

Association of *pvl* gene with incomplete hemolytic phenotype in clinical *Staphylococcus aureus*

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Purpose: This experiment aimed to evaluate the correlation between the hemolytic phenotype of *Staphylococcus aureus* and *pvl* gene in terms of characteristics of antibiotic resistance.

Materials and methods: Two-hundred and eleven strains of hospital-acquired *S. aureus* and their bacterial susceptibility to 20 antibiotics were determined by MicroScan WalkAway96. All strains were cultured on Columbia sheep blood agar plates for 24 hours and then underwent ten passages for investigation of their hemolytic phenotypes. *S. aureus* produced incomplete β -hemolytic phenotype, termed as *S. aureus* strains with incomplete hemolytic phenotype (SIHP). The *pvl* gene was identified by PCR amplification followed by DNA sequencing. Statistical analyses of the data were performed using SPSS version 16.0 software.

Results: Fifty-two (24.64%) strains were confirmed to maintain the incomplete hemolytic phenotype of *S. aureus* (SIHP). Meanwhile, 15 (7.11%) of 211 strains were found to carry the *pvl* gene, and eight of the 15 strains were SIHP. Compared with *S. aureus* strains with complete hemolytic phenotype (SCHP), SIHP showed higher susceptibility to seven of the 20 antibiotics (oxacillin, ciprofloxacin, gentamicin, ceftriaxone, ceftiofur, levofloxacin, and moxifloxacin) ($P < 0.05$). The *pvl*-positive bacteria had a higher rate of resistance to four antibiotics (rifampin, ciprofloxacin, levofloxacin, and moxifloxacin) in comparison with the *pvl*-negative strains ($P < 0.05$).

Conclusion: SIHP had a high frequency of *pvl* gene. The *pvl*-positive isolates showed less resistance to rifampin, ciprofloxacin, levofloxacin, and moxifloxacin. Additionally, the majority of SIHP isolates (61.54%) were methicillin-resistant *S. aureus*. SIHP strains had significantly higher antibiotic resistance to ceftiofur when compared with SCHP, while SCHP strains had a high rate of antibiotic resistance to ciprofloxacin, gentamicin, ceftriaxone, levofloxacin, and moxifloxacin. The results may help to provide medical advice for selection of antibiotics for patients with SIHP-associated infections.

Keywords: *S. aureus*, incomplete hemolytic phenotype, *pvl*, antimicrobial drug resistance, virulence factor

Introduction

Staphylococcus aureus is one of the most common pathogens isolated from both healthy individuals and patients worldwide, which can produce a variety of secreted toxins and cause various infections in humans.^{1–3} PVL is one of the toxins of synergohymenotropic exotoxin produced by *S. aureus* and belongs to the pore-forming toxin family.^{4–6} PVL shows strong lytic activity against host defense cells such as human polymorphonuclear neutrophils,⁷ monocytes, macrophages, and rabbit neutrophils but not murine neutrophils in vitro.^{8–10} Pore formation requires

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the presence of the two components of the toxin, LukS-PV and LukF-PV. Previous studies demonstrated that PVL aggravated many infections,¹¹ such as skin and soft tissue infection,¹ necrotizing pneumonia, bone joint infections, and even bacteremia.^{12–14}

It has been found that the *pvl* gene can spread from strain to strain by bacteriophages and plasmids.^{15,16} As a result of the *pvl* gene's transmission mechanism, the prevalence of *pvl*-positive strains has gradually increased.¹⁷

The prevalence of the *pvl* gene has been less common in methicillin-susceptible *S. aureus* isolates than in methicillin-resistant *S. aureus* (MRSA).¹⁸ The *pvl* gene locus represents a stable genetic marker of community-acquired MRSA (CA-MRSA) strains.¹⁹ Studies have shown that PVL had a high proportion in CA-MRSA.²⁰ The PVL genes have also been reported to be present in 77% of the CA-MRSA isolates, but only 4% of HA-MRSA isolates were *pvl*-positive.²¹ Although the toxin may be a highly linked epidemiological marker for CA-MRSA strains, PVL is not the major virulence determinant of CA-MRSA.²²

