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Preliminary study on small angle X-ray scattering patterns of intact vancomycin susceptible and non-susceptible *Staphylococcus aureus* cells

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ABSTRACT: Low-level vancomycin-resistant *S. aureus* designated as heterogeneous vancomycin-intermediate *S. aureus* (hVISA) have been associated with treatment failure and unable to detect by routine disk diffusion method. As small angle X-ray scattering (SAXS) can be used for studying size, shape, and biology structure of bacterial cells; thus, this study aimed to investigate the SAXS patterns of biomolecules of vancomycin susceptible *S. aureus* (VSSA), hVISA, and, VISA cells. A total of 9 *S. aureus* isolates, 3 each from VSSA, hVISA, and VISA groups, were cultured on brain heart infusion agar with and without vancomycin. The cultured cells were kept overnight to reach their exponential phase and subjected to the SAXS using beamline 1.3W: SAXS. Under vancomycin untreated condition, the VISA cells showed different SAXS pattern from those of the VSSA and the hVISA, whereas the vancomycin treated cells of hVISA and VISA displayed similar SAXS patterns. The ribosome of VSSA was significantly smaller than that of hVISA under the untreated condition but showed no statistically different under the treated condition. In addition, when compared the ribosome and the DNA of VSSA with the VISA's and the DNA of VISA with the hVISA's, they were different only under the untreated condition. This preliminary study showed that under the stress condition mediated by vancomycin, the vancomycin non-susceptible *S. aureus* (hVISA and VISA) had similar SAXS patterns; while under the non-stress condition, the VSSA and hVISA showed similar patterns. This study provides preliminary information on bacterial adaptation under vancomycin-mediated stress condition.

KEYWORDS: *Staphylococcus aureus*, small angle X-ray scattering, biological macromolecules, vancomycin

INTRODUCTION

Staphylococcus aureus is the most common Gram-positive cocci pathogen in community and health care associated infections especially the methicillin-resistant *S. aureus* (MRSA) strains. It causes various infections including skin and soft tissue infections, pneumonia, bone infection, and blood stream infection [1]. Vancomycin is a main drug for treatment of serious MRSA infections. Wide uses of this antibiotic led to increasing vancomycin non-susceptible MRSA, resulting in the difficulty in clinical management [2, 3]. The low-level vancomycin-resistant *S. aureus*, such as vancomycin-intermediate *S. aureus* (VISA) and heterogeneous vancomycin-intermediate *S. aureus* (hVISA), had increased cell wall thickness which did not relate to any specific genetic determinant. They were typically associated with extended hospitalization and persisting bacterial infection, leading to prolong therapy and treatment failure [4–6].

Small angle X-ray scattering (SAXS) is an X-ray spectroscopy technique used for studying the size, shape, and biology structure on scale of 1 to 100 nanometers [7]. The application of SAXS has developed during the past decade. The most application is the study of shape and function of macromolecules and nanocomposites in solutions [8]. For example, SAXS has been applied to examine human bone tissue, breast cancer tissue, micelles, and hemoglobin as well as to study the changing of intracellular structure of *Escherichia coli* cells induced by certain antibiotics [9, 10]. Currently, there is no SAXS information of *S. aureus* especially among isolates with reduced susceptibility to vancomycin. Therefore, the application of SAXS in this study may provide scattering patterns of the morphological impact of vancomycin on *S. aureus*. Here, the X-rays scattering patterns of bacterial cell suspensions obtained from 3 *S. aureus* groups: VSSA, hVISA, and VISA, were measured and, then, used to investigate bacterial intracellular components under the effect of vancomycin.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Nine *S. aureus* isolates, 6 isolates derived from Srinagarind Hospital and 3 references strains, were used in this study (Table 1). The 9 isolates, all from frozen

Table 1 Bacterial isolates and their half vancomycin MIC used in this study.

