Research Article

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Phenotypic and molecular characterisation of Staphylococcus aureus with reduced vancomycin susceptibility derivated in vitro

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Abstract: Vancomycin has been the primary agent used to treat serious Methicillin-resistant *Staphylococcus aureus* (MRSA) infection for many years. However, the rise of MRSA infection rates and the extensive use of vancomycin have led to the emergence of reduced vancomycin susceptibility. Therefore, four typical *Staphylococcus aureus* (*S. aureus)* strains from different clinical specimens were derivated by vancomycin in vitro to better clarify their phenotypic and molecular characteristics. Some experiments, such as stepwise selection of vancomycin-resistant strains, pulsed-field gel electrophoresis (PFGE), antimicrobial susceptibility test, population analysis profile-area under the curve (PAP-AUC), molecular typing, transmission electron microscopy, δ-hemolysin expression, autolysis assay, biofilm assay and quantitative real-time polymerase chain reaction (qPCR) for gene expression were carried out to compare the derivated bacteria with their parental strains. Results showed that the observed phenotypes of vancomycin-resistant strains such as hemolysin, autolysis and biofilm significantly reduced, which were associated with vancomycin resistance capability of the selected strain. The changes of phenotype and regulatory genes expression were inversely proportional to the vancomycin minimum inhibitory concentration (MICvan). Most heterogeneous vancomycin intermediate *Staphylococcus aureus* (hVISA) or VISA strains belonged to *spa* type t570 and *agr* group II. In summary, the clinical isolated vancomycin susceptible *Staphylococcus aureus* (VSSA), hVISA and VISA could be derivated into high vancomycin-resistant VISA in vitro, but it was difficult for them to develop into vancomycin resistant *Staphylococcus aureus* (VRSA). VISA and hVISA could gradually adapt to the environment with the vancomycin concentration that continuously elevates.

Keywords: molecular characterisation; phenotype; *Staphylococcus aureus*

1 Introduction

Vancomycin has been the primary agent used to treat serious MRSA infection for many years. However, the rise of MRSA infection rates and the extensive use of vancomycin have led to the emergence of reduced vancomycin susceptibility. Vancomycin resistant *S. aureus* strains are associated with inefficient vancomycin treatment [1]. Nowadays more and more clinical *S. aureus* strains with reduced vancomycin susceptibility have been reported frequently, including hVISA and VISA [2-7]. In 2008, researchers from peking union medical college surveyed 1012 strains of MRSA collected from 14 cities in China, showed that the incidence of hVISA was between 13% and 16% [8]. In addition, our previous studies have shown that the four-year period (2007-2010) of hVISA prevalence is from 8.2% to 11.7% in Northeast China [7].

Cell wall thickening with structural abnormality is the most common feature of hVISA comparing with VSSA [9,10]. Studies have shown that genetic changes also

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lead to low-level vancomycin resistance in *S. aureus*. *Spa* typing has emerged as an effective and rapid method for typing MRSA to moderate discrimination and exhibit high throughput inter-laboratory reproducibility [11]. Moreover, the two-component regulatory system of the accessory gene regulator (*agr*) has been linked to low-level vancomycin resistance in *S. aureus* as well [12].

Although *S. aureus* with reduced vancomycin susceptibility are clinically relevant, most of the data comes from patients with bacteremia. Therefore, four typical *S. aureus* strains from different clinical specimens were selected in this experiment, a VSSA from blood, a hVISA from sputum, a hVISA from blood, and a VISA from drainage liquid. To better clarify the phenotypic and molecular characteristics these strains were induced with vancomycin by an in vitro multi-step treatment.

2 Material and methods

2.1 Bacterial strains and culture conditions

From 2007 to 2011, 757 strains of *S. aureus* were collected in two affiliated hospitals (2249 beds and 4818 beds respectively) of China Medical University and stored at -80°C in brain heart infusion (BHI) broth (Becton Dickinson, Sparks, MD, USA) containing 20% (v/v) glycerol. From each patient only one isolated strain was used in this study. The strains of *S. aureus* were identified with coagulase test using test tube and a VITEK 2 automation system (bioMerieux, France). Subsequently they were screened for hVISA or VISA with BHI agar containing 3 μg/ml vancomycin (BHIA-3V) by PAP-AUC methods. Finally, 20 VSSA, 76 hVISA and 4 VISA strains were derivated. The MIC_{VAN} of strains was detected by antimicrobial susceptibility test. Based on the results of the test, four representative strains, with the number of 11, 143, 187 and 18 from different clinical specimens were selected for further experiments, and their MIC_{VAN}s were 1 μg/ml, 1 μg/ml, 2 μg/ml, and 4 μg/ml, respectively.