Incomplete hemolytic phenotype has an opaque, darker hemolytic ring in contrast with the transparent hemolytic phenotype of *S. aureus* strains with complete hemolytic phenotype (SCHP).²³ Recently, Zhang et al proposed that a number of *S. aureus* strains with incomplete hemolytic phenotype (SIHP) strains belong to the class of *S. aureus*. The authors demonstrated that SIHP strains were MRSA which highly expressed β -hemolysin and carried the *tst* gene. Because toxic shock syndrome toxin (expressed by *tst*) causes toxic shock syndrome,^{24,25} SIHP possesses high virulence potential.²³ Here we determined the prevalence of SIHP, antimicrobial resistance, and PVL genes carried in *S. aureus*, and compared the diversity of these factors between SIHP and SCHP strains.

Materials and methods

Bacterial strains

After excluding strains from repeated sources, a total of 211 strains were selected randomly and isolated from patients in the First Affiliated Hospital of Anhui Medical University from January 2016 to December 2017. Constituent ratios of departments where strains were isolated are shown in Table 1. The specimens included 30 samples of blood (14.22%), 70 of secreta (33.18%), 45 of sputa (21.33%), nine of shunt fluids (4.27%), 25 from

wounds (11.85%), eight from interstitial fluids (3.79%), and 24 others (11.37%). The Medical Ethics Committee of the First Affiliated Hospital of Anhui Medical University approved this study (no Quick-PJ2018-07–29) and all isolates were collected with written informed consent from the patients, conducted in accordance with the Declaration of Helsinki.

Antimicrobial sensitivity testing

The bacteria were cultured on Columbia sheep blood agar plates at 35°C in an atmosphere containing 5% CO₂ (v/v) for 24 hours. According to the manufacturer's instructions, 1–2 single colonies were added to Mueller-Hinton broth (MHB), followed by proper adjustment of bacteria concentration. Based on the broth microdilution method and the Clinical and Laboratory Standards Institute criteria, bacterial identification and antimicrobial susceptibility testing were performed using MicroScan WalkAway 96 (Beckman Coulter, Brea, CA, USA).²⁶ The bacterial isolates were subjected to 20 antibiotics for sensitivity testing. Antibiotics included: tetracycline, beta-lactams (oxacillin, ceftriaxone, cefoxitin), fluoroquinolones (levofloxacin, ciprofloxacin, moxifloxacin), gentamicin,

Table 1 Constituent ratios of departments where strains were isolated

Department	Strains, n (%)
Neurology	9 (4.27)
Orthopedic	21 (9.95)
Pediatrics	5 (2.37)
ENT	3 (1.42)
Out-patient	11 (5.21)
Infectious diseases	11 (5.21)
Oncology	2 (0.95)
Intensive Care Unit	24 (11.37)
General surgery	15 (7.11)
Respiration	3 (1.42)
Plastic surgery	6 (2.84)
Cerebral surgery	3 (1.42)
Urology	5 (2.37)
Endocrinology	14 (6.64)
Dermatology	24 (11.37)
Burns	12 (5.69)
Nephrology	18 (8.53)
Hematology	6 (2.84)
Obstetrics and gynecology	1 (0.47)
Plastic surgery	9 (4.27)
Rheumatism and Immunological Disease	9 (4.27)
Total	211(100.00)

ampicillin, amoxicillin, daptomycin, clindamycin, nitrofurantoin, linezolid, penicillin, rifampin, sulfamethoxazole–trimethoprim, quinupristin–dalfopristin, erythromycin, and vancomycin.

Detection of hemolytic phenotype

All the strains were identified as *S. aureus* by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS VITEK V3.0, BioMérieux, Craponne, France). The strain ATCC25923 with the complete hemolytic phenotype was taken as SCHP reference. All the isolates were cultured on Columbia sheep blood agar plates from different companies (OXOID, UK; BioMérieux; BD Biosciences, San Jose, CA, USA; TianDa, People's Republic of China) at 35°C in an atmosphere containing 5% CO₂ (v/v) for 24 hours, and 1–2 single colonies from one agar plate were taken to the next Columbia sheep blood agar plates from the same company and cultured for 24 hours, and then underwent ten serial passages. The hemolytic phenomenon was observed. Strains with incomplete hemolytic phenotype on all four blood agar plates and their phenotypes stable to ten passages were identified as SIHP and otherwise, as SCHP. Several SIHP colonies gathered and formed a grid-like hemolytic phenotype.