S. aureus strain	Phenotype	Half MIC (μg/ml)
ATCC29213	VSSA	0.25
MR2	VSSA	0.5
MR3	VSSA	0.5
ATCC700698 (Mu3)	hVISA	1
MR9	hVISA	0.5
70–97	hVISA	0.5
ATCC700699 (Mu50)	VISA	2
150	VISA	2
127	VISA	1.5

stocks, consisted of 3 isolates each of VSSA, hVISA, and VISA. They were first sub-cultured on blood agar (Oxoid, Hampshire, England), incubated at 37 °C for 24 h, and then grown on Tryptic Soy Agar (TSA: Oxoid, Thermo Fisher Scientific, MA, USA) with vancomycin concentration equal to half MIC of each isolate and without vancomycin. The TSA plates were incubated at 37 °C for 24 h. Each bacterial isolate was cultured on 3 sets of TSA plates representing 3 biological replicates.

The three reference strains of *S. aureus*: ATCC29213, ATCC700698 (Mu3), and ATCC700699 (Mu50), were controls for VSSA, hVISA, and VISA phenotypes, respectively.

Minimum inhibitory concentration (MIC)

The vancomycin MIC values of all isolates were determined using agar dilution method according to the CLSI 2019 [11].

Modified population analysis profile with an area under the curve (PAP-AUC) for identification of hVISA phenotype

A modified PAP-AUC was conducted according to Wootton et al [12]. Briefly, a serial ten-fold dilution of 0.5 McFarland suspensions to 10^{-6} of each isolate was prepared. An aliquot of 20 μl from each dilution was spread on brain heart infusion (BHI) (Oxoid) agar plate containing vancomycin (Sigma-Aldrich, MO, USA) concentrations of 0, 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 μg/ml. After 48 h of incubation, the number of bacteria in each plate was mathematically calculated, and the results were presented as colony forming unit per milliliter (CFU/ml). Then, the $\log_{10}$ of CFU/ml grown on each plate was plotted versus the vancomycin concentrations using the Graph Pad Prism software version 5.0.1 (GraphPad Software Inc., CA, USA). The PAP-AUC ratio was calculated from areas under the curve of the test isolate and 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 being VSSA, hVISA, and VISA phenotypes, respectively [12].

Sample preparation for SAXS

The bacterial cells from exponential phase culture were suspended in 0.1 M phosphate buffer saline (PBS) pH 7.0 at 10^8 CFU/ml, washed 3 times with Piperazine-N, N′-bis (2-ethanesulfonic acid) (PIPES) buffer (0.1 M, pH 7.0), and fixed with 2.5% glutaraldehyde solution in PIPES buffer for 1 h. After centrifugation at 9000 rpm, the pellet was washed 3 times with PBS buffer (10 mM, pH 7.0), then resuspended in 100 μl of PBS buffer and stored at 4 °C until subjected to the SAXS experiment.

Small angle X-ray scattering experiments

The SAXS experiments were carried out at the beamline 1.3W: SAXS/WAXS of Synchrotron Light Research Institute (SLRI), Nakhon Ratchasima, Thailand. Beamline 1.3 W delivers a total photon flux of 2×10^9 Ph/s focused to a sample spot size of 2 mm × 1 mm (horizontal × vertical) [13]. To get a homogeneous suspension, the bacterial cells were gently resuspended prior to the SAXS measurement. A 60 μl sample of bacterial cell suspension was applied into a liquid cell, and the SAXS data were recorded using the Mar SX165 CCD detector at sample-detector distance of 4.6 m and at a wavelength of 1.38 Å. The range of momentum transfer $0.055 < q < 1.28$ nm^{-1} was covered ($q = (4\pi \sin\theta)/\lambda$; where 2θ is the scattering angle, and λ is the X-ray wavelength). Each sample solution was measured at 20 °C and 10 min exposure time. Before measuring the sample, PBS buffer was measured as a background for each sample. The 2D SAXS images were reduced and radially averaged by the program SAXSIT, which was developed by SLRI staff, to obtain 1D scattering curves.

