

Vancomycin-Intermediate *Staphylococcus aureus* (VISA) in Malaysia: A Case Study

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Abstract

Vancomycin intermediate *Staphylococcus aureus* (VISA) associated infection is low globally despite an increase in the methicillin resistance *Staphylococcus aureus* (MRSA) infection. To date there is no record of VISA in Malaysia. We are reporting the case of VISA isolated from a 66-year-old patient with history of MRSA infection receiving prolonged vancomycin treatment. The isolate was identified using conventional, commercial biochemical test and whole genome sequencing for analysis of 16s RNA region. Antimicrobial susceptibility testing was confirmed using broth microdilution. The organism developed mutations for *graR*, *graS*, and *walR*, *vraR* genes and exhibited thickened cell wall when examined under transmitted electron microscopy. This case is highlighted to promote judicious use of antibiotic and implement stringent infection control measure to prevent the spread of the resistant phenotype.

Keywords: *Staphylococcus aureus*; Vancomycin; Malaysia; Whole genome sequencing; Mutation

Introduction

MRSA continues to be the main pathogen associated with hospital infection, causing high mortality rates among patient. Vancomycin remains as an important agent used in the treatment of the infection. Similarly, increase reliance was seen to other glycopeptide antibiotics such as teicoplanin. These drugs remained as the last agents for intravenous therapy to combat MRSA infection. Hence, the discovery of first strain of *Staphylococcus aureus* with reduced susceptibility to VISA in Japan rippled a global alarm [1].

Vancomycin was first introduced for the treatment of gram-positive organisms in 1958. To date it continues to be the drug of choice for treating most methicillin-resistant *Staphylococcus aureus* (MRSA) infections globally. Increased reliance on vancomycin for the past 20 years was observed, synchronized with the increase in the prevalence of both coagulase-negative staphylococci and *Staphylococcus aureus* [2].

National antimicrobial resistance surveillance programme in Malaysia was initiated in 1989. To date the programme continues to do routine surveillance of the bacteria that were isolated from clinical samples. The susceptibility data was analyzed and the presence of organism with rare susceptibility phenotype will be further confirmed by reference laboratory. Until today no cases of VISA/VRSA has been reported. We are reporting the first encounter of VISA isolated from a referral hospital in Malaysia.

Case Report

RAR has underlying diabetes with end stage renal disease and was on ambulatory peritoneal dialysis. She was initially hospitalized in January 2017 for fever, difficulty in breathing and bilateral leg swelling. Upon examination, although the Glasgow coma scale was full, the patient appeared very lethargic, with temperature of 38.4°C, pulse rate of 163/min and respiratory rate of 40/min. She was dyspnoea but was not cyanosed. There was bilateral pitting oedema up to the upper calves. The abdomen was slightly distended with evidence of shifting dullness, liver size was normal. The respiratory examination showed evidence of basal crepitation indicating fluid overload. The other systems were unremarkable.

Blood investigation upon admission showed severe metabolic acidosis with hyperkalemia. Septic workout failed to isolate any positive culture however serology test was positive for IgM leptospira. The titre was 1 in 640. Intravenous ceftriaxone 1 g every 12 hourly was administered and the temperature settled on day three post treatment. Antibiotic course was continued for another seven days. During this admission, intra-jugular catheter (IJC) was inserted for hemodialysis. Patient was well until day twelfth of admission when she developed spike of temperature and blood culture grew MRSA. Intravenous vancomycin 1gram stat dose and later continued with renal adjustment dose (250-500 mg daily) to combat the infection. The source of MRSA infection was possibly from the IJC, so catheter was removed later. IV vancomycin was continued for total of 25 days from the isolation of MRSA and patient was discharged well with new IJC reinserted before discharge for hemodialysis.

In March 2017, two weeks after the discharge, the patient presented to the hospital again with fever, chills and rigor. The temperature was 39°C upon admission, blood pressure was 134/92 mmHg and heart rate were 95/min. Oxygen saturation was 99%. Blood culture grew MRSA with increased minimum inhibitory concentration (MIC) to vancomycin (4 µg/mL). Thus, the patient was having VISA infection. She was initially given vancomycin 1gm stat followed by renal adjustment dose (500 mg daily) however; treatment was change to intravenous linezolid 600 mg and later daptomycin as she was not responding well to the initial vancomycin treatment. Blood culture became negative of VISA on day 26th of daptomycin 6 mg/kg IV infusion daily treatment. Treatment was continued for another six weeks after the culture became negative. During the treatment platelet level was closely monitored.

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In total the patient had received a 31-day course of intravenous vancomycin before the organisms became intermediate to vancomycin before the treatment was switched to daptomycin.