Detection of *pvl* genes

The strains were inoculated on Columbia blood agar plates and cultured at 35°C in an atmosphere containing 5% CO₂ for 24 hours. Four bacterial colonies were selected and suspended in 8 mL of MHB and cultured at 37°C with shaking at 200 rpm for 12 hours. One mL of MHB bacterial suspension was moved to a sterile centrifuge tube with 200 µL lysostaphin (Sangon, Shanghai, People's Republic of China), boiled at 37°C for 1 hour. After that, DNA were extracted and purified using DNA Extraction Kit (TianGen, Beijing, People's Republic of China) according to the manufacturer's instructions. The DNA were stored at –20°C and prepared for PCR detection.

PCR was run as follows: pre-denaturation at 93°C for 3 minutes, denaturation at 93°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute. PCR products were sequenced and analyzed by BLAST for the expected sequences of peptides. Primers for *pvl* gene (PVL-F, 5'–ATCATTAGGTTAAAATGTCTGGACATGATCCA–3'; and PVL-R, 5'–GCATCAAGTGTATTGGATAGCAAAGC–3') were used to generate an internal control with an amplicon size of 433 bp.²⁷

Statistical analysis

Chi-squared test and Fisher's exact test were used for the statistical analysis and $P < 0.05$ was considered statistically significant. All the data were analyzed by using SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA) and Figures 3–7 were made by GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Hemolytic phenotype of SIHP strains on sheep agar blood plates

One-hundred and fifty-nine strains cultured on blood agar plates for 24 hours presented complete hemolytic rings (β-hemolytic phenotype) (Figure 1B). The incomplete hemolytic phenotype formed by a single colony of bacteria looked like a donut in which a small, lighter circular hemolytic ring located at the center and the outside was wrapped in an opaque, darker hemolytic ring (Figure 1A). When the two colonies grew close, a transparent linear hemolysis zone was formed at the junction of the two colonies on the hemolytic ring. Several SIHP colonies aggregated, forming a grid-like hemolytic phenotype (Figure 1C). In addition, after ten serial passages, the incomplete hemolytic phenotype still remained (Figure 1D). Fifty-two of 211 hospital-acquired *S. aureus* were identified as SIHP strains.

Testing for the *pvl* gene

Among the 211 clinical isolates, 15 (7.11%) yielded positive *pvl* gene amplification (Figure 2). Eight (53.33%) of the 15 *pvl*-positive isolates belonged to SIHP strains and seven (3.57%) of 196 *pvl*-negative isolates were SCHP strains. The prevalence of *pvl* gene in SIHP was significantly higher than that in SCHP ($P < 0.05$) (Figure 3).

Drug resistance of *S. aureus*

Antimicrobial characteristics of 211 *S. aureus* isolates are demonstrated in Figure 4. It shows that *S. aureus* strains presented high resistance to oxacillin (45.02%), cefoxitin (46.45%), ampicillin (76.30%), penicillin (82.46%), and erythromycin (65.88%) when compared with the other antibiotics tested such as tetracycline (31.28%), ceftriaxone (21.80%), levofloxacin (27.01%), ciprofloxacin (32.70%), moxifloxacin (17.06%), gentamicin (28.44%), amoxicillin (24.64%), daptomycin (0.47%), clindamycin (37.91%), nitrofurantoin (0.95%), linezolid (0.95%), rifampin (13.74%), sulfamethoxazole–trimethoprim (9.00%), and quinupristin–dalfopristin (2.84%). Among 211 *S. aureus*