2.2 Stepwise selection of vancomycin-resistant strains

In order to select vancomycin-resistant strains, the clinical parental strains were subjected to a stepwise selection in the presence of vancomycin [13]. About 100 μl of 10^5 colony-forming unit (CFU)/ml bacterial suspension cultured in BHI medium harboring 1/2 MIC vancomycin was plated on brain heart infusion agar (BHIA) plate which was supplemented with 4% sodium chloride and 1 MIC vancomycin. These strains were then plated onto BHIA plates with the vancomycin concentration of 1 μg/ml which was higher than 1 MIC. This selection procedure was repeated, and during each round of selection the used vancomycin concentration increased 1 μg/ml than that of last time until no further colonies could be isolated. Figure 1 shows the induction process of four parental strains. The total selection time for strain 11, 143, 187, and 18 was 45 days, 55 days, 60 days, and 60 days respectively. Therefore, the obtained derivative strains were named 11*45d, 143*55d, 187*60d, and 18*60d correspondingly. All strains were validated for their isogenicity by PFGE and stocked in 20% glycerol at -80°C.

2.3 PFGE

PFGE was carried out as described previously [14]. Chromosomal DNAs of the parental and derivated strains were digested with SmaI and separated using a Gene Path system (CHEF-DR, PULS WAVE.760; Bio-Rad, Hercules, USA) according to the manufacturer's instructions, and a 48.5 kb ladder was used as the DNA size marker (Bio-Rad). Electrophoresis was performed for 22 h with an initial pulse time of 5 s and final pulse time of 40 s.

2.4 Antimicrobial susceptibility test

The MIC_{VAN} of strains were detected by trace cation-supplemented Mueller-Hinton broth (Oxoid) dilution method which was recommended by the guidelines of the Clini-

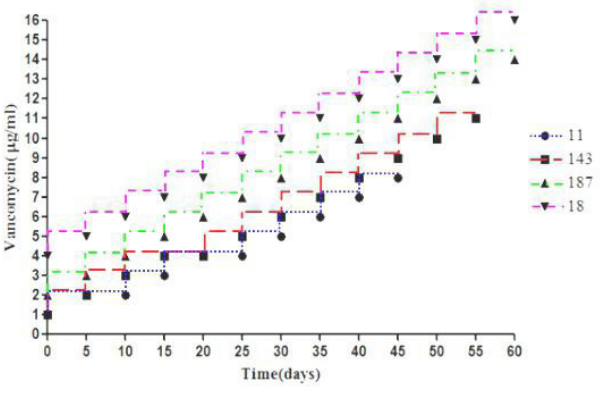

Figure 1: The induction process of four parental strains 11, 143, 187 and 18 with an initial vacomycin concentration of 1 μg/ml, 1 μg/ml, 2 μg/ml and 4 μg/ml respectively. Each round of selection lasted for 5 days with an increase of 1 μg/ml vancomycin concentration repeatedly.

cal And Laboratory Standards Institute (CLSI) [15]. And the MIC_{VAN} assays was determined both by the parental strain and the derivated strain. In general, MH medium was the only medium used in antimicrobial susceptibility test. However to simulate different growth environment, BHI media was used together with MH media in this study [16,17]. Vancomycin was obtained from Sigma Chemical, St. Louis, MO, USA.

2.5 PAP-AUC test

The vancomycin-resistant subpopulations of both parental and derivated strains were measured by PAP-AUC test [18]. In brief, after overnight growth in BHI broth, 50 µl bacterial suspension of seed culture was transferred into 4 ml pre-warmed BHI broth and incubated at 37°C, 200 rpm shaking speed. The new culture was cultivated in same growth condition to a status of approximately OD_{600} 0.7, and diluted with BHI media to OD_{600} 0.3 (approx. 10^8 CFU/ml). The cultures were then diluted in saline to 10^{-7} CFU/ml and spreaded onto BHIA plates containing 0, 1, 2, 3, 4, 5, 6, 8, 10 or 12 mg/L of vancomycin respectively. Colonies were counted after 48 h incubation at 37°C and the viable count was plotted against vancomycin concentration using GraphPad Prism 5 (GraphPad; San Diego, CA, USA). The plotted data was then used to calculate AUC. To distinguish VISA, hVISA and vancomycin-susceptible MRSA, a ratio that the AUC of tested MRSA divided by the corresponding AUC of Mu 3 was calculated. The criteria used for detection of hVISAs were the AUC ratio of 0.9. In this test, the hVISA strain Mu3 (ATCC 700698) and VISA strain Mu50 (ATCC 700699) were used as positive controls while the VSSA strain ATCC29213 was used as a negative control. The result of each experiment was adopted only when the positive and negative controls were confirmed.

2.6 Molecular typing

A standard protocol for DNA extraction was used to provide template in PCR experiments [19]. The *spa* gene encoding protein A of *S. aureus* was amplified and the DNA product was sequenced by Beijing Genomics Institute (Beijing, China) [20]. RIDOM Staph-Type software (Ridom GmbH,Germany) was used to analyze the *spa* sequence and define the *spa* type. In multiplex PCR, 5 primers were used to analyze the *agr* type [21]. The primers used in this experiments are summarized in Table 1.

