The basis of α-hemolysis Negative Methicillin-resistant Staphylococcus Aureus Isolates from Beijing Children’s Hospital

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Abstract

Background. Methicillin-resistant *Staphylococcus aureus* (MRSA) Clonal Complex 59 (CC59) clone has spread among Chinese children, resulting in many *Staphylococcus aureus* infections. α-hemolysin (Hla) is an important virulence factor of *Staphylococcus aureus*; but little research has been done on CC59 isolates with negative α-hemolysis.

Results. During the 4 periods (2009-2011, 2012-2013, 2016, 2017), 291 MRSA isolates were collected. Isolates with β and δ hemolysis accounted for 60.47% among the MRSA isolates in 2009-2011; 56.41% in 2012-2013; 77.14% in 2016; and 56.25% in 2017. Most ST59 isolates (94.38%), 9 ST338 isolates (100%), 13 ST22 isolates (92.86%), and 6 ST398 isolates (85.71%) showed α and δ hemolysis. α-hemolysin in most clinical isolates is highly conservative, each showed one amino acid locus variation, the most common mutation was one at position 275 instead of isoleucine, then glutamic acid replaced aspartic acid at 208. Seventeen ST59 and 2 ST338 isolates had no hemolysis, 3 ST59 isolates showed single mutation (C448G), and only one ST59 isolate showed multilocus mutation. Other ST typing, such as ST1, ST5, ST88, ST20, ST239 and ST398, all had multilocus mutations, sites were from 3 to 8, no conservative sequence was found among isolates with the same ST typing. The carrying rates of RNA III, Rot, agrA, SarR, SarU and SigB were all over 93%, the carrying rates of SarZ and SarA genes were 41.86% and 34.88% respectively. Transcriptional levels of *hla* in isolates showed α and δ hemolysis and β and δ hemolysis were equal. USA300 and R23 produced Hla, R23 didn't show α-hemolysin phenotype.

Conclusions. Most clinical CC59 isolates from children in China were α-hemolysis negative. There was no statistically significant difference in hla gene and RNA expression, they produced the protein. The reason for the phenotypic deletion probably related to α-hemolysin (Hlβ).

Background

*Staphylococcus aureus* (S. aureus) is an opportunistic pathogen causing a wide range of disease in humans from skin and soft tissue infections to bacteremia, osteomyelitis, toxic shock syndrome and staphylococcal enterotoxins [1]. *Staphylococcus* virulence can enable the organism to resist host defenses, attach to the tissue matrix, degrade macromolecules and lyse cellular elements. *S. aureus* secretes a group of polypeptides capable of damaging the host cell plasma membrane including α, β, δ and γ-hemolysin. These hemolysins can produce α, β, δ and γ-hemolytic reaction, respectively [2]. In 1944, Christie, Atkins, and Munch-Petersen reported the lytic phenomenon which was called CAMP reaction [3]. Then CAMP test had been used to identify subtypes of staphylococci and streptococci groups [4]. We had been reported the hemolytic patterns of our MRSA isolates, we aimed to know the basis of the disappeared α-hemolysis in CC59 clone [5].

α-hemolysin (Hla) is one of the most prominent virulence of *S. aureus*. Hla also exhibits distinct responses to intoxication and has been recognized as an important cause of injury in skin necrosis, corneal infections, intracellular bacterial invasion and sepsis [6]. Hla has specific forms of interaction with membranes containing sphingomyelin and cholesterol, which are plasma membrane lipid, facilitating pore formation, cellular membrane injury, and the tissue barrier damage. β-hemolysin (Hlβ) is a neutral sphingomyelinase hydrolyzing sphingomyelin. Sphingomyelin is the site where they work together [7].