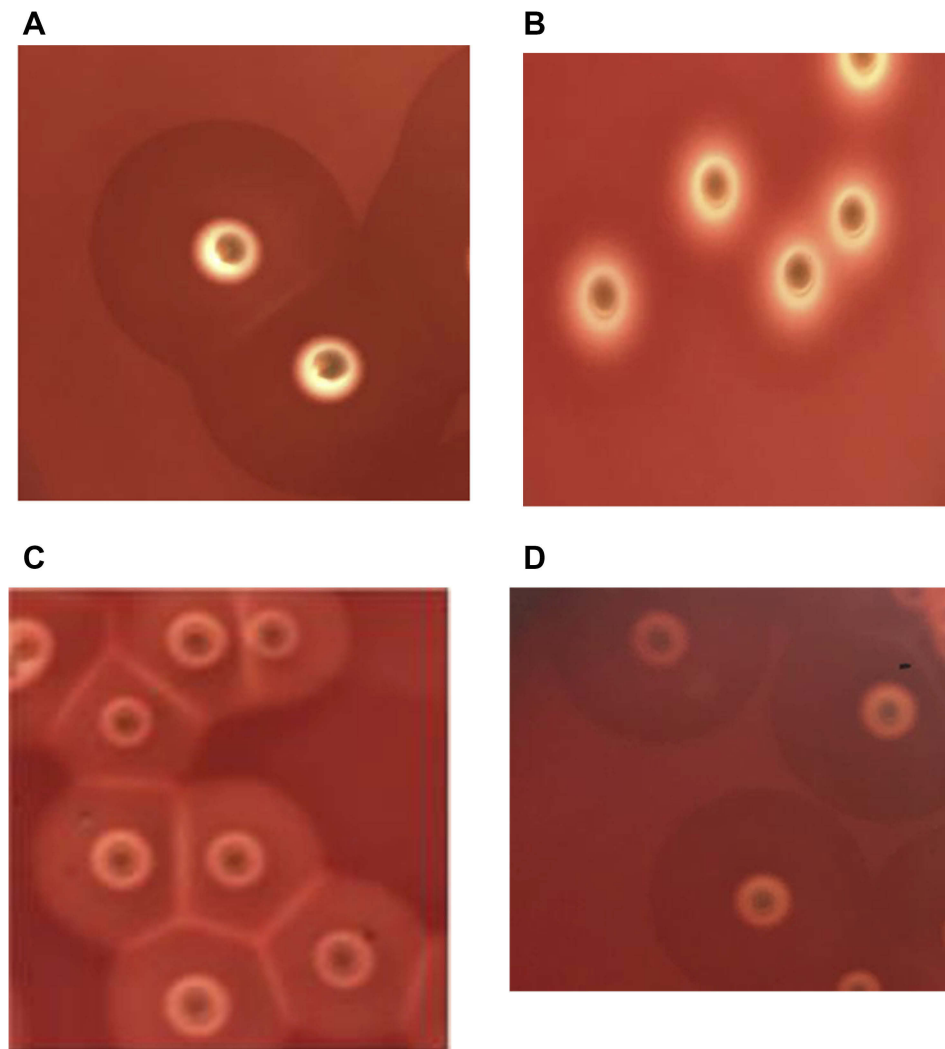


Figure 1 Hemolytic phenotype comparative analyses of *Staphylococcus aureus* on blood agar plates.

Notes: (A) *S. aureus* strains with incomplete hemolytic phenotype (SIHP) strains growing in the microaerophilic condition. (B) *S. aureus* strains with complete hemolytic phenotype (SCHP) control strain of *S. aureus* ATCC25923. (C) Several SIHP colonies aggregated, forming a grid-like hemolytic phenotype. (D) SIHP strains after ten serial passages.

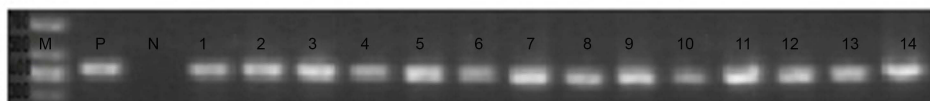


Figure 2 Detection of the *pvl* gene.

Notes: ATCC49775. Due to limitations of instrument, only 14 *pvl*-positive strains are shown here.

Abbreviations: M, marker; P, *pvl*-positive strain; N, *pvl*-negative strain.

isolates, the minimum inhibitory concentrations (MICs) of vancomycin were found to be MIC =0.5 (two strains), MIC =1.0 (109 strains), MIC 2.0 (99 strains), and MIC =4.0 (one strain). The MICs of *S. aureus* are shown in Figure 5. No statistically significant difference in MICs was noted between SIHP and SCHP strains. Similarly, no difference was seen between *pvl*-positive and -negative isolates.