Data analysis

The 1D SAXS scattering profile, ploted between the intensity and the scattering vector q, was fitted to log normal size distribution to investigate size and size distribution of particle using software package SASfit produced by Paul Scherrer Institute (PSI) [14]. The form factor was used as a model of spherical particle and 3 sphere models were applied to fit the scattering profile. Three sphere models with decreasing sizes (from big to small) were used to represent ribosome, DNA, and small protein, respectively [10].

The paired t-test was used for statistical analysis to evaluate any difference between the size distribution of internal composition of *S. aureus* isolates grown in media with and without vancomycin (vancomycin treated and untreated conditions) (VSSA-(U) vs. VSSA-(T), hVISA-(U) vs. hVISA-(T), and VISA-(U) vs. VISA-(T)), while independent t-test was used to compare the size distribution of *S. aureus* between each phenotype (VSSA vs. hVISA, VSSA vs. VISA, and hVISA vs. VISA). The p-value of less than 0.05 was considered as statistically significant.

RESULTS

MIC and PAP-AUC of *S. aureus*

The vancomycin MIC levels before and after culture in medium plus vancomycin of each isolate were equal. The MIC levels of individual 3 isolates of VSSA, hVISA, and VISA groups were 0.5–1, 1–2, and 3–4 μg/ml, respectively; while their PAP-AUC were 0.51, 0.63, 0.67; 0.92, 0.96, 1.14; and 1.46, 1.58, 1.62, respectively (Table 1).

Comparison of scattering patterns

Among the cells of VSSA, hVISA, and VISA groups, the vancomycin-untreated cells of VSSA and hVISA showed similar SAXS patterns, but they were different from that of the VISA (Fig. 1A). In contrast, the vancomycin-treated cells of hVISA displayed SAXS patterns similar to that of the VISA-treated cells (Fig. 1B).

When comparing the scattering patterns between vancomycin treated and untreated cells within the VSSA, hVISA, and VISA groups; the patterns of VSSA, hVISA, and VISA cells after treatment with vancomycin were similar to that of their corresponding untreated cells (Fig. 2).

Size distribution among VSSA, hVISA, VISA cells

In the present study, we found that all bacterial isolates, including the reference strains, showed similar scattering patterns. These patterns yielded similar form factor which were fitted to a previous report [10]. The intracellular components of *S. aureus* isolates were modeled as filled spheres from literature data on the elementary composition and density [10]. In our results, a minimum of 3 populations were found: the largest size the ribosomes, followed by the DNA, and the small proteins. Each population had a mean diameter range of 58.80–92.20 nm, 11.00–17.10 nm, and 1.40–4.30 nm, respectively (Table 2).

Comparing within each corresponding bacterial group, there was no difference in the size of each component between the vancomycin treated and untreated cells ($p > 0.05$). The treated VSSA and hVISA had mean ribosome size larger than that of the untreated cells, whereas the treated VISA had mean ribosome size smaller than the treated cells. However, none were statistically different ($p > 0.05$).

Comparing between the bacterial groups under vancomycin untreated condition, the ribosome of VSSA vs. hVISA, the ribosome and DNA of VSSA vs. VISA, and the DNA of VISA vs. hVISA were statistically different ($p < 0.05$). The ribosome of VSSA was smaller than those of hVISA and VISA. The DNAs of VSSA and hVISA were larger than that of VISA. In addition, the small proteins of VSSA were larger than those of hVISA and VISA. On the other hand, under the vancomycin treated condition, only small proteins of VSSA were significantly larger than those of VISA ($p < 0.05$) (Table 3).