2.7 Transmission electron microscopy

The *S. aureus* cells in logarithmic phase were fixed in 2.5% glutaraldehyde of 0.1 M PBS (pH 7.4) for 1 h, and treated with 1% osmium tetroxide at 4°C for 1 h. These cells were then dehydrated with different graded concentrations of ethanol, and embeded in Epon812. Ultra-thin sections were stained with uranium acetate and lead citrate, then examined with JEM-1200EX transmission electron microscope (Jeol, Japan). The cell wall thickness was evaluated by photographic images at a magnification of 30,000 times and measured as Cui described previously [22]. Ten cells of each strain were measured in this study.

2.8 δ-hemolysin expression

RN4220 is a standard strain of *S.aureus* which can only produce β-hemolysin on sheep blood agar plate. While β-hemolysin production in RN4220 could be enhanced by δ-hemolysin, the expression tests were carried out on sheep blood plate. One linear *S.aureus* RN4220 cells was firstly streaked out on the plate, and then the parental and derivated strains were cross-streaked perpendicularly to RN4220 on sheep blood agar plate to detect the δ-hemolysin they produced [23]. The VSSA strain ATCC29213 was used as a positive control and the hVISA strain Mu3 (ATCC 700698) and VISA strain Mu50 (ATCC 700699) were used as negative controls respectively.

2.9 Autolysis assay

For autolysis assay, *S.aureus* was inoculated in BHI media at 37°C to make a seed culture. After overnight growth, 200 µl bacterial suspension was added to 20 ml BHI broth and incubated at same culture condition until OD_{600} was raised to 0.8. Then the culture was chilled rapidly, washed in ice-cold PBS and resuspended in 50 mM glycine-0.01%Triton X-100 buffer (pH 8.0) to an OD_{600} of about 1.0. Samples were incubated with gentle shaking, and the OD_{600} was measured hourly using a U-2900 double beam spectrophotometer (Hitachi) [24]. Each assay was performed in triplicates respectively.

2.10 Biofilm assay

The ability of strains to adhere to a polystyrene microtiter plate was assessed as described before [25]. After overnight growth in 4 ml BHI broth, the *S.aureus* culture was

Table 1: Primers used in molecular typing experiments

Fragment	Primer Orientation	Sequence
spa	Forward	5'-TAAAGACGATCCTTCGGTGAGC-3'
	Reverse	5'-CAGCAGTAGTGCCGTTTGCTT-3'
agr	Sa	5'-ATGCACATGGTGCACATGC-3'
	1Sa	5'-GTCACAAGTACTATAAGCTGCGAT-3'
	2Sa	5'-TATTACTAATTGAAAAGTGCCATAGC-3'
	3Sa	5'-GTAATGTAATAGCTTGTATAATAATACCCAG-3'
	4Sa	5'-CGATAATGCCGTAATACCCG-3'

diluted to a1:100 ratio in 4 ml fresh BHI broth, and grew to a OD_{600} of about 0.3. Then the newly obtained culture was diluted again to the concentration of about 10^5 CFU/ml, and 200 µl of which was added to each well of the 96-well polystyrene microtiter plate (Greiner bio-one), with eight replicates per experiment. The plate was incubated at 37°C with shaking for 24 h. Unbound cells were removed carefully by inversing the plate and tapping on an absorbent paper. The adherent cells were stained with 200 µl of 0.1% crystal violet, and the excess dye was removed by rinsing three times with 250 µl PBS. The solvent was then removed from the cells and the crystal violet was dissolved by adding 200 µl of ethanol-acetone (80:20, wt/wt). The UV-Vis absorbance of the sample was measured at 578 nm with a VarioskanTM flash multimode reader (Termo Fisher). Each assay was performed in triplicates respectively.

2.11 Quantitative real-time PCR for gene expression

Total RNA from each sample in early-exponential phase was extracted using the RNAiso Plus Kit (Takara) following the manufacturer's instructions [26]. To remove DNA contamination, each RNA sample was digested with DNase I using standard DNase protocol (Takara), and cDNA was reverse-transcripted using PrimeScript® RT reagent Kit with gDNA Eraser (Takara). Six genes (16S ribosomal RNA, RNAIII, icaA, icaR, lytM and atl) were monitored by qPCR reactions, which were performed with 2 µl of cDNA, 10µl of SYBR® Premix Ex TaqTM II (Takara), 0.8 µl of forward primer (0.4 µM), 0.8 µl of reverse primer (0.4 µM), and RNase free water added to a final volume of 20 µl at last. Reactions were carried out with an ABI 7500 Real-Time PCR system (Applied Biosystems). The primers used in the experiments are summarized in Table 2. The transcription of each gene was normalized on the basis of 16S rRNA level. Each assay was performed in triplicate and repeated twice.

2.12 Statistical analysis

Statistical analyses were performed using SPSS version 16.0 (SPSS Inc., USA). Statistical significance was determined with the unpaired Student's t-test. P-value less than 0.05 was considered statistically significant.