Antimicrobial characteristics of *pvl*-positive and -negative strains

In comparison with the sensitivity of *pvl*-negative strains to quinolones (levofloxacin 29.08%, ciprofloxacin 35.20%, and moxifloxacin 18.37%) and rifampin (14.29%), the sensitivity of *pvl*-positive *S. aureus* to quinolones (levofloxacin 0%, ciprofloxacin 0%, and moxifloxacin 0%) and rifampin

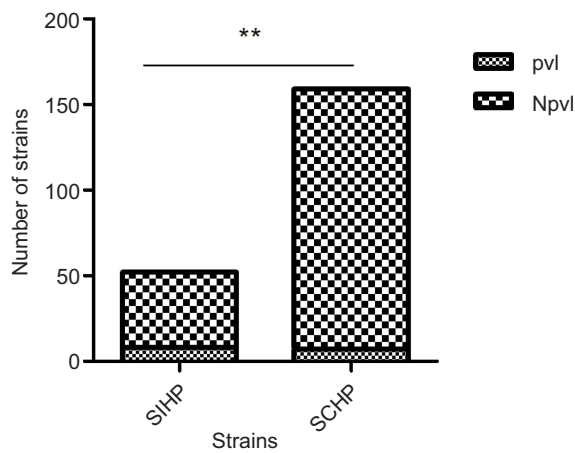


Figure 3 Comparison of *pvl* gene between *Staphylococcus aureus* strains with incomplete hemolytic phenotype (SIHP) strains and *S. aureus* strains with complete hemolytic phenotype (SChP) strains.

Notes: ** $P < 0.01$.

Abbreviations: pvl, *pvl*-positive *S. aureus*; Npvl, *pvl*-negative *S. aureus*.

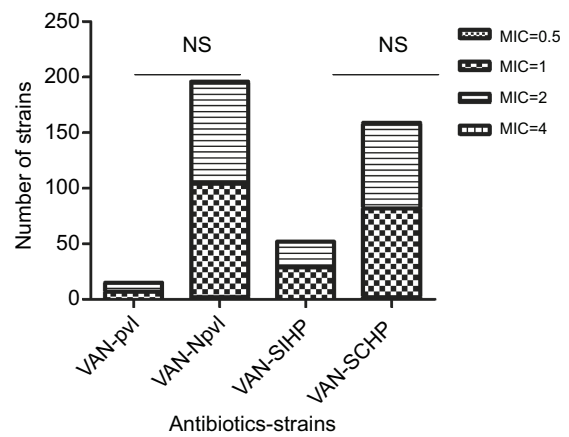


Figure 5 Comparison of MIC to vancomycin between *Staphylococcus aureus* strains with incomplete hemolytic phenotype (SIHP) strains and *S. aureus* strains with complete hemolytic phenotype (SChP) strains as well as *pvl*-positive and -negative strains.

Notes: (NS), $P > 0.05$, difference was not statistically significant.

Abbreviations: MIC, minimum inhibitory concentration; VAN, vancomycin; R, resistant; I, intermediate; S, susceptible; pvl, *pvl*-positive *S. aureus*; Npvl, *pvl*-negative *S. aureus*; NS, not significant.

(6.67%) was significantly elevated ($P < 0.05$) (Figure 6). No statistically significant difference in antibacterial sensitivity was noted in the remaining 16 antibiotics between *pvl*-positive and -negative strains ($P > 0.05$).

Antimicrobial characteristics of SIHP and SChP strains

Compared with SChP strains, SIHP strains had a remarkably higher susceptibility to levofloxacin, ciprofloxacin, and moxifloxacin. Additionally, SIHP strains were more susceptible to gentamicin and ceftriaxone than SChP strains, but more resistant to ceftioxin than SChP strains. There were 61.54% of SIHP strains but only 39.62% of SChP strains found to be MRSA ($P > 0.05$). All statistical differences between SIHP and SChP strains are shown in Figure 7.