DISCUSSION

SAXS technique has been used to study various interesting model of bacteria. SAXS experiments using a synchrotron source are mostly optimized to study components with size ranges of around 1–100 nm [15]. A report of SAXS scattering of *S. aureus* cells showed a complex model assumed that phosphoglucosamine mutase enzyme (GlmM) inhibited di-adenylate cyclase enzymes (DacA) by masking the active site of the cyclase and preventing higher oligomer formation. This report provided an important mechanism of how cyclic di-adenosine monophosphate (c-di-AMP) production can be regulated in bacterial cell [16]. Study on an important virulence factor of *S. aureus*, Staphylococcal protein A (SpA) using SAXS provided the statistical conformation of a flexible protein [17]. It was found that using SAXS scattering to study ultrastructural changes in MRSA responding to broad-spectrum antimicrobial peptides was superior over electron microscope for contributing towards the development of drugs against resistant bacteria [18].

The SAXS scattering patterns of VSSA, hVISA, and VISA isolates between the vancomycin treated and untreated cells were not significantly different. This may be due to the low concentration of vancomycin (half MIC, Table 1) used that might not have any significant effect to these isolates. In contrast, there were significant differences on scattering patterns among the bacterial groups. Under the vancomycin untreated condition, VSSA and hVISA had similar scattering patterns which were different from that of VISA (Fig. 1A). This was in accordance with the phenotypic characteristics of VSSA and hVISA which expressed as vancomycin susceptible results (MIC 0.5–2 μg/ml) when tested using the routine susceptibility method. However, the hVISA isolates, which had PAP-AUC value higher than that of the VSSA, were suggested to be a primary stage in the transformation from VSSA to VISA phenotypes [19]. Therefore, under the condition without vancomycin pressure, the hVISA isolates tended to have similar performance to that of the VSSA. On the other hand, under vancomycin treated condition, the hVISA cells had scattering pattern similar to that of the VISA (Fig. 1B) suggesting that the hVISA tended to have similar behavior to the VISA under vancomycin pressure. This may be due to their similarity in tolerance to low level vancomycin (both the hVISA and the VISA had higher PAP-AUC values than those of the VSSA) [12]. In addition, our previous study and a recent report showed that the hVISA isolates also had thickening cell wall, though not as thick as that of the VISA isolates [20, 21]. The vancomycin MIC of the VISA isolates in this study was slightly higher than those of the hVISA and the VSSA isolates. It has been reported that the cell wall thickness of *S. aureus* had high correlation with the vancomycin MIC [22]. Thus, the vancomycin MIC

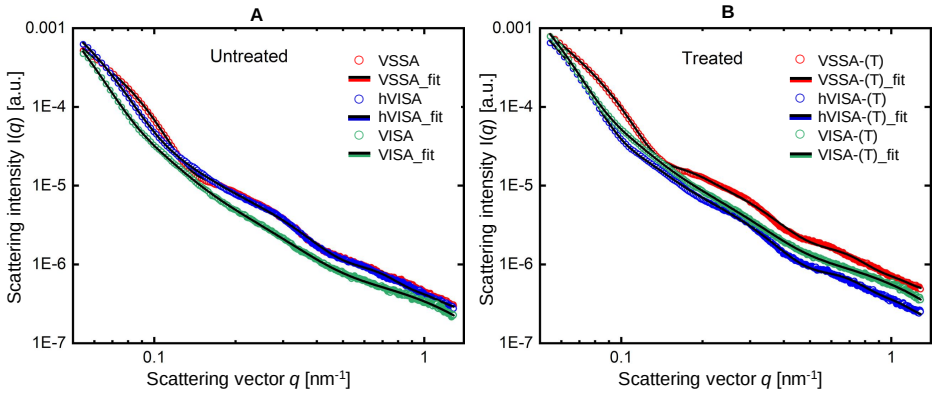

Fig. 1 Comparison of small angle X-ray scattering (SAXS) curves of vancomycin untreated cells (A) and treated cells (B) of VSSA, hVISA, and VISA groups.

