Identification of hemolytic activity and hemolytic genes of Methicillin-resistant *Staphylococcus aureus* isolated from Chinese children

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To the Editor: Methicillin-resistant *Staphylococcus aureus* (MRSA) are a group of gram-positive bacteria that cause a wide range of diseases in children.[1] Surveillance of MRSA is particularly important since certain clones have spread over wide geographical regions. The MRSA clones that have spread in North China over the past decade include ST59-SCC*mec*IVa-t437, ST59-SCC*mec*V-t437, and ST239-SCC*mec*III-t030.

MRSA toxins that can damage the host cell membrane include α, β, δ, and γ hemolysin. The hemolytic activities of these toxins were described in 1944 by Christie, Atkins, and Munch-Petersen (which was called the CAMP reaction).[2] The hemolytic activities of MRSA isolated from Chinese children have not yet been determined. The aim of this study was to provide novel insights into hemolytic activities of these isolates.

The key laboratory routinely received MRSA-positive samples that were isolated from patients in the bacteriology room (≤18 years old) of Beijing Children's Hospital (BCH) over three different periods (2005–2011, 2012–2013, 2016). Invasive MRSA isolates were included as previously described.[3]

The CAMP test was performed for hemolysis. Briefly, 5% sheep blood agar (SBA) plates were prepared using defibrinated sheep blood (5% in tryptone soy broth [TSB; Becton, Dickinson and Company, Franklin Lakes, NJ, USA], 25 mL per plate). A *S. aureus* strain of RN4220 that produced β-hemolysis was streaked down at the centre of an SBA plate. Test strains were streaked perpendicular to the center streak, but without touching it. The plates were incubated at 37°C for 24 h before analysis.

DNA isolation and polymerase chain reaction amplification were used for multi-locus sequence typing (MLST), SCC*mec* typing, accessory gene regulator (*agr*) typing, *spa* typing, the intact *hlβ* gene, the *int* gene, the *hlα*, and *hlδ* gene as previously described.

During the three periods, the key laboratory of BCH collected 259 MRSA isolates (2005–2011: 70 isolates, 2012–2013: 90 isolates, 2016: 99 isolates). Fourteen MLSTs were identified among the MRSA isolates in 2005 to 2011; 16 MLSTs in 2012 to 2013; and 16 MLSTs in 2016. The frequencies of different MLSTs varied across the three periods. The percentage of ST59 MRSA significantly increased from 48.6% (34/70) in 2005 to 2011 and 46.7% (42/90) in 2012 to 2013 to 79.8% (79/99) in 2016 ($P < 0.001$). The ST239 clone disappeared in 2016. There was an increase in the frequency of SCC*mec*IV cases (2005–2011: 61.4%; 2012–2013: 45.6%; and 2016: 84.8%, $P < 0.001$) and of *spa*-t437 cases (2005–2011: 31.4%; 2012–2013: 43.4%; and 2016: 63.6%, $P < 0.001$) over the periods covered by the study, especially in 2016. The SCC*mec*III type was not detected in 2016. Four *agr* types were detected during the study; the main type was *agr*I, with incidence increasing from 72.9% in 2005 to 2011 to 88.9% in 2012 to 2013, and increasing further to 92% in 2016 ($P < 0.01$). Our results revealed that isolates sharing the genetic characteristics of ST59-SCC*mec*V-*spa*-t437-*agr*I increased from 24.3% (17/70) in 2005 to 2011 and 31.1% (28/90) in 2012 to 2013 to 59.6% (59/99) in

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2016 ($P < 0.001$). On the contrary, the ST239-SCC*mec*III-t030-*agr*I clone disappeared in 2016.