Discussion

Bacteriophages are responsible for spreading *pvl* gene among bacterial strains.¹⁵ Recently, phage-therapy as a therapeutic strategy in some infections has been proposed.²⁸ Bacteriophages are able to completely prevent biofilm formation through this strain and eliminate the staphylococcal biofilms within 4 hours.²⁹ In in vitro inhibitory assays, phage κ lysed a range of clinically isolated MRSA strains successfully.³⁰ We speculate that bacteriophages might inhibit the resistance of *pvl*-positive strains to quinolones and rifampin.

The *pvl* gene prevalence of SIHP strains is significantly higher than that of SChP strains. Moreover, SIHP strains have high susceptibility to quinolones and the susceptibility pattern is similar to *pvl*-positive strains. We believe that bacteriophages might inhibit the resistance of *pvl*-positive

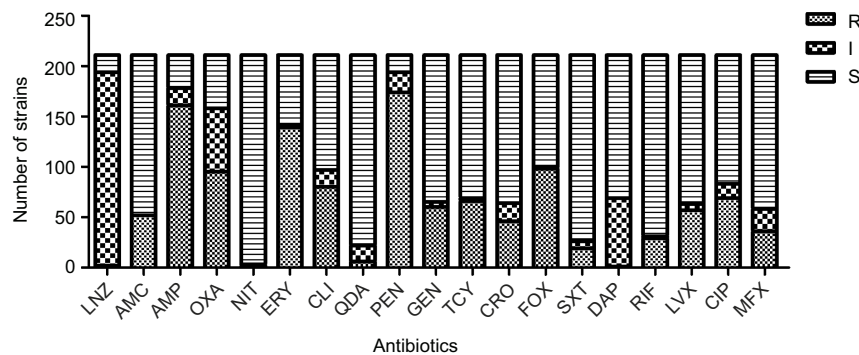


Figure 4 Antimicrobial characteristics of 211 *Staphylococcus aureus* in terms of 19 antibiotics.

Abbreviations: AMC, amoxicillin; AMP, ampicillin; CIP, ciprofloxacin; CLI, clindamycin; CRO, ceftriaxone; DAP, daptomycin; ERY, erythromycin; FOX, ceftioxin; GEN, gentamicin; I, intermediate; LNZ, linezolid; LVX, levofloxacin; MPX, moxifloxacin; NIT, nitrofurantoin; OXA, oxacillin; PEN, penicillin; QDA, quinupristin-dalfopristin; R, resistant; RIF, rifampin; S, susceptible; SXT, sulfamethoxazole–trimethoprim; TCY, tetracycline.

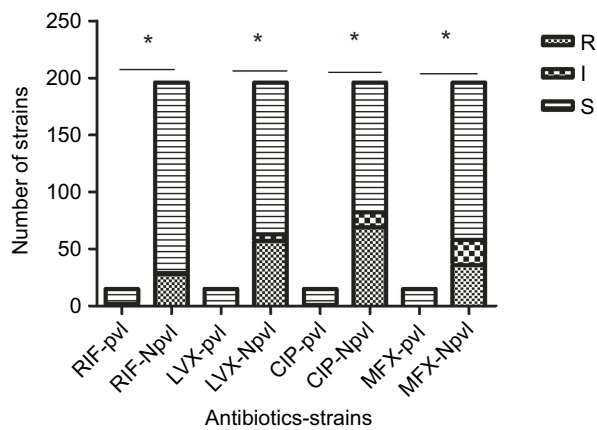


Figure 6 Comparison of drug resistance to four antibiotics in *pvl*-positive and -negative strains.

Notes: * $P < 0.05$. *pvl*, *pvl*-positive *Staphylococcus aureus*; *Npvl*, *pvl*-negative *S. aureus*. **Abbreviations:** CIP, ciprofloxacin; I, intermediate; LVX, levofloxacin; MFX, moxifloxacin; R, resistant; RIF, rifampin; S, susceptible; *pvl*, *pvl*-positive *Staphylococcus aureus*; *Npvl*, *pvl*-negative *S. aureus*.

strains to quinolones, and the correlation between *pvl* gene and incomplete hemolytic phenotype may be responsible for quinolones sensitivity of SIHP strains.