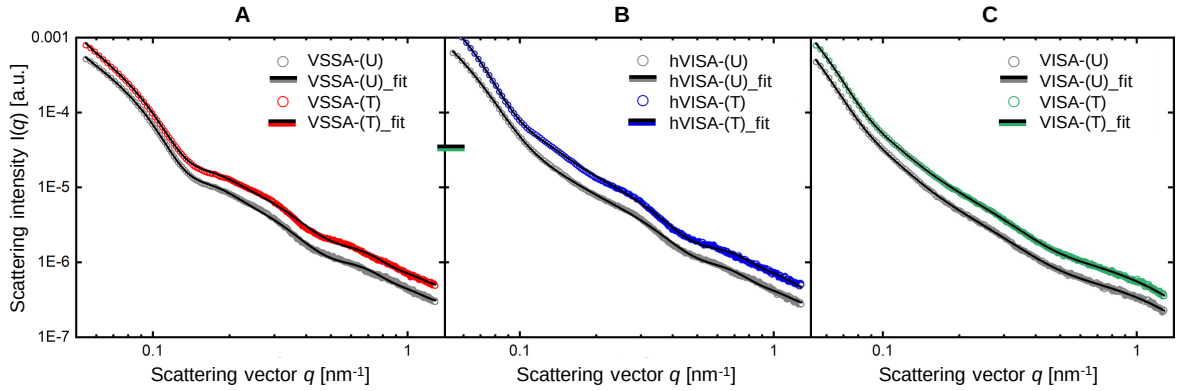

Fig. 2 Comparison of small angle X-ray scattering (SAXS) between vancomycin untreated cells and treated cells of VSSA (A), hVISA (B), and VISA (C) groups.

and PAP-AUC value may have indirect effect to their different SAXS performance. However, further study is required for verification.

For the intracellular components generated from the SAXS scattering curves, significant differences were found among the bacterial groups. Under the untreated condition, the ribosome of VSSA was significantly smaller than that of the hVISA suggesting that they might have discrepancy in performances even under condition without vancomycin pressure, as they

Table 2 Size distribution of internal composition of *S. aureus* modeled from SAXS scattering.

Phenotype	Size distribution* (nm) ± SD					
	1	*p*-value	2	*p*-value	3	*p*-value
VSSA-(U)	58.8 ± 5.2	0.279	17.1 ± 0.1	0.060	3.4 ± 0.6	0.144
VSSA-(T)	65.5 ± 7.6		14.3 ± 0.1		4.3 ± 0.5	
hVISA-(U)	72.1 ± 10.9	0.389	17.1 ± 0.2	1	1.8 ± 0.5	0.443
hVISA-(T)	80.3 ± 9.9		17.1 ± 0.2		2.2 ± 0.5	
VISA-(U)	92.2 ± 13.8	0.748	11.0 ± 1.7	0.408	1.6 ± 0.3	0.818
VISA-(T)	88.7 ± 12.7		12.3 ± 1.7		1.5 ± 0.3	

* Size distributions were calculated by the average diameter in nanometer: 1 = sphere model for ribosome; 2 = sphere model for DNA; 3 = sphere model for small proteins (a few nanometers); VSSA, vancomycin-susceptible *S. aureus*; hVISA, heterogeneous vancomycin-intermediate *S. aureus*; VISA, vancomycin-intermediate *S. aureus*; T, treated with vancomycin; U, untreated with vancomycin.

Table 3 Comparing the size of sphere models between the bacterial groups under vancomycin untreated and treated conditions.

