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Phenotypic characteristics and comparative proteomics of *Staphylococcus aureus* strains with different vancomycin-resistance levels

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ABSTRACT

Reduced vancomycin susceptibility of methicillin-resistant *Staphylococcus aureus* (MRSA) is a worldwide problem. Unfortunately, its genetic marker and molecular mechanisms remained unknown. This study investigated differential phenotypic characteristic and protein expression profiles among three groups of MRSA isolates, including vancomycin-susceptible *S. aureus* (VSSA), heterogeneous vancomycin-intermediate *S. aureus* (hVISA) and vancomycin-intermediate *S. aureus* (VISA) ($n = 7$ isolates/group). Phenotypic characteristic revealed significant greater number of isolates with non-spreading colony in VISA as compared to both VSSA and hVISA groups. 2-DE followed by nanoLC-MS/MS analyses revealed increased glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in both hVISA and VISA, whereas 50S ribosomal protein L14 (RplN) and DNA-binding protein II (Hup) were increased only in VISA. The non-spreading colony and GAPDH level of MRSA may be used as the markers for differentiation of VSSA, hVISA and VISA.

1. Introduction

Staphylococcus aureus is one of the problematic microbes causing serious infections in humans and has become a critical public health concern. This bacterium has developed a critical resistance to various antimicrobials. Methicillin-resistant *S. aureus* (MRSA) was first reported in Jevons (1961). Vancomycin, a glycopeptide antimicrobial, is the recommended treatment for serious infections caused by MRSA. However, as a consequence, the first set of isolates of MRSA with reduced susceptibility to vancomycin including, vancomycin-intermediate *S. aureus* (VISA) and heterogeneous VISA (hVISA), had been reported in 1997 (Hiramatsu et al., 1997a, 1997b). The hVISA isolates show vancomycin MICs within the susceptible range but contain a subpopulation (approximately one in 10^5–10^6 cells or more) with reduced susceptibility to vancomycin (MIC of ≥4 μg/ml).

Since the first report of MRSA with reduced susceptibility to vancomycin, number of similar reports has been increasing rapidly throughout the world. These strains are often associated with vancomycin treatment failure and cannot be discriminated from the sensitive strain by routine laboratory methods, such as disk diffusion and MIC determination. The population analysis profile with area under the curve (PAP-AUC) is considered as a gold standard procedure for hVISA detection (Wootton et al., 2001). However, it is time-consuming, labor-intensive, and may be not suitable for overloaded routine service. Moreover, the genetic basis of low-level vancomycin resistance is not clearly understood. In the post-genomic era, proteomics has been developed and serves as a highly powerful tool for studying proteins on a large-scale and may demonstrate the differences in microbial protein expression. Additionally, the study of proteomics provides information regarding biological, pathological and physiological properties (Francois et al., 2010). Therefore, proteomics may provide significant information for understanding mechanisms underlying antimicrobial resistance and for defining marker(s) for detection of the low level vancomycin-resistant strains. In the present study, we thus compared phenotypic characterizations and comparative proteomic analysis of VISA, hVISA, and vancomycin-susceptible *S. aureus* (VSSA).

2. Materials and methods

2.1. Ethics approval

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Khon Kaen University (project number HE552272).

Abbreviations: MRSA, methicillin-resistant *S. aureus*; VSSA, vancomycin susceptible *S. aureus*; VISA, vancomycin-intermediate *S. aureus*; hVISA, heterogeneous vancomycin-intermediate *S. aureus*; nanoLC-MS/MS, nanoscale liquid chromatography coupled to tandem mass spectrometry; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RplN, 50S ribosomal protein L14; Hup, DNA-binding protein II.

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2.2. Bacterial isolates

A total of 21 MRSA isolates composed of 7 sets of parents and their mutants including 7 parent VSSA isolates, 7 induced-hVISA and 7 induced-VISA which derived by culturing the strains in brain heart infusion media supplemented with an increasing vancomycin concentration as previously described (Wongthong et al., 2015). All the isolates were identified by routine laboratory methods and confirmed by detection of specific MRSA genes (*mecA* and *femA* genes) by polymerase chain reaction (PCR) (Kondo et al., 2007). The minimum inhibitory concentrations (MICs) to vancomycin (Sigma, St. Louis, MO, USA) of all isolates were determined by an agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guideline (CLSI, 2012). Bacterial isolates were confirmed as hVISA strains ($n = 7$ isolates/set) by testing a population analysis profile with area under the curve (PAP-AUC) (Wootton et al., 2001). The isolates were kept at −20 °C in skimmed milk (Difco Laboratories, Detroit, MI, USA) plus 20% glycerol. The reference strains of *S. aureus* ATCC 29213 (VSSA), ATCC 700699 (Mu50, VISA) and ATCC 700698 (Mu3, hVISA) were used as the control strains for vancomycin susceptible, vancomycin-intermediate and heterogeneous vancomycin-intermediate *S. aureus*, respectively.

2.3. Molecular typing

Bacterial DNA was extracted by using achromopeptidase enzyme (Sigma) method (Sasaki et al., 2010), and the supernatant was used as DNA template for PCR. The MRSA accessory gene regulator (*agr*) grouping was analyzed according to Lina et al. (2003), and the staphylococcus cassette chromosome *mec* (SCC*mec*) type was determined by using a multiplex PCR as described by Kondo et al. (2007).

2.4. Other phenotypic characteristics

The phenotypic characteristics of all isolates such as colony spreading, urease activity and the thickness of cell wall were determined according to protocols published previously (Sirichoat et al., 2016).

2.5. Bacterial protein extraction and two-dimensional electrophoresis (2-DE)

Bacterial cells were harvested from an overnight culture by a centrifugation at 5000×*g* and 4 °C for 15 min. Protein extraction and 2-DE were performed according to methods described previously (Tavichakorntrakool et al., 2009) with slight modification as follows. The cell pellets were washed twice with low-salt phosphate buffer saline and resuspended in 500 μl of lysis buffer containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 2% IPG buffer (pH 3–10) and 40 mM dithiothreitol (DTT). The undissolved particulates were further disrupted using an ultrasonic homogenizer (Sonic & Materials Inc., Newtown, CT, USA) while being kept on ice. The protein solutions were cleaned with 2-D Clean-up kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. Protein concentration was quantified by the Bradford method using bovine serum albumin (BSA) as a protein standard. The first-dimensional separation or isoelectric focusing (IEF) was performed by using 100 μg of total protein, which was premixed with a rehydration buffer and applied to an immobilized pH gradient (IPG) strip (pH 3–10 linear, 7-cm-long) (GE Healthcare) on EttanIPGphor II Isoelectric Focusing (IEF) Unit (GE Healthcare) at 20 °C using a step-wise mode until reaching 6500 Vh. The second-dimensional separation was performed by transferring the equilibrated IPG strip to a 12% polyacrylamide slab gel in Hoefer mini-Vertical Electrophoresis Unit (GE Healthcare), where the system was run at 150 V for approximately 2 h. The resolved protein spots were visualized by Colloidal Coomassie Blue G-250 staining. The 2-DE of each sample was performed in triplicate.

2.6. Protein spot matching and quantitative analysis

Protein extracts of bacterial isolates were resolved in 2-D gel, individually, for analysis. All gel images were scanned at a resolution of 300 dpi using Image Scanner III with Lab Scan 6.0.1 software (GE Healthcare). Protein spots were then matched and analyzed by using Image Master 2D Platinum 7.0 software (GE Healthcare). The reference gel was then used for matching the corresponding protein spots among different gels. Background subtraction was performed, and the % volume of each spot was normalized with total volume of all spots in each image.

2.7. In-gel tryptic digestion and protein identification by nanoLC-MS/MS

The protein spots with significantly differed intensity levels among groups were excised from the 2-D gels and subjected to in-gel tryptic digestion as described previously (Tavichakorntrakool et al., 2009). Separation of the digested peptides was performed using EASY-nLC II (Bruker Daltonics; Bremen, Germany). Briefly, peptides were loaded from a cooled (7 °C) auto sampler into an in-house, 3-cm-long pre-column containing 5-μm C18 resin (Dr. Maisch GmbH; Ammerbuch, Germany) and then to a 10-cm-long analytical column packed with 3-μm C18 resin (Dr. Maisch GmbH) using mobile phase A (0.1% formic acid). The peptides were then separated by mobile phase B (acetronitrile/0.1% formic acid) gradient elution with three steps as follows: 0–35% for 30 min, 35–80% for 10 min, and then 80% for 10 min at a flow rate of 300 ml/min. Peptide sequences were then analyzed by amaZonspeed ETD (Bruker Daltonics) with ESI nanosprayer ion source (spray capillary: fused silica with outer diameter of 90 μm and inner diameter of 20 μm) controlled by HyStar version 3.2 and trapControl version 7.1. Mass spectrometric parameters were set as follows: electrospray voltage = 4500 V, high-voltage end-plate offset = 500 V, nebulizer gas = 0.55 bar, dry gas =5.0 l/min, and dry temperature = 150 °C. Precursors were scanned from the 400 to 2200 *m/z* range with enhanced resolution mode (speed =8100 m/z/s), ICC (Ion Charge Control) target = 200,000, and maximal accumulation time = 50 ms. The three most intense signals in every MS scan were selected for MS/MS analysis, whereas singly charged ions were excluded. For MS/MS experiments, fragmented peptides from 150 to 3000 *m/z* range were scanned with XtremeScan mode (speed = 52,000 m/z/sec), ICC target =

Table 1
Phenotypic characteristics of VSSA, hVISA and VISA isolates.

	VSSA	hVISA	VISA	*P*-value		
	($n = 7$ isolates)	($n = 7$ isolates)	($n = 7$ isolates)	VSSA vs. hVISA	VSSA vs. VISA	hVISA vs. VISA
PAP-AUC ratio	0.731 ± 0.039	1.003 ± 0.035	2.276 ± 0.422	NS	<**0.001**	**0.002**
Vancomycin MIC of each isolates (μg/ml)	1, 1, 1, 1, 1, 1, 1	2, 2, 2, 2, 2, 2, 2	4, 4, 5, 7, 12, 12, 16	NS	<**0.001**	<**0.001**
No. of isolates with non-spreading colony (%)	1 (14.3)	0 (0)	7 (100)	NS	**0.005**	**0.001**
Urease activity (absorbance unit)	1.078 ± 0.418	1.487 ± 0.347	2.019 ± 0.274	NS	NS	NS
Cell wall thickness (nm)	34.948 ± 0.352	38.606 ± 0.377	46.186 ± 0.362	<**0.001**	<**0.001**	<**0.001**

Abbreviations used: **VSSA**, vancomycin-susceptible *S. aureus*; **hVISA**, heterogeneous vancomycin-intermediate *S. aureus*; **VISA**, vancomycin-intermediate *S. aureus*; **PAP-AUC**, population analysis profile with area under the curve; **NS**, not significant.