TL, Fowler VG Jr (2019) Methicillin-resistant *Staphylococcus aureus*: an overview of basic and clinical research. *Nat Rev Microbiol* **17**, 203–218.
10. Mahjabeen F, Saha U, Mostafa MN, Siddique F, Ahsan E, Fathma S, Tasnim A, Rahman T, et al (2022) An update on treatment options for methicillin-resistant *Staphylococcus aureus* (MRSA) bacteremia: a systematic review. *Cureus* **14**, e31486–31495.
11. Tong SYC, Lye DC, Yahav D, Sud A, Robinson JO, Nelson J, Archuleta S, Roberts MA, et al (2020) Effect of vancomycin or daptomycin with vs without an antistaphylococcal β-lactam on mortality, bacteremia, relapse, or treatment failure in patients with MRSA bacteremia: a randomized clinical trial. *JAMA* **323**, 527–537.
12. Hiramatsu K, Kayayama Y, Matsuo M, Aiba Y, Saito M, Hishinuma T, Iwamoto A (2014) Vancomycin-intermediate resistance in *Staphylococcus aureus*. *J Glob Antimicrob Resist* **2**, 213–224.

13. Hidayat LK, Hsu DI, Quist R, Shriner KA, Wong-Beringer A (2006) High-dose vancomycin therapy for methicillin-resistant *Staphylococcus aureus* infections: efficacy and toxicity. *Arch Intern Med* **166**, 2138–2144.
14. Cui J, Zhang H, Mo Z, Yu M, Liang Z (2021) Cell wall thickness and the molecular mechanism of heterogeneous vancomycin-intermediate *Staphylococcus aureus*. *Lett Appl Microbiol* **72**, 604–609.
15. Kaito C, Sekimizu K (2007) Colony spreading in *Staphylococcus aureus*. *J Bacteriol* **189**, 2553–2557.
16. Lin MH, Ke WJ, Liu CC, Yang MW (2016) Modulation of *Staphylococcus aureus* spreading by water. *Sci Rep* **6**, 25233–25241.
17. CLSI (2023) *Performance Standards for Antimicrobial Susceptibility Testing*, 33rd edn, CLSI supplement M100, Clinical and Laboratory Standards Institute, USA.
18. Lulitanond A, Engchanil C, Chaimanee P, Vorachit M, Ito T, Hiramatsu K (2009) The first vancomycin-intermediate *Staphylococcus aureus* strains isolated from patients in Thailand. *J Clin Microbiol* **47**, 2311–2316.
19. Wootton M, Howe RA, Hillman R, Walsh TR, Bennett PM, MacGowan AP (2001) A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in *Staphylococcus aureus* in a UK hospital. *J Antimicrob Chemother* **47**, 399–403.
20. Tabuchi F, Lulitanond A, Lulitanond V, Thunyaharn S, Kaito C (2020) Epidemiological study on the relationship between toxin production and *psm-mec* mutations in MRSA isolates in Thailand. *Microbiol Immunol* **64**, 219–225.
21. Omae Y, Sekimizu K, Kaito C (2012) Inhibition of colony-spreading activity of *Staphylococcus aureus* by secretion of δ-hemolysin. *J Biol Chem* **287**, 15570–15579.
22. Wongthong S, Kamonsutthipaijit N, Kaewhan C, Chanawong A, Tippayawat P, Lulitanond A (2022) Preliminary study on small angle X-ray scattering patterns of intact vancomycin susceptible and non-susceptible *Staphylococcus aureus* cells. *ScienceAsia* **48**, 37–42.
23. Cafiso V, Bertuccio T, Spina D, Purrello S, Campanile F, Di Pietro C, Purrello M, Stefani S (2012) Modulating activity of vancomycin and daptomycin on the expression of autolysis cell-wall turnover and membrane charge genes in hVISA and VISA strains. *PLoS One* **7**, e29573.
24. Peschel A, Vuong C, Otto M, Götz F (2000) The D-alanine residues of *Staphylococcus aureus* teichoic acids alter the susceptibility to vancomycin and the activity of autolytic enzymes. *Antimicrob Agents Chemother* **44**, 2845–2847.
25. Hu J, Ma XX, Tian Y, Pang L, Cui LZ, Shang H (2013) Reduced vancomycin susceptibility found in methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* clinical isolates in Northeast China. *PLoS One* **8**, e73300.
26. Harigaya Y, Ngo D, Lesse AJ, Huang V, Tsuji BT (2011) Characterization of heterogeneous vancomycin-intermediate resistance, MIC and accessory gene regulator (*agr*) dysfunction among clinical bloodstream isolates of *Staphyloccocus aureus*. *BMC Infect Dis* **11**, 287–293.
27. Howden BP, Smith DJ, Mansell A, Johnson PD, Ward PB, Stinear TP, Davies JK (2008) Different bacterial gene expression patterns and attenuated host immune responses are associated with the evolution of low-level vancomycin resistance during persistent methicillin-resistant *Staphylococcus aureus* bacteraemia. *BMC Microbiol* **8**, 39–52.
28. Brignoli T, Douglas E, Duggan S, Fagunloye OG, Adhikari R, Aman MJ, Massey RC (2022) Wall teichoic acids facilitate the release of toxins from the surface of *Staphylococcus aureus*. *Microbiol Spectr* **10**, e01011–01022.
29. Reichmann NT, Gründling A (2011) Location, synthesis and function of glycolipids and polyglycerolphosphate lipoteichoic acid in Gram-positive bacteria of the phylum Firmicutes. *FEMS Microbiol Lett* **319**, 97–105.
30. Aoyagi T, Kaito C, Sekimizu K, Omae Y, Saito Y, Mao H, Inomata S, Hatta M, et al (2014) Impact of *psm-mec* in the mobile genetic element on the clinical characteristics and outcome of SCC*mec*-II methicillin-resistant *Staphylococcus aureus* bacteraemia in Japan. *Clin Microbiol Infect* **20**, 912–919.
31. Howe RA, Monk A, Wootton M, Walsh TR, Enright MC (2004) Vancomycin susceptibility within methicillin-resistant *Staphylococcus aureus* lineages. *Emerg Infect Dis* **10**, 855–857.
32. Kaito C, Saito Y, Ikuo M, Omae Y, Mao H, Nagano G, Fujiyuki T, Numata S, et al (2013) Mobile genetic element SCC*mec*-encoded *psm-mec* RNA suppresses translation of *agrA* and attenuates MRSA virulence. *PLoS Pathog* **9**, e1003269.