At least 3 global regulatory systems: the accessory gene regulator (agr), the staphylococcal accessory gene regulator (sar), and the staphylococcal accessory protein effector (saec) control hla gene expression [8]. The agr system as a quorum-sensing system provides the primary control of Hla production via RNAIII molecule. The agr system produces autoinducer peptide (AIP), AIP binding to AgrC on cell surface, activates AgrA in the cell, AgrA binds to the P3 promoter of the agr locus and activates the production of the RNAIII molecule, which can up-regulate the transcription of *hla* gene. The agr locus exerts a direct positive impact on hla expression, whereas the SarA protein family (SarA, SarL, SarR, SarZ) positively affects hla expression by both agr-dependent and agr-independent pathways, two sarA homologues, rot and sarT, repress hla expression. Signal-transduction system encoded by saeS (sensor) and saeR (response regulator) positively regulates the expression of hla at the transcriptional level, the sae activation is influence by agr and sigma factor B (sigB). Because the regulation of Hla is complex, it involves multiple regulatory systems and there are interactions among multiple regulatory systems, previous experiments have demonstrated the essential role for Hla in *S. aureus*, people often choose to examine Hla expression levels to determine the regulators in these systems. This study was designed to make further efforts to compare the hemolytic activity of different clones of *S. aureus* clinical isolates from Beijing Children's Hospital, to clarify the mechanism of α hemolysis deletion in clinical CC59 isolates. We had detected the carriage of *hla* regulator genes, analyzed the sequence of hla gene and its promoter, the transcription and protein expression of Hla, it provided a theoretical basis for the regulation of hla in clinical MRSA. This may also be one of the reasons for the popularity of CC59 clone.

Results

Hemolytic activities of MRSA isolated from BCH during 4 periods

Fig 1 showed that USA300 had α and δ hemolysis, R23 showed β and δ hemolysis, R23Ahlα showed β and δ hemolysis, R23Aag exhibited β hemolysis. During the 4 periods (2009-2011, 2012-2013, 2016, 2017), the key laboratory of BCH collected 291 MRSA isolates. Four hemolysis patterns were observed in our study: α and δ, β and δ, β and δ alone. Isolates with β and δ hemolysis accounted for 60.47% among the MRSA isolates in 2009-2011; 56.41% in 2012-2013; 77.14% in 2016; and 56.25% in 2017, the proportions were more than 50%. The frequencies of α and δ hemolysis varied across the four periods covered by the study, from 15.24% to 41.03% (Fig 2 and Table 1). Very few isolates expressed a single β or δ hemolytic phenotype. Three isolates could not be typed.

Hemolysis patterns of different MRSA types isolated from BCH
There were 26 ST typing of 291 isolates, the most common types in the top 7 were ST59, ST239, ST88, ST5, ST22, ST338 and ST398. There were 178 ST59 isolates, most ST59 isolates (94.38%), 9 ST338 isolates (100%) showed β and δ hemolysis, both ST59 and ST338 clone belong to CC59 clone. Twenty-two ST239 isolates (73.33%), 8 ST88 isolates (100%), 4 ST5 isolates (100%), 13 ST22 isolates (92.86%) and 6 ST398 isolates (85.71%) showed α and δ hemolysis. A few isolates exhibited β or δ hemolysis, as shown in Table 2 and Fig 3.

Table 1

| Hemolysis | Four periods | 2009-2011 | 2012-2013 | 2016 | 2017 |
|-----------|--------------|-----------|-----------|------|------|
| βδ        | 26(60.47)    | 44(56.41) | 81(77.14) | 36(56.25) |
| αδ        | 13(30.23)    | 32(41.03) | 16(15.24) | 18(28.13) |
| β         | 4(9.3)       | 0(0)      | 5(4.76)   | 2(3.13) |
| δ         | 0(0)         | 0(0)      | 3(2.86)   | 5(7.81) |
| NT        | 0(0)         | 2(2.56)   | 0(0)      | 3(4.68) |
| Total     | 431(100)     | 78(100)   | 105(100)  | 64(100) |