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board or equivalent committee.

Informed consent: Informed consent has been obtained from all individuals included in this study.

3 Results

3.1 Stepwise selection of vancomycin-resistant strains

During the induction process (Fig.1), it was interesting to see that the survival of strain 11 became harder and harder when the concentration of vancomycin increased gradually, and fewer vancomycin-resistant subgroups emerged compared to other three strains. After 45 days' continuous induction of vancomycin in vitro, strain 11 could eventually grow on BHIA plate with vancomycin concentration as high as 8 µg/ml. Before the concentration of vancomycin in BHIA plate was increased to 4 µg/ml, a few subgroups of strain 143 could survive, but after repeating the induc-

Table 2: Primers used in quantitative real-time PCR experiments

Fragment	Primer Orientation	Sequence
RNAIII	Forward	5'-TTCACTGTGTCGATAATCCA-3'
	Reverse	5'-TGATTTCAATGGCACAAGAT-3'
icaA	Forward	5'-CTTGCTGGCGCAGTCAATAC-3'
	Reverse	5'-GCGTTGCTTCCAAAGACCTC-3'
icaR	Forward	5'-GGAGTATGACGGTACAACAC-3'
	Reverse	5'-CGCCTGAGGAATTATCTGGA-3'
lytM	Forward	5'-ATACATTCGTAGATGCTCAAG-3'
	Reverse	5'-ATCATGGCTGTTATACGCTTG-3'
atl	Forward	5'-GGCAAGCGTTATCCGGAATT-3'
	Reverse	5'-GTTTCCAATGACCCTCCACG-3'
16SrRNA	Forward	5'-GGCAAGCGTTATCCGGAATT-3'
	Reverse	5'-GTTTCCAATGACCCTCCACG-3'

tion steps in the same vancomycin concentration for multiple times, vancomycin-resistant subgroups increased abundantly. Strain 143 could eventually grow on BHIA plate with vancomycin concentration as high as 11 μg/ml by increasing the vancomycin concentration continuously for 55 days. The induction processes of strain 187 and strain 18 were relatively smooth compared with strains 11 and 143. The final tolerated vancomycin concentration of strain 187 and strain 18 was 14 μg/ml and 16 μg/ml after 60 days' induction respectively.

3.2 PFGE results

Strains were considered identical in genotype when their PFGE patterns contained the same number and sizes of fragments. PFGE patterns with less than four band differences from an existing genotype were defined as its subtypes [21]. The PFGE results (Fig.2) showed that the electrophoretic bands of all the derivated strains were consistent with their parental strains, which indicated that the derivative strains were homologous to their parental strains. The electrophoretic band of each derivated strain was not identical compared with each other, which illustrated the different clinical origin of the four derivated strains.

3.3 Antimicrobial susceptibility results

It was interesting to see that the MIC_{VAN}s of all strains in BHI medium were higher than that in MH medium (Table 3). The parental strains 11 and 143 had the same MIC_{VAN} of 1 μg/ml in MH medium and 2 μg/ml in BHI medium, their corresponding derivated strains 11*45d and 143*55d also displayed the same MIC_{VAN} of 4 μg/ml in MH medium and 8 μg/ml in BHI medium respectively. The MIC_{VAN} of parental strain 187 was 2 μg/ml in MH medium and 6 μg/ml in BHI medium, while the MIC_{VAN} of 187*60d was 6 μg/ml in MH medium and 12 μg/ml in BHI medium. The MIC_{VAN} of 18 was 4 μg/ml in MH medium and 6 μg/ml in BHI medium, while the MIC_{VAN} of 18*60d was 6 μg/ml in MH medium and 16 μg/ml in BHI medium.

3.4 Molecular typing

Sequence analysis revealed that the *spa* and *agr* genes of parental strains 143, 187, 18 and their derivated strains belonged to *spa* type t570 and *agr* group II respectively while strain 11 and 11*45d had the same genotype of *spa* type t3932 and *agr* group I (Table 1).

3.5 Transmission electron microscopy

As expected, the cell wall of derivated strains was much more thicker than the corresponding parental strains (Fig.3). The cell wall thickness in nm of parental strains 11, 143, 187, and 18 were 20.34±5.46, 25.63±3.78, 27.21±4.43, and 28.89±6.12 respectively, while the number of their corresponding derivated strain 11*45d, 143*55d, 187*60d, and 18*60d had cell wall thickness 40.67±4.52 ($p<0.05$), 45.92±5.81 ($p<0.05$), 63.54±2.37 ($p<0.01$), and 78.62±7.98 ($p<0.01$) respectively.