Materials and Methods

The suspected VISA isolate was sent to our laboratory in April 2017 from a referral hospital.

Identification method

Identification of the organisms was done based on conventional method by taking into consideration the gram stain characteristic, colonial morphology, basic biochemical tests and commercial Vitex System (Biomérieux, France).

Antimicrobial susceptibility testing

Antimicrobial susceptibility test (AST) was performed according to the Clinical Laboratory Standards Institute guidelines and breakpoints were ascertained based on the guideline [3]. Disc diffusion AST was done for penicillin (10 µg), cefoxitin (30 µg), gentamicin (10 µg), tetracycline (30 µg), clindamycin (2 µg), rifampicin (5 µg) and teicoplanin (30 µg) (Oxoid, USA). The minimal inhibitory concentration (MIC) values of vancomycin were determined using the -test (Biomérieux, France) method. Broth microdilution was also performed to confirm the MIC value of the organism [3].

S. aureus ATCC 29213 and *Enterococcus faecalis* ATCC 29212 strains were used as vancomycin susceptible, *E. faecalis* ATCC 51299 as vancomycin resistant and *S. aureus* Mu50 strain was included as vancomycin intermediate controls.

Electron microscopy

The isolate was subjected to imaging under electron microscopy. Briefly, fixation of isolate using glutaraldehyde 2.5% for 10 minutes was performed followed by osmium tetroxide for another 10 minutes. Washing steps were included after every fixation followed by dehydration process using 50,70,90 and 100% of acetone for five minutes each. Later polymerization using ultracut, 90 nm was done before the isolate was viewed under transmission electron microscope (TEM). The wall thickness between *Staphylococcus aureus*, MRSA and the organism under investigation was measured.

PCR analysis

DNA was extracted from this isolate using DNA extraction kit for blood and cell (Promega, USA) according to manufacturer's protocol. The DNA was used as template for amplification process using molecular method based on specific primers targeting the SCC *MecA* and *PVL* genes as previously described [4]. *VanA* genes detection were using specific primer pair as describe earlier [5]. PCR products were visualized using gel electrophoresis on a 1.2% agarose (BioRad, United States) gel, stained with red safe and gel image was viewed using Gel documentation system.

Whole genome analysis

Genomic DNA of the isolate was extracted using commercial DNA extraction kit following the manufacturer's protocol (Illumina®, USA). Genomic DNA purity was measured at absorbance 260: 280 targeting ratio of 1.8 to 2.0. The concentration of genomic DNA was quantified using the Qubit™ dsDNA HS Assay kit (Invitrogen, Life Technologies, USA).

The isolate was then subjected to whole genome sequencing. The genome library preparation was performed by using Nextera® XT DNA

Library Preparation kit (Illumina®, USA) following the manufacturer's protocol. The DNA was purified using Ampere XP Beads (Beckman Coulter, USA). The library quality and concentration were assessed and determined using Agilent Bioanalyzer High Sensitivity DNA chips (Agilent, USA). Then, sequencing template was prepared using Illumina® MiSeq v2 reagent kit (Illumina®, USA) following the manufacturer's protocol. The genome sequencing was carried out using Miseq sequencer (Illumina®, USA).

Sequencing reads were obtained in FASTQ format text file from the NGS sequencer. Phred scores were used as the quality scores for each base. The platforms encode the quality scores using ASCII characters equivalent to Q^{+33} which supported encoding of Phred scores from 0 to 93.

The Phred score was also used to calculate the probability of error using the formula $P=10^{-Q/10}$ where P is the probability of error for bases and Q is the Phred quality score. Poor-quality reads were filtered out using BBTools (version 36) in two sequential steps [6]. First, removing any reads with two or more ambiguous bases or mean Phred quality score less than 20. Second, low-quality bases representing by Phred quality score 19, were trimmed from the 30-end, and if after the trimming the read length was less than 70 bp or mean Phred quality score of the read was less than 50, they were discarded.

The paired end reads passing above quality filters were mapped against *Staphylococcus aureus* N315 reference genome (GenBank accession number NC_002745) using BWA short read aligner version 0.7.2 [7].

Genome assembly

The sequencing reads were assembled using de Bruijn graphs to collapse identical reads and SPAdes [8] to obtain contigs. The high-quality reads obtained at the above step were assembled to form contigs. SPAdes uses k-mers for building the initial de Bruijn graph [9], which detects and removes chimeric reads. Then it estimates the distances between k-mers in the genome and constructs paired assembly before finally constructing the contigs. During the multiple-stages assembly process, SPAdes also adjust for errors iteratively, making it more tolerant to whole genome sequencing data with nonuniform read coverage, variation in insert lengths, higher levels of sequencing errors or chimeric reads.