MRSA occurs more frequently in SIHP strains than SCHP ones, our results are similar to those in the study of Zhang et al.²³ They demonstrated that SIHP isolates carried the *tst* gene which expressed toxic shock syndrome toxin and caused toxic shock syndrome. In the present study, we noted that the *pvl* gene was frequently carried by SIHP strains and they were more resistant to cefoxitin. Therefore, we suggest that SIHP might be a class of *S. aureus* with potentially high virulence, that commonly

carries virulence factors and presents relatively high resistance to oxacillin and cefoxitin.

SIHP strains are more susceptible to gentamicin and ceftriaxone, and more resistant to oxacillin and cefoxitin than SCHP strains. There may be a series of common regulatory genes that regulate antibiotic sensitivity and hemolytic phenotype. The mechanism of susceptibility, however, remains unclear.

As described previously, SIHP strains with *tst* and *pvl* gene cause toxic shock syndrome and aggravate many infections.^{11,24} They have high susceptibility to quinolones, gentamicin, and ceftriaxone as well. Unfortunately, identification of antibiotic susceptibility from the isolation and culture of bacteria is time-consuming. Therefore, for patients with severe *S. aureus* infection, selection of antibiotics will be preferentially based on clinical experience. These results may assist when providing medical advice on the appropriate use of antibiotics when the *S. aureus* isolated from patients has been defined as incomplete hemolytic phenotype. The relationship between the *pvl* gene and drug resistance provides microbiologists with a way to explore how the PVL gene reduces drug resistance in *S. aureus*. Our study, however, may not reflect a general situation of hospital-acquired *S. aureus* in central China, due to the limitation of district and the number of *S. aureus* strains collected. Further approaches would be needed to clarify the significance of PVL gene in formation of incomplete hemolytic ring and increased antibiotic resistance of *S. aureus*.

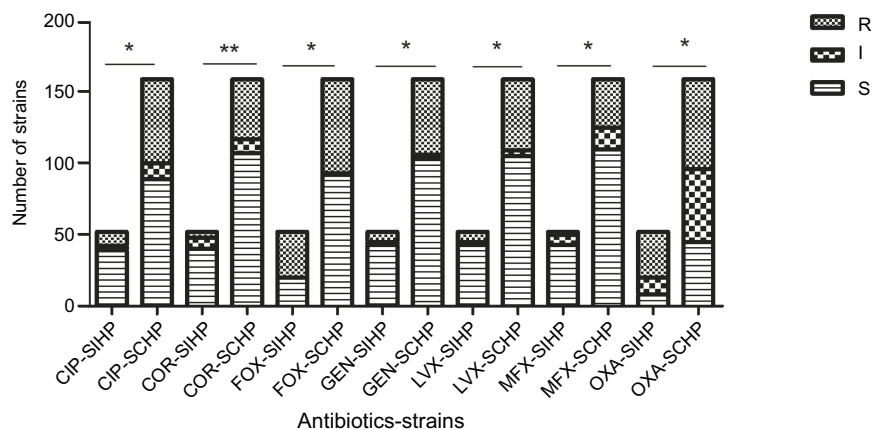


Figure 7 Comparison of drug resistance to seven antibiotics between *Staphylococcus aureus* strains with incomplete hemolytic phenotype (SIHP) strains and *S. aureus* strains with complete hemolytic phenotype (SCHP) strains.

Notes: ** $P < 0.005$; * $P < 0.05$.

Abbreviations: CIP, ciprofloxacin; CRO, ceftriaxone; FOX, cefoxitin; GEN, gentamicin; I, intermediate; LVX, levofloxacin; MFX, moxifloxacin; OXA, oxacillin; R, resistant; S, susceptible.

Conclusion

Taken together, the frequency of *pvl* gene is likely associated with the incomplete hemolytic ring of *S. aureus* strains. *pvl*-positive *S. aureus* shows a high sensitivity to quinolones and rifampin. SIHP strains are susceptible to quinolones, ceftriaxone, and gentamicin but have a higher resistance to oxacillin and ceftioxin when compared with SCHP strains, which may be helpful for correct selection of antibiotics in patients with SIHP-associated infections.

Ethical approval

The Medical Ethics Committee of the First Affiliated Hospital of Anhui Medical University approved this study (no Quick-PJ2018-07-29) and all isolates were collected with written informed consent from the patients, in accordance with the Declaration of Helsinki.

Data availability

All data generated or analyzed during this study are included in this published article.

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Author contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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