3.6 PAP-AUC results

Compared with parental strains, the numbers of vancomycin-resistant subgroups of derivated strains increased significantly (Fig.4). Meanwhile their resistance to vancomycin was significantly enhanced. The parental strain 11 was classified to be VSSA because of the ratio AUC11/AUCMu3 <0.9, while the corresponding derivated strain 11*45d was classified to be VISA because of the ratio value of AUC11*45d/AUCMu3>1.3. The parental strain 143 was classified to be hVISA because the ratio value ranges from 0.9≤ AUC143/AUCMu3 ≤1.3, while the corresponding derivated strain 143*55d was VISA. Similarly, the parental strain 187 and the derivated strain 187*60d were hVISA and VISA respectively while the both the parental strain 18 and the derivated strain 18*60d were VISA.

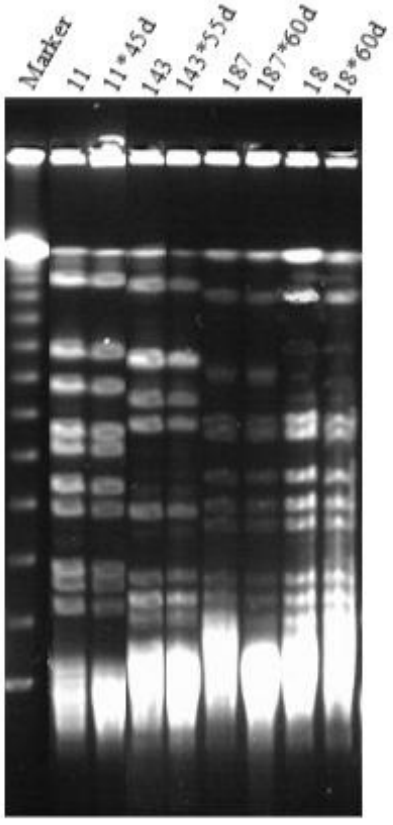

Figure 2: The electrophoretic bands of parental and derivated strains. DNA marker is Bio-Rad CHEF DNA size marker (#170-3635).

3.7 Autolytic assay

Compared with the parental strains, the autolysis activity of all derivated vancomycin-resistant VISA strains were decreased, but the reduction level of autolysis was different (Fig.5). The autolysis level of strain 11*45d, 143*55d and 187*60d was between that of standard hVISA strain Mu3 and Mu50, close to that of Mu50 and higher than that of Mu50 respectively. However, the autolysis level of parental VISA strain 18 was less than that of Mu50 while its derivated strain 18*60d did not decreased significantly but increased to be a little higher that that of Mu50.

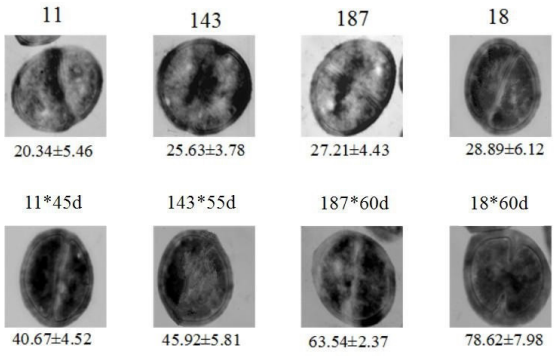

Figure 3: The cell wall thickness (nm) of representative parental strains and their vancomycin-resistant derivated strains. Microscope magnification: ×30,000

Table 3: The MICvan and molecular type of four pairs of strains determined by CLSI broth microdilution method with BHI and MH medium

Strains	Source	Oxacillin resistance	Glycopeptide phenotype	Vancomycin MIC (μg/ml) BHI	MH	Molecular type spa type	agr group
11	blood	MSSA	VSSA	2	1	t3932	I
11*45d				8	4		
143	sputum	MRSA	hVISA	2	1	t570	II
143*55d				8	4		
187	secretion	MRSA	hVISA	6	2	t570	II
187*60d				12	6		
18	drainage	MRSA	VISA	6	4	t570	II
18*60d				16	6		

3.8 δ-hemolysin expression

Fig. 6 shows the δ-hemolysin production of all the tested strains. Similar to positive control (strain ATCC29213), there was an enhanced zone of hemolysis at the border of VSSA strain 11 and quality control strain RN4220. However, there was no enhanced hemolysis zone at the borders of vancomycin-resistant parental or derivated strains with strain RN4220, just like the results of negative controls (hVISA strain Mu3 and VISA strain Mu50).

3.9 Biofilm assay

The results of biofilm assay revealed a reduction in adhesive ability for all derivated vancomycin-resistant strains compared to that of the parental strains ($p<0.05$). Moreover, the biofilm formation ability of parental VISA strain 18, hVISA strains 143 and 187 reduced significantly compared with VSSA strain 11 (Fig.7).