Table 2

| Hemolysis patterns | STs       | ST59 | ST239 | ST88 | ST5 | ST22 | ST338 | ST398 | Others* | P     |
|--------------------|-----------|------|-------|------|-----|------|-------|-------|---------|-------|
| βδ                 | 168(94.3) | 0(0.00) | 1(10.00) | 0(0.00) | 0(0) | 9(100.00) | 0(0) | 9(23.08) | <0.0001 |
| αδ                 | 6(3.37)   | 22(73.33) | 8(80.00) | 4(100.00) | 13(92.86) | 0(0.00) | 6(85.71) | 20(51.28) |       |
| β                  | 3(1.69)   | 4(13.33) | 1(10.00) | 0(0.00) | 0(0.00) | 0(0.00) | 0(0.00) | 3(7.69) |       |
| δ                  | 1(0.56)   | 1(3.33)  | 0(0.00) | 0(0.00) | 1(7.14) | 0(0.00) | 0(0.00) | 5(12.82) |       |
| NT                 | 0(0.00)   | 3(10.00) | 0(0.00) | 0(0.00) | 0(0.00) | 0(0.00) | 1(14.29) | 2(5.13) |       |
| Total              | 178(100.00) | 30(100.00) | 10(100.00) | 4(100.00) | 14(100.00) | 9(100.00) | 7(100.00) | 39(100.00) |       |

*ST1, ST6, ST9, ST25, ST30, ST72, ST97, ST120, ST121, ST509, ST585, ST630, ST769, ST869, ST965, ST1224, ST1295, ST1777, ST1821

Amino acid site variation of Hlα

The amino acid sequence of Hlα of wild S. aureus is as follows: the length of the gene is 960bp (X01645.1), the first 78 bp encodes 26 amino acids (precursor peptide), the remaining 882 bp encodes 293 amino acids, the sequence is as follows (KT 27954.1): MKTRIVSSVTMLNLGNSLMPVANA (serial number: K27954.1) ADSDINIKTGTTIGSNTTVGTGGTLTYTDKENMHKKVFYSFIDDKHNKLLVIRTGTIAQGYRVYSEEGANKSGAWPSAFKQVLQPDNEVAQISDYPRNSIDT

We selected 43 representative isolates from 291 clinical strains in four periods for α hemolysin gene sequencing, these sequences were converted to amino acid sequences through BLAST, compared with wild strain, it showed that the amino acid sequence of α hemolysin in most clinical isolates is highly conservative, each showed one amino acid locus variation, the most common mutation was threonine at position 275 instead of isoleucine, then glutamic acid replaced aspartic acid at 298. There was no correlation between amino acid locus variation and hemolytic activities. The specific amino acid locus variation was shown in the Table 3.