S. aureus produces at least four hemolytic activities, α, β, δ, and γ. α-Hemolysis generates a wide zone of complete hemolysis with blurred edges on sheep blood agar (SBA). β-Hemolysis produces a wide zone of incomplete hemolysis with sharp edges. δ-Hemolysis is a narrow zone of incomplete hemolysis with blurred edges. γ-Hemolysis cannot be seen on SBA, as it is inhibited by agar. β-Hemolysis partially inhibits α-hemolysis, which leads to a more turbid zone than that produced by α-hemolysis alone [Figure 1E]. δ-Hemolysis is strongly synergistic with β-hemolysis, producing a turbid zone that is broader than that produced by δ-hemolysis [Figure 1D]. These hemolytic patterns are illustrated in Figure 1.

Four hemolytic patterns were observed in our study: α and δ; β and δ; β and δ alone. 143 ST59 isolates, 118 t437 isolates, 134 SCC*mec*IV and 151 *agr*I isolates exhibited

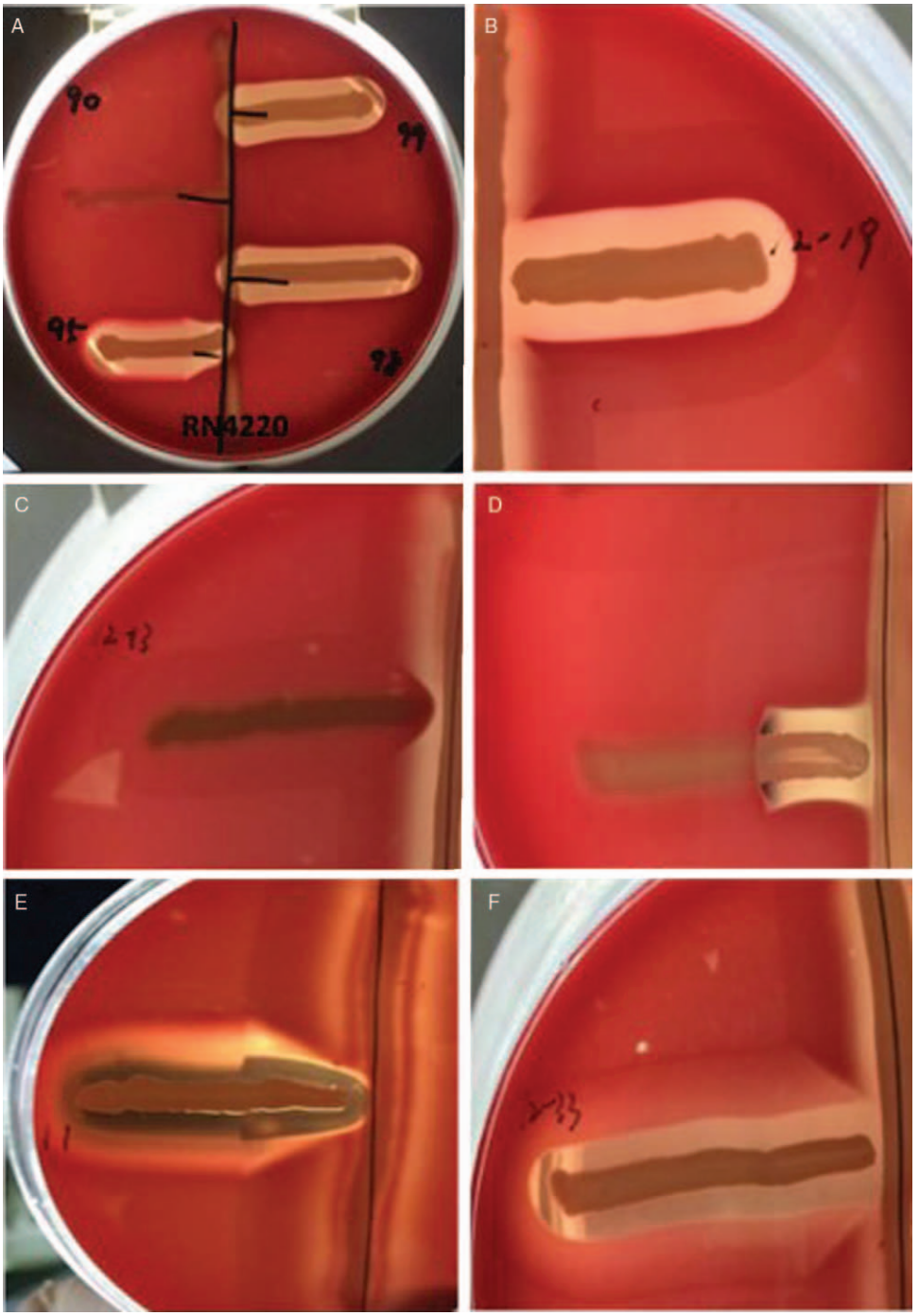

Figure 1: Hemolysis. Bacteria for testing were streaked at a right angle to RN4220 (A: vertical bar), and the plate was incubated for 24 h. (B and F) exhibited β- and δ-hemolysis. (C) produced β-hemolysis. (D) exhibited δ-hemolysis. (E) exhibited α- and δ-hemolysis.

β- and δ-hemolysis, which accounted for 93.5%, 77.1%, 87.6%, and 98.7% of all β- and δ-hemolytic isolates, respectively. Among isolates that showed β- and δ-hemolysis, 100 (65.4%) were ST59-SCC*mec*IV-t437-*agr*I clones.

All isolates carried the *hlα* and *hlδ* genes, and the intact *hlβ* gene was found in 97.4% (149/153) of isolates that exhibited β-hemolysis. The *int* gene was detected in 94.1% (80/85) of the isolates that showed α-hemolysis.

Among 259 children, 40 (15.4%) were classified as cases of invasive *S. aureus* infection. These patients came from all over the country, with cases coming from the provinces of Anhui, Hubei, Hebei, Shandong and Shanxi as well as from Beijing city. There was no significant correlation between hemolysis type of MRSA isolates and the periods of study, age, sex of infected children, admission to pediatric intensive care unit (PICU), and diagnosis ($P > 0.05$).