3.10 Quantitative real-time PCR results

The results of quantitative real-time PCR showed that the relative expression levels of *atl* gene which related to autolysis for the derivated strains 11*45d, 143*55d, 187*60d, and 18*60d were 0.21, 0.77, 0.29, and 0.89 times fold changes compare with their corresponding parental strains, and that levels of another autolysis related gene *lytM* for the four derivated strains were 0.41, 0.78, 0.62, and 0.93 times respectively. The relative expressions of *icaA* gene which related to the biofilm formation were 0.35, 0.64, 0.19, and 0.72 times, while the relative expressions of another that related gene *icaR* were 0.36, 0.53, 0.56, and 0.67 times respectively. The relative expressions of RNAIII gene, an effect factor in *agr* regulating system, were 0.49, 0.01, 0.03, and 0.83 times respectively in the derivated strains compared with their parental strains (Fig.8).

4 Discussion

The vancomycin's mode of action is well known as the inhibition of bacteria cell wall biosynthesis by binding with the terminal D-alanyl-D-alanine moieties via hydrogen bond interactions formations [27]. The interaction of the five hydrogen bonds form the binding network in the vancomycin dimer conformation [28,29]. Since the first VISA and hVlSA were reported in Japan [30,31], the *S. aureus* with reduced vancomycin susceptibility have attracted more and more attention from scientists. The reported vancomycin resistance mechanism is the induced alteration of terminal amino acid residues from D-alanyl-D-alanine to a D-alanyl-D-lactate variation which results in the loss of one hydrogen-bonding interaction [32]. The evi-

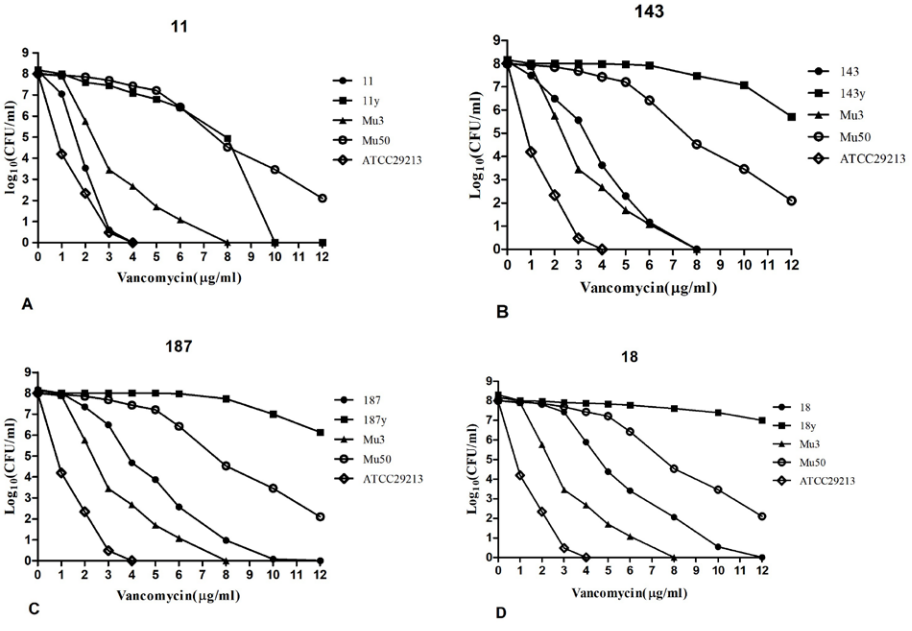

Figure 4: Population analysis of four pairs of parental strains and derivated strains. The hVISA strain Mu3 (ATCC 700698) and VISA strain Mu50 (ATCC 700699) were used as positive controls. The VSSA strain ATCC29213 was used as a negative control.

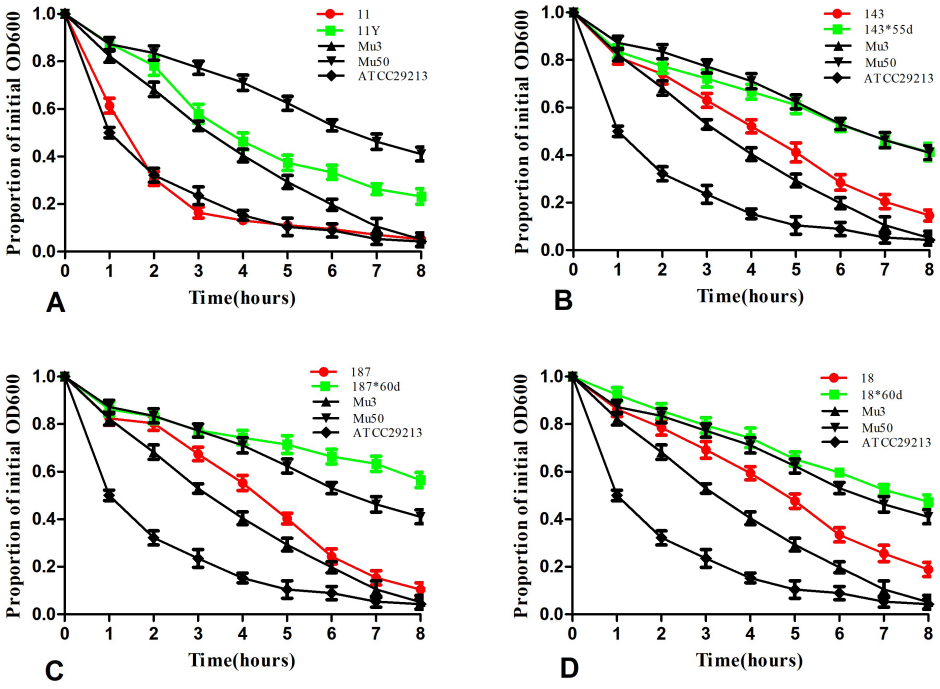

Figure 5: Autolytic assay results of four pairs of parental strains and derivated strains. The results which described as the mean OD values were done in triplicates.