Comparison	Size*	Size distribution (nm) ± SD		*p*-value	
		Untreated	Treated	Untreated	Treated
VSSA-hVISA	1	58.8 ± 5.2 vs. 72.1 ± 10.9	65.5 ± 7.6 vs. 80.3 ± 9.9	0.00001	0.108
	2	17.1 ± 0.1 vs. 17.1 ± 0.2	14.3 ± 0.1 vs. 17.1 ± 0.2	0.405	0.088
	3	3.4 ± 0.6 vs. 1.8 ± 0.5	4.3 ± 0.5 vs. 2.2 ± 0.5	0.029	0.070
VSSA-VISA	1	58.8 ± 5.2 vs. 92.2 ± 13.8	65.5 ± 7.6 vs. 88.7 ± 12.7	0.017	0.060
	2	17.1 ± 0.1 vs. 11.0 ± 1.7	14.3 ± 0.1 vs. 12.3 ± 1.7	0.004	0.109
	3	3.4 ± 0.6 vs. 1.6 ± 0.3	4.3 ± 0.5 vs. 1.5 ± 0.3	0.110	0.001
hVISA-VISA	1	72.1 ± 10.9 vs. 92.2 ± 13.8	80.3 ± 9.9 vs. 88.7 ± 12.7	0.119	0.446
	2	17.1 ± 0.2 vs. 11.0 ± 1.7	17.1 ± 0.2 vs. 12.3 ± 1.7	0.004	0.080
	3	1.8 ± 0.5 vs. 1.6 ± 0.3	2.2 ± 0.5 vs. 1.5 ± 0.3	0.140	0.115

* Size distributions were calculated by the average diameter in nanometer: 1 = sphere model for Ribosome; 2 = sphere model for DNA; 3 = sphere model for small proteins (a few nanometers); VSSA, vancomycin-susceptible *S. aureus*; hVISA, heterogeneous vancomycin-intermediate *S. aureus*; VISA, vancomycin-intermediate *S. aureus*; Treated, treated with vancomycin; Untreated, untreated with vancomycin.

had different PAP-AUC values. However, no significant difference under vancomycin treated condition was found. This finding suggested a different response of the bacteria to vancomycin. It is possible that the vancomycin susceptible cells (VSSA) has higher stress response to vancomycin than those of the vancomycin non-susceptible (hVISA and VISA) cells leading to a drastic change of ribosome in the VSSA cells to be similar to those of the vancomycin non-susceptible (hVISA and VISA). (The ribosomes of the hVISA and the VISA were rarely changed.) As a result, no difference of ribosome among these three bacterial groups under the treated condition (Table 3). In addition, the ribosome and DNA of VSSA vs. VISA, and the DNA of hVISA vs. VISA were different only under the untreated condition, suggesting that even in the condition with no pressure from vancomycin, the VISA cells have usual different performance from those of the VSSA and the hVISA.

Interestingly, the sizes of ribosomes and small proteins in the VSSA and the hVISA groups increased after exposed to vancomycin, in contrast to the reducing size in the VISA group. This may be due to the VSSA and the hVISA cells had higher stress response to vancomycin for their survival, whereas the VISA cells could continue their life cycles as usual. However, further investigation with larger sample size is needed to verify and to understand more on the bacterial performance.

Though the target site of vancomycin is D-alanyl-D-alanine residue of the cell wall [23], it may have indirect effect to other intracellular components of the bacterial cells such as cell membrane, ribosome, and DNA [23]. The VISA and the hVISA cells had thickening cell wall and increasing free D-alanyl-D-alanine residues, leading to vancomycin blockage from reaching its target site [21, 24]. This may be the reason of lower stress response of the VISA and the hVISA to vancomycin than that of the VSSA. The SAXS is a useful technique for investigating the changes of intracellular components of bacteria under the effects of antimicrobials. The different degree in stress responses may be strategically applied to combat the low-level vancomycin resistant bacteria like hVISA and VISA.

Since this is the preliminary study with a limitation of sample size, further studies with larger sample size of each bacterial group, testing under various vancomycin concentrations, and using various antimicrobials are necessary. The studies' results could be used to elucidate the adaptation of bacterial intracellular components during the antimicrobial stress response.

CONCLUSION

This is a preliminary study of VSSA, hVISA, and VISA cellular changes under stress from vancomycin compared with the non-stress treated cells. Using the SAXS, scattering patterns were different among the vancomycin susceptible and non-susceptible *S. aureus*. The SAXS can be used as a method to study the effects of antimicrobials on bacteria to investigate the changes of bacterial ultra-structures.

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