Since the first detection of MRSA, different STs have emerged as predominant clones in different geographical regions. ST239 has become the predominant hospital-associated (HA-) MRSA clone, while ST59-IV and ST30-IV have become the predominant community-associated (CA-) MRSA clones in Asia. In our study, ST59-t437-IV was the dominant CA- and HA-MRSA clone in the hospital instead of ST239. However, the factors contributing to its epidemic are still unclear.

MRSA virulence factors include exotoxins that can damage the host plasma membrane such as pore-forming toxins (α-, δ-, and γ-hemolysin) and a neutral sphingomyelinase (β-hemolysin). Among these exotoxins, α-hemolysin, in particular, has been shown to have critical role in MRSA associated with severe disease.[4] As shown in this study, the dominant MRSA ST59 clone did not produce α-hemolysis, thus suggesting a dysfunction of α-hemolysin. Meanwhile, our data showed that the ST239 clone, which had decreasing incidence in the study, produced α-hemolysis. The shift in the STs coincided with differences in hemolytic patterns.

In *S. aureus*, *agr* is a two-component regulatory system that separately controls the production of α- and δ-hemolysin.[5] Previous studies have shown that the β-hemolysin gene is non-functional in most clinical *S. aureus* strains due to the insertion of the bacteriophage phi 13, while most bovine *S. aureus* isolates express β-hemolysin. In contrast, in this study, most isolates (97.4%) with the β and δ phenotype carried the intact *hlβ* gene, indicating that the bacteriophage phi 13 was absent in these strains.

There was no correlation between the hemolytic phenotype of invasive isolates and the clinical characteristics of the isolates, which may also be related to the small sample size and result bias.

In conclusion, the predominant ST59 clone produced β-hemolysis instead of α-hemolysis. The α-hemolysis defective ST59 infections took place both in the community and at the hospital.

Declaration of patient consent

All participants provided their written informed consent before participating in the study. In the forms, all the guardians of the patients have given their consents for the images and other clinical information to be reported in the journal. The guardians of the patients understand that the names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

None.

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