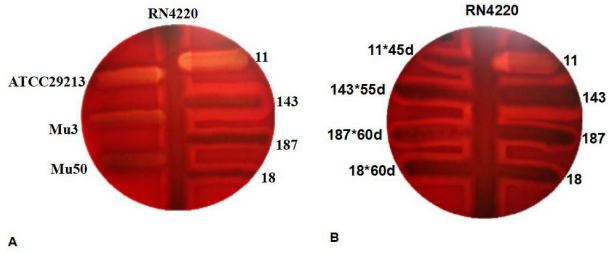

Figure 6: The δ-hemolysin production in parental and derivated strains. β-hemolysin producing strain RN4220 is streaked vertically and the test strains are streaked horizontally. The VSSA strain ATCC29213 was used as a positive control, the hVISA strain Mu3 (ATCC 700698) and VISA strain Mu50 (ATCC 700699) were used as negative controls.

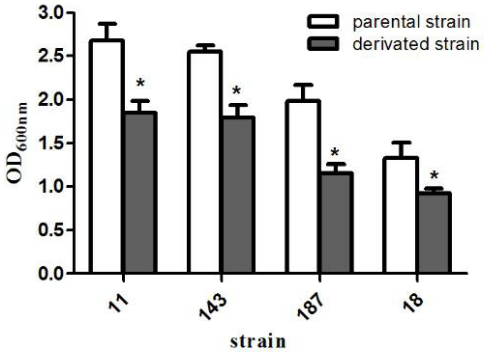

Figure 7: The biofilm formation of four pairs of parental and derivated strains (*P<0.05). The results were done in triplicates.

dence about phenotypic and molecular characterization of vancomycin-resistant *S. aureus* mainly come from clinical cases [33-36], however the complex environment of the human body will affect the exploration of relationship between VSSA and vancomycin-resistant hVISA or VISA. In this study, the in vitro stepwise selection of vancomycin-resistant strain was used to simulate the process of clinical inducing treatment. It is interesting that none of the derivated strains developed into VRSA in highest vancomycin concentration during up to 60 days of persistent vancomycin stimulation. PFGE analysis and molecular typing suggested that the parental strains were genetically homologous to their corresponding derivated strains .

We also found that the MIC_{van}s of *S. aureus* strains in BHI medium were higher than that in MH medium. These results indicate that the vancomycin resistance level of *S. aureus* isolates is closely related to the nutritional status of environment. BHI medium which can provide better nutrition for *S. aureus* is similar to the environment of human body, while MH medium which just like the external environment does not provide enough nutrition to support bacteria growth. This could be the reason why the derivated strains of *S. aureus* can resist high concentration of vancomycin in BHI culture medium but the MIC_{van}s detected with MH media can not rise to VRSA level. It is

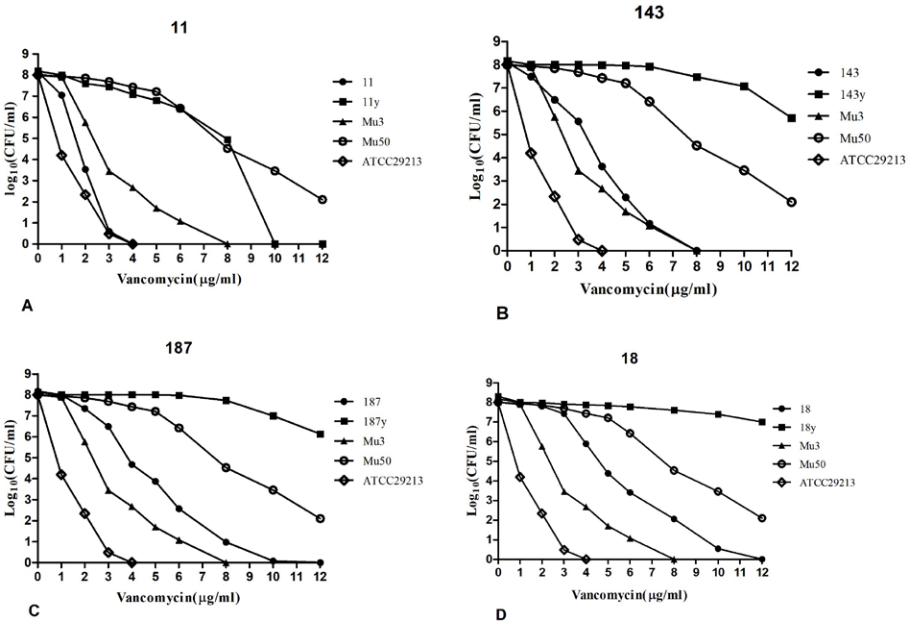

Figure 8: The relative gene expression (A. *Atl*, B. *lytM*, C. *icaA*, D. *icaR*, E. RNAIII) of derivated strains compared to that of their parental strains (*P<0.05, **P<0.01).