Table 3

| Mutations of MRSA Hlα amino acid sequences based on hemolytic activities |
| Isolates | spa | SCCmec | ST   | Haemolysis | Mutations |
|----------|-----|--------|------|------------|-----------|
| 2012-2   | t437 | V      | 338  | β          | I275T     |
| 2012-3   | t437 | V      | 59   | β          | I275T     |
| 2012-4   | t7637 | NT    | 88   | aδ         | D208E     |
| 2012-14  | t186 |        | 88   | aδ         | I275T     |
| 2012-16  | t437 |        | 398  | aδ         | -         |
| 2012-17  | t437 |        | 398  | aδ         | -         |
| 2012-24  | t8660 | V     | 120  | aδ         | I275T     |
| 2012-28  | t172 |        | 59   | β          | I275T     |
| 2012-32  | t008 |        | 239  | aδ         | -         |
| 2012-38  | t034 | V      | 398  | aδ         | -         |
| 2012-43  | NT   |        | 5    | aδ         | D208E     |
| 2012-44  | t127 | V      | 1    | aδ         | I275T     |
| 2012-48  | t030 | III    | 585  | aδ         | R-22H     |
| 2013-59  | t114 |        | 1    | aδ         | I275T     |
| 2013-74  | t4549 | V     | 630  | β          | -         |
| 2013-82  | t078 |        | 59   | β          | I275T     |
| 2013-90  | t078 |        | 59   | aδ         | T261-     |
| 2013-92  | t10555 | IV    | 72   | aδ         | D208E     |
| 2012-96  | t037 | III    | 239  | aδ         | R-22H     |
| 2012-97  | t081 | NT     | 25   | aδ         | D208E     |
| 2012-9   | t437 |        | 59   | β          | D208E     |
| 2012-12  | t441 |        | 59   | β          | I275T     |
| 2012-26  | t437 |        | 59   | β          | -         |
| 2013-85  | t437 |        | 59   | β          | I275T     |
| 2013-86  | t437 |        | 59   | β          | I275T     |
| 2012-49  | t437 |        | 59   | β          | I275T     |
| 2012-53  | t437 |        | 59   | β          | I275T     |
| 2010-25  | t127 |        | 88   | β          | -         |
| 2011-32  | NT   |        | 965  | β          | I275T     |
| 2011-46  | NT   |        | 59   | aδ         | L157-     |
| 2012-52  | t437 |        | 59   | aδ         | -         |
| 2016-005 | t437 |        | 59   | β          | I275T     |
| 2016-045 | t437 |        | 338  | β          | I275T     |
| 2016-047 | t437 |        | 1224 | β          | I275T     |
| 2016-065 | t3515 |        | 59   | aδ         | I275T     |
| 2016-078 | t2049 | NT    | 769  | aδ         | I275T     |
| 2016-085 | t437 |        | 59   | β          | I275T     |
| 2016-090 | t172 |        | 59   | β          | -         |
| 2016-114 | t437 |        | 59   | δ          | S99P, I275T |
| 2016-122 | t172 |        | 59   | β          | -         |
| 2017-039 | t437 |        | 59   | β          | I275T     |
| 2017-033 | t437 |        | 59   | β          | -         |
| 2017-110 | t2755 |        | 1295 | β          | I275T     |
Analysis of polymorphisms in the *hla* promoter

To elucidate the potential mechanisms involved in down-regulating *hla* expression in selected isolates, we performed single nucleotide polymorphisms (SNPs) analysis of *hla* promoter region based upon the published *S.aureus* strain S15 (CP040801.1) genomes in the NCBI genome database. The promoter gene sequence of *hla* is 484 bp, 43 representative isolates were sequenced and then were compared with the template, we found that the DNA sequences of *hla* promoter region were almost identical among the CC59 isolates. 17 ST59 and 2 ST338 isolates had no mutation, 3 ST59 isolates showed single mutation (C448G), and only one ST59 isolate showed multilocus mutation, it suggested that the promoter sequence of CC59 clone is relatively conservative. Other ST typing, such as ST1, ST5, ST88, ST20, ST239 and ST398, had multilocus mutations, sites were from 3 to 8, no conservative sequence was found among isolates with the same ST typing. Mutations in promoter sites were not associated with hemolytic phenotype (Table 4).