Genome annotation

The tRNAs and rRNAs genes were predicted using ARAGORN (v1.2.34) [10] and RNAmmer (v1.2) [11]. The protein-coding genes were first predicted using Prodigal [12], and the predicted sequences were used to predict their function by using BLAST [13] and HMMER [14] to search against various sequence or domain databases.

16S rRNA similarity search

The 16S rRNA Similarity Search were performed using BLASTN (v2.2.25+) and the data derived were searched against the 16S rRNA database in NCBI [15].

Whole-genome sequencing and gene mutation

The sequence under study were further compare with four other VISA isolates COL_00690, JH9_00693, Mu50_00656, N315_00636, where the databases were obtained from the NCBI to look for mutation presence within the *vraSR*, *graSR*, *walRS*, *vraSR*, *rpoB* genes which are candidate genes most commonly associated with VISA [16,17]. The mutations were verified by visualizing the alignment files in Clustal W and Clustal X multiple sequence alignment programs (version 2.0).

Genes	Function	Mutation point	Previous study
graR gene	Response regulator	D148Q	[15,16]
graS gene	Sensor histidine kinase	S104L, E108D, E112D, K146E, Y156E, D218N, Y219H, T224I, F245Y, I247V, E248D, G250S, N254I, S259A, Y261I, Y265H, N302D, H310Q, H310Q, V317I, R324K	[17]
vraR gene	Response regulator	E059D	[15]
walR gene	Response regulator	P216S	[18]

Table 1: Gene mutations in candidate genes associated with VISA observed for the isolate.

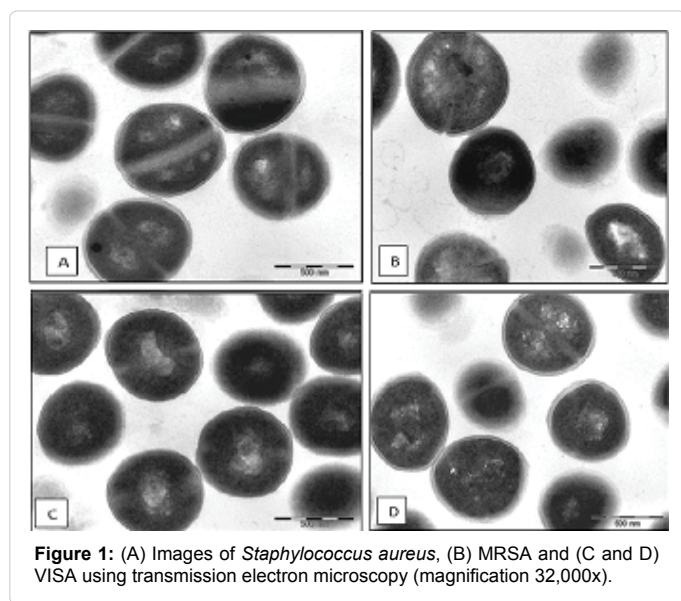


Figure 1: (A) Images of *Staphylococcus aureus*, (B) MRSA and (C and D) VISA using transmission electron microscopy (magnification 32,000x).

Result

Overnight incubation of blood sample at $36 \pm 2^\circ\text{C}$ on blood agar revealed whitish, round colonies with well demarcated edge and surrounding area of beta lysis were observed [18]. It was a gram-positive coccus in cluster. The organism produced positive reaction to catalase, coagulase slide and tube tests. Further identification by using GP card Vitex System (Biomérieux, Inc, France) confirmed the isolate was *Staphylococcus aureus* 99.9%. DNA sequence similarity search for 16s RNA region also further confirmed the identification with score value of 100% and E value 1.

CLSI redefined vancomycin breakpoints as susceptible at a vancomycin broth MIC of $\leq 2 \mu\text{g/ml}$, intermediate at a vancomycin broth MIC of 4 to $8 \mu\text{g/ml}$ and resistant at a vancomycin broth MIC of $\geq 16 \mu\text{g/ml}$. The isolate was confirmed to be vancomycin intermediate *Staphylococcus aureus* with MIC using commercial Etest, $4 \mu\text{g/ml}$. Similar value was also derived using microbroth dilution for confirmatory test. The organism was resistant to penicillin, cefoxitin, gentamicin, tetracycline and clindamycin. It was intermediate to teicoplanin but was sensitive to linezolid with MIC of $3.0 \mu\text{g/ml}$.

The organism harboured SCC *Mec A* and *PVL* genes however does not demonstrate *Van A* gene. Visualization under electron microscope revealed a thicker cell wall compared to the MRSA and Methicillin sensitive *Staphylococcus aureus* which explained the increase in vancomycin MIC reading (Figures 1A-1D).

Approximately 1.8 million paired end reads were obtained for this isolate with read lengths ranging from 35 to 251 bp, and median coverage of 171 was achieved. The numbers of paired reads before and after quality control were 1,863,764 and 1,552,008.

De novo assembly of the reads was performed using IDBA-UD genome assembler (v1.0.9) that generated 84 contigs with total size of 2.86 Mbp. The assembly coverage was satisfactory (171%). The longest contig was 290,695bp in length. GC content was 32.7%.

The protein-coding gene prediction showed a total of 2668 protein-coding genes sequences per genome. In total 24 gene mutations were observed for the isolate under study involving *graR*, *graS*, and *walR*, *vraR* genes. No significant mutation was detected for *vraS*, *walK*, *rpoB* genes (Table 1).

The whole genome sequence was deposited in DDBJ/ENA/GenBank under the accession number PKLD00000000.

Discussion

This case report indicated a hospital acquired VISA infection in patient admitted for leptospiral infection. Similar finding of VISA infection has been reported in other Asian countries [19,20], Australasia [21], European [22,23] and American Continent [24].

Increased use of vancomycin or history of previous exposure to vancomycin for MRSA treatment in patients has been identified as the predisposing factors for the emergence of VISA which lead to vancomycin treatment failure [20]. This patient has been exposed to intravenous vancomycin course for 31 days prior to the increased vancomycin MIC level organism was detected. The resistant entity evolved *in vivo* upon exposure towards vancomycin [25]. *Scc MecA* gene and *PVL* genes that were associated with MRSA were detected for this organism. In contrast, lack of *Van* genes for this isolate is expected as this gene is associated with vancomycin resistant strain (VRSA).

It was postulated and confirmed that mutation/s that occurs in vast genetic loci involving genes encoding for global regulators of *Staphylococcus aureus* were responsible explanation of the emergence of resistance among VISA strains [26]. The mutation arose in sequential manner suggesting that it was partly a defence process of the bacteria for survival in the presence of antibiotic. A report by Howden et al. [21] stated that mutations in regulatory genes such as *walKR*, *vraRS*, and *graRS*, and the gene encoding RNA polymerase subunit B (*rpoB*) were mostly link to VISA phenotype. The VISA isolate in this study showed mutations in *graR*, *graS*, *vraR* and *walR* genes. Most of the mutations were found within *graS* gene, an important protein in gibberellin (GA) signaling, which regulates various aspects of bacterial growth and development. However, no mutation was seen among *vraS*, *walK* and *rpoB* genes.

Generally, VISA isolates exhibit significantly thicker wall compared to *Staphylococcus aureus* sensitive strain and MRSA isolates [27]. This isolate demonstrated similar finding upon electron microscopic examination, which explained the increased in MIC value.

Laboratory ability to confirm VISA infection is very crucial in the management of patients. Microbroth dilution (MDB) is the recommended confirmatory susceptibility method. However, majority of laboratories in Malaysia are using commercial gradient method (E

test) for vancomycin MIC. There is possibility of error as gradient MIC has been known to give slightly higher reading than MBD [28]. Hence, suspected isolates will need to be referred to reference laboratory for further verification.

Clinical implication of VISA infection was vastly studied [29,30] and no direct correlation has been observed in increasing mortality of patients. However, the VISA infection will lead to vancomycin treatment failure, prolonged hospitalization and extended treatment using alternative expensive antibiotics. This patient survived the infection after receiving an alternative treatment agent though she was an immuno-compromised patient. Nevertheless, there has been report stating that some heterogenous VISA infection may still be cured by glycopeptides agents [31].

Emergence of VISA strain in Malaysia also emphasized the importance of prudent use of antimicrobial to avoid escalation of problem and the emergence of VRSA, and infection-control measures to prevent the transmission among hospitalized patients. Proper infection control measures may impede the spread of the infection [32].

Conclusion

The finding from our report highlights the importance of laboratories to be on the alert for this resistant phenotype. Caution may need to be imposed especially on patients with MRSA infection who received prolonged vancomycin therapy and showed clinical evidence of poor respond to the therapy. This finding also warrants a more extensive epidemiological and surveillance study to really ensure that number of cases are not under/missed diagnosed. Further investigation on the clonality of the strain is required as previous reports has described the selection of subpopulations of VISA colonies from previously vancomycin-sensitive strains with common clonal based on pulse-field-gel-electrophoresis patterns [33].

Ethical Issue

The patient has verbally consented for this case report. To avoid other issues, we have kept the name anonymous.

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