also worth to mention that BHI medium was used to detect the differences of vancomycin resistance between parental and derivated strains, which displayed similar results in PAP-AUC test. Under the same vancomycin concentration, the quantity of vancomycin-resistant subgroups of the derivated strains was close to or more than that of the standard VISA strain Mu50, but none of the strains developed into VRSA. A significant increase in the cell wall thickness demonstrated in all hVISA and VISA strains was consistent with previous reports [10,23], from which the cell wall thickness was confirmed positively to be associated with the degree of vancomycin resistance (Fig. 3).

The results of molecular typing revealed that the parental hVISA or VISA strains 143, 187, 18 and their derivated strains belonged to *spa* type t570 and *agr* group II, while the parental VSSA strain 11 and its derivated strain belonged to the same *spa* type t3932 and *agr* group I (Table 1). These findings are consistent with the previous reports, in which the *agr* type II strains and loss of *agr* function are associated with VISA [12].

δ-hemolysin production was another function for *agr* operon [37]. The *agr* operon consists two divergent promoters P2 and P3, and the expression of P3 controlled operon produces the *agr* effector molecule RNAIII which is responsible for post-transcriptional regulation of multiple virulence factors, such as α-haemolysin and δ-haemolysin (encoded within the RNAIII transcript) [38,39]. The lack of δ-hemolysin expression was most likely due to the loss of *agr* [12]. In this study the only clinical parental VSSA strain 11 produced strong δ-hemolysin, however, all the other hVISA and VISA strains did not produce δ-hemolysin. Furthermore, the expressions of RNAIII in the derivated strains significantly reduced compared with their corresponding clinical parental strains (Fig.8). In these cases, we infer that hVISA and VISA can produce less toxin than VSSA due to the lowly expressed regulatory genes for producing the main toxin δ-haemolysin.

In addition, the loss or defection of *agr* function is also associated with autolysis level of *S. aureus* [23,25]. Reduced autolytic activity has already been established as a common symptom to the hVISA and VISA phenotype [25]. In our study, autolytic activity reduction was observed in all of the four VISA derivated strains, however the degree of autolysis reduction was different in every strain pair (Fig.5). Autolytic activity is generally affected by cell wall composition, autolysin enzymatic activities, autolysin gene transcription and post translational processing of autolysins [40]. Some studies reported that VISA emerged to hVISA when VISA acquired a reduced autolysis activity caused by a down-regulation of autolysin genes (*atl*/*lytM*) [41]. We thus examined the expression of *atl* and *lytM* genes. The expression levels of these two genes reduced in all derivated strains.

Loss of *agr* function could also have significant effects on exoprotein and adhesion expression which could potentially promote biofilm formation [12,23]. Compared

with parental VSSA, the biofilm formation ability of derivated hVISA or VISA decreased remarkably [25]. One of the major constituents of staphylococcal biofilms is poly N-acetyl glucosamine (PIA/PANG) which is synthesized by icaADBC complex [42]. According to our results, the biofilm formation and relative expression of *icaA* gene in all derivated strains were reduced while the relative expression of *icaR* was also decreased in each group [43]. Some reports [42-44] demonstrated that *icaR* was a negative regulator for icaADBC expression. Other regulatory factors, such as *sarA*, *sigmaB*, *rbf*, and *ccpA* etcs were also reported to affect the *ica* gene expression as well as the PIA/PANG production. It is obvious that the regulation is extremely complex and multi-factorial which is similar to the consequence of previous reports [7, 25].

5 Conclusion

This study revealed that the clinical isolates VSSA, hVISA and VISA could be derivated into high vancomycin-resistant VISA but difficult to develop into VRSA in vitro. Meanwhile, the changes of phenotype and regulated genes expression were inversely proportional to the MICvan value in derivated strains. In addition, the results of this study also showed that hVISA and VISA could gradually adapt to the living pressure changes via the continuous elevation in vancomycin concentration, and grow eventually with relatively higher vancomycin concentration. These findings provide comprehensive information that build up the detailed process of vancomycin resistance evolution and pave the way for keeping vancomycin susceptibility in further clinical applications.

Authors' contribution: JX, LP and XM participated in the design of the study; JH, YT, YY and DS performed experiments; JX, LP and XM analyzed data; JX wrote the manuscript; JX and XM reviewed the manuscript.

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