Table 4

| Identification of SNPs in the *hla* promoter region of MRSA isolates from Chinese children |
| Isolates   | Hemolysis | ST | spa | SNP1 | SNP2 | SNP3 | SNP4 | SNP5 | SNP6 | SNP7 | SNP8 | SNP9 | SNP10 | SNP11 | SNP12 |
|------------|-----------|----|-----|------|------|------|------|------|------|------|------|------|-------|-------|-------|
| 2013-59    | α δ (n=20) | 1  | t114| A3G  | C18T |      | T44C | C93G | T170C| A227G| C381T|       |       |       |       |
| 2012-44    |           | 1  | t127| A3G  | C18T |      | T44C | C93G | T170C| G171A| A227G| C381T|       |       |       |
| 2012-43    |           | 5  | t1895| A3G | C18T | T26A | T44C | C93G |      | G171A|       |       |       |       |       |
| 2012-97    |           | 25 | t1081| A3G | C18T |      | T44C | C93G |      | C381T|       |       |       |       |       |
| 2011-46    |           | 59 | NT  | -    |      |      |      |      |      |      |       |       |       |       |       |
| 2013-90    |           | 59 | t078| -    |      |      |      |      |      |      |       |       |       |       |       |
| 2016-065   |           | 59 | t3515| -   |      |      |      |      |      |      |       |       |       |       |       |
| 2012-52    |           | 59 | t437| -    |      |      |      |      |      |      |       |       |       |       |       |
| 2017-039   |           | 59 | t437| -    |      |      |      |      |      |      |       |       |       |       |       |
| 2013-92    |           | 72 | t10555| A3G | C18T | T44C | C93G |      | A227G| C381T|       |       |       |       |       |
| 2012-14    |           | 88 | t1186| A3G | C18T | T44C | C93G |      | G171A|       |       |       |       |       |       |
| 2012-04    |           | 88 | t17637| A3G | C18T | T26A | T44C | C93G |      | G171A| C381T|       |       |       |       |
| 2012-24    |           | 120| t18660| A3G | A17T |      | T44C | C93G |      | G171A|       |       |       |       |       |
| 2012-32    |           | 239| t1008| A3G | C18T |      | T44C |      | G171A|       |       |       |       |       |       |
| 2012-96    |           | 239| t1037| A3G | C18T |      | T44C |      |       |       |       |       |       |       |       |
| 2012-38    |           | 398| t1034| A3G |      |      |      |      |      | C93G |       |       |       |       |       |
| 2012-16    |           | 398| t437 | A3G | C18T | T44C | C93G |      | G171A|       |       |       |       |       |       |
| 2012-17    |           | 398| t437 | A3G | C18T | T26A | T44C | C93G |      | G171A|       |       |       |       |       |
| 2012-48    |           | 585| t1030| A3G | C18T |      | T44C |      | G171A|       |       |       |       |       |       |
| 2016-078   |           | 769| t12049| A3G | C18T | T44C | C93G |      | G171A| T477  |       |       |       |       |       |
| 2016-122   | β (n=3)   | 59 | t1163|      |      |      |      |      |      | C448G|       |       |       |       |       |
| 2016-090   |           | 59 | t172 |      |      |      |      |      |      | C448G|       |       |       |       |       |
| 2016-085   |           | 59 | t437 | -    |      |      |      |      |      |       |       |       |       |       |       |
| 2013-82    | βδ (n=19) | 59 | t078 | -    |      |      |      |      |      |       |       |       |       |       |       |
| 2012-28    |           | 59 | t172 |      |      |      |      |      |      | C448G|       |       |       |       |       |
| 2017-033   |           | 59 | t437 | A3G | C18T | T44C |      |      |       |       |       |       |       |       |       |
| 2012-03    |           | 59 | t437 | -    |      |      |      |      |       |       |       |       |       |       |       |
| 2012-09    |           | 59 | t437 | -    |      |      |      |      |       |       |       |       |       |       |       |
| 2012-26    |           | 59 | t437 | -    |      |      |      |      |       |       |       |       |       |       |       |
The detection of genes regulating \textit{hla}

In this study, we detected eight genes regulating \textit{hla}, including \textit{SarZ}, \textit{RNA III}, \textit{Rot}, agrA, SarA, SarR, SarU and \textit{sigB}. The carrying rates of \textit{RNA III}, \textit{Rot}, agrA, SarR, SarU and \textit{sigB} were all over 93\%, the carrying rates of \textit{SarZ} and \textit{SarA} genes were 41.86\% and 34.88\% respectively. The 8 regulatory genes were not related to the hemolytic phenotype of the isolates (Table 5).

| Table 5 | Distribution of genes regulating \textit{hla} grouped by hemolysis of MRSA |
|---------|----------------------------------|
| hemolysis | SarZ No (%) | RNA III No (%) | Rot No (%) | agrA No (%) | SarA No (%) | SarR No (%) | SarU No (%) | sigB No (%) | P |
| α and δ (n=20) | 9 (45\%) | 20 (100\%) | 18 (90\%) | 20 (100\%) | 8 (40\%) | 20 (100\%) | 20 (100\%) | >0.05 |
| β (n=3) | 1/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | >0.05 |
| β and δ (n=19) | 7 (36.84\%) | 16 (84.21\%) | 18 (94.74\%) | 19 (100\%) | 3 (15.79\%) | 19 (100\%) | 18 (94.74\%) | 19 (100\%) | >0.05 |
| δ (n=1) | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | >0.05 |
| Total (n=43) | 18 (41.86\%) | 40 (93.02\%) | 40 (93.02\%) | 43 (100\%) | 15 (34.88\%) | 42 (97.67\%) | 42 (97.67\%) | 43 (100\%) | >0.05 |

Expression of Hlα among isolates with different hemolysis phenotypes

Isolate 2016R23 showed β and δ hemolysis, we knocked out its \textit{hla} gene to form R23Δhla. Isolates with β and δ hemolysis, α and δ hemolysis and R23Δhla were selected for qRT-PCR to determine a differential expression of \textit{hla} gene, the result was shown in Fig 4. Isolates with β and δ hemolysis did not change the transcription of \textit{hla} gene (P=0.84). The expression of R23Δhla was 0. Transcription level of Hlα wasn’t related to α hemolysis in clinical isolates.

Production of Hlα in USA300, R23 and R23Δhla
We selected USA300 (α and δ hemolysis) and R23 (β and δ hemolysis) and R23Δhla (β and δ hemolysis) for Western blot to detect Hla. R23Δhla didn’t produce Hla. USA300 and R23 produced Hla, indicating that even though R23 didn’t show a hemolysis phenotype, Hla protein were still produced.

Overexpression of Hla restores a hemolysis of the R23Δhla isolate We considered that a hemolysis could be restored by augmenting the expression of Hla. As the R23Δhla isolate maintained a defect in Hla production compared to the R23 clinical isolate, we utilized a high-copy-number plasmid containing the hla gene under the control of its native promoter. While this approach might increase basal expression levels of Hla simply due to high copy numbers, then multiple copies may effectively restore production of Hla by the R23Δhla mutant. R23Δhla contained multicopy plasmid display a 5-fold level of over expression of the toxin relative to the endogenous expression in the R23Δhla isolate, overexpression of the hla gene under the control of its own promoter restored Hla hemolysis, leading to α, β and δ hemolysis in complement R23 isolate (chla), within the region of intersection, the α and β-hemolysin zone is more turbid with sharper edges on agar than seen with α-hemolysin alone because of inhibition by β-hemolysin (Fig. 6, bottom, right).

Discussion

Hla has been shown to intoxicate a wide range of human cell types, including not only epithelial cells, endothelial cells, but also T cells, monocytes, macrophages and neutrophils [9]. Researchers used to think S. aureus produces the characteristic α hemolysis, but in our study, the clinical CC59 isolates expressed β and δ hemolysis, they didn’t produce α hemolysis. Both the representative R23 isolate and its mutation (with hla gene deletion) showed β and δ hemolysis. On the other hand, other STs showed α and δ hemolysis without β hemolysis. We didn’t find isolates express α and δ hemolysis at the same time.

Aim to find out the reason for negative α hemolysis, hla gene was sequenced, the converted amino acid sequences were conservative. In 1994, Barbara E. Menzies and Douglas S. Kernodle reported that substitution of histidine 35 with leucine produced a mutant toxin (H35L) without hemolytic or lethal activity [10]. We didn’t have H35L mutant in this study, our R-22H, S99P, L157-, D208E, T261- and I275T mutants exhibited no impact on hemolytic patterns, which were consistent with Ying-Chun Xu’s results in 2016 [11]. The mutants were different with Barbara Walker and Hagan Bayley’s results in 1995 [12]. These changes in amino acid residues didn’t result in loss of α hemolysis.

To elucidate potential regulatory mechanisms, we analyzed the hla promoter regions and identified predominant SNPs at positions 3, 17, 18, 26, 44, 93, 170, 171, 227, 381, 448, 477 and 478 from the start codon in selected isolates besides ST59, some of them were the same as Ana Tavares’ results [13]. Sequence of ST59 hla promoter was relatively conservative, but these changes didn’t contribute to the differential control of hla expression among isolates.

The expression of Hla is simultaneously regulated by various global regulatory systems, including the accessory gene regulator (agr system). The agr locus consists of five genes (agrA, agrC, agrD, agrB and hlaδ). AgrA function as sensor and response regulator protein. RNA II is the effector of agr system, it modulates Hla expression both at the transcriptional and translational levels. RNAIII can directly or indirectly regulate numerous transcription regulators (SarA, SarR, SarU and SarZ). In contrast, the homologues of staphylococcal accessory regulator Rot repress the expression of Hla [14]. In addition, it has been revealed that Hla transcription is also affected by sigma B factor (sigB) [15]. The detection of Hla regulators were agreement with Li Shipeng and Sun Jing’s results [16]. Among 43 isolates, 41.86% (18/43) were positive for SarZ, 34.88% (15/43) harbored the SarA gene. More than 90% isolates carried RNAII, Rot, agrA, SarR, SarU and sigB genes. The carriage of these regulatory genes wasn’t found associated with the hemolytic patterns.

Meanwhile, the level of hla RNA between the β and δ hemolysis and α and δ hemolysis isolates had no difference. Gel-electrophoresis revealed that the total amount of Hla on both USA300 and R23 was the same. The loss of α hemolysis in R23 isolate had no impact on Hla production. Over production of Hla could restore the α hemolytic activity. Our results indicated that the α-hemolytic impairment happened after the translation of Hla.

People had found some S. aureus toxins may act in synergistic fashion, of which example is the strong hemolysis exhibited by the synergism of β-toxin and δ-toxin. Sometimes, they act in antagonistic way, Richard P. Novick etc reported α-hemolysin and β-hemolysin are mutually inhibitory in 2007 [17]. Staphylococcal Hla is one of pore forming toxins (PFTs) of pathogenic bacteria, it causes cell death by altering the apical membrane permeability of the targeting cells by insertion of a number of water-soluble single-chain polypeptides into the membrane bi-layer and the formation of hydrophilic transmembrane pores. The monomers of Hla bind to specific plasma membrane receptors of host cells at low concentrations, whereas at high concentrations (> 1 μmol/L) they can also bind nonspecifically to phosphocholine headgroups of phospholipids like sphingomyelin or phosphatidylcholine of the plasma membrane [18]. The Hlβ could hydrolyze the sphingomyelin, which is the bancer of Hla. Our ST59 isolates harbored the two hemolysins together. These results brought forward a hypothesis that in CC59 isolates the Hlβ inhibits a hemolysis by hydrolyzing Hla’s target (sphingomyelin) on membrane.

Conclusions

We had demonstrated the gene expression, transcription and translation of Hla in MRSA ST 59 isolates from Chinese children, we found that the negative α hemolysis wasn’t due to Hla itself. We put forward the hypothesis that due to Hlβ hydrolyzing sphingomyelin, Hla couldn’t bound to the monomers on membrane which resulted in negative α hemolysis in CC59 isolates. Nevertheless, there are a lot of work to do to prove the relationship among Hla, Hlβ and sphingomyelin on membrane.

Methods

Bacterial isolates

The Key Laboratory routinely received MRSA-positive samples that the bacteriology room isolated from patients (≤18 years old) of Beijing Children’s Hospital (BCH) during 4 periods (2009-2011, 2012-2013, 2016, 2017). BCH is a tertiary hospital in North China with more than 1,000 beds. This study was approved by the Ethics Committee of Beijing Children’s Hospital affiliated to the Capital Medical University (No. 2016–93, 23/06/2016), and obtained
clearance from the Institutional Biosafety Committee (IBSC) ([2017] No.43). MRSA isolates were collected and identified as described previously by Xin Yang et al [19].

**Determination of hemolytic activities**

The CAMP test for hemolysis was performed. 5% sheep blood agar (SBA) plates were prepared using defibrinated sheep blood (Becton Dickinson, 5% in tryptone soy broth, 25 ml per plate). A S. aureus strain of RN4220 that produced β-hemolysis only was streaked down the center of the SBA plate. Test strains were streaked perpendicular to, but not touching, the center streak. The plates were incubated in 37 °C for 24 h before analysis.

**DNA isolation and PCR reactions**

DNA isolation and polymerase chain reaction (PCR) amplification were used for multilocus sequence typing (MLST), SCCmec typing, and spa typing as previously described [8]. PCR screens of the MRSA isolates were used to identify the presence of genes of hla, hla promoter, SarZ, RNA III, Rot, agrA, SarA, SarR, SarU and SigB (Table 6). PCR products of hla and hla promoter were sequenced and sequences were analyzed using Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast).

| Table 6 | Primers used in the present study |
|---------|----------------------------------|
| gene    | Forward primer (5′–3′)           | Reverse primer (5′−3′) | Reference |
| hla     | TTAGCGGAAAACATTTTC               | TTATTCCGAGGAATTTCAAA  | 2014 [13] |
| hla-pro | CACTATTTTTTTTTTTTTTTTCC       | GTTGTTACACCTGTGAC     | 2014     |
| AgrA    | GAATCATGATGAAAAATTTCATTTGGAGAGCAGTCC | GAACCTCGAGTTATTTTTTTACGTTTCA | sunjing [16] |
| RNA III | CACAGAGATTGGTGGAAATAG             | CATGACTAAACATATGTTATGAG | sunjing   |
| SarA    | ACGCGTACGGTCGAAGCAGTTTGGGTAACA  | GCAGGATCAGGGATGCCCCATTGAGTGAACCTTTAAACCT | This study |
| SarR    | ATGAGTTAAAAATATTGAAATAT        | TCGTTCAATTTTAAACG      | This study |
| SarU    | TGGGGACATGAAAAATTTAGGGAGGAGGAG  | TCTGCAAGGATCGCTCTTTT | This study |
| SarZ    | CGCGGATCCCGAGGAAAGGGAATTCTAGCCAC | CCAAGCTTTCAAGAACCTAGCAGATCGAGTTT | This study |
| SigB    | GCCAGGTTGCGCTAGTTTTTA          | GCCGTTCCTGAGATCGTGCA  | This study |
| Rot     | TGGGATTTGCGGTGATTGTTG           | TCTTAAGCTTTTGGTGCCT    | This study |

**Gene knockout**

We selected 2016R23 isolate to knock out hla gene by Biofeng company (www.biofeng.com), the complete deletion of hla gene was confirmed by PCR, sequencing and alignment with the existing genomic sequences (CP003166.1).

**RNA isolation and cDNA synthesis**

Overnight cultures were diluted 1:100 in 10 mL tryptic soy broth and incubated at 37 °C with shaking at 220 rpm until grown to the stationary growth phase. Cell aliquots harvested during the stationary phase were pelleted by centrifugation at 12000 rpm for 5 min at −4 °C. Each pellet was washed in an equal volume of Tris-EDTA buffer [10 mM TrisHCl, 1 mM EDTA (pH 8)]. Each pellet was then resuspended in TE buffer (pH 8.0) containing 10 g/L lysozyme and 40 mg/L lysostaphin and incubated at 37 °C for 15 min. Total bacterial RNA was isolated using RNAiso (Takara, Japan) according to the manufacturer’s instructions. DNA contaminants were removed by incubating the total bacterial RNA with RNase-free DNase I (Takara, Japan) at 30 U/100 µg total RNA for 1 hour at 37 °C. The amount of RNA recovered was determined using a NanoDrop fluorospectrometer (NanoDrop Technologies, Wilmington, DE, USA). The absence of DNA was verified using PCR. Samples were then stored at −80°C. Complementary DNA (cDNA) was synthesized from total RNA using a PrimeScript RT Reagent Kit (Takara, Japan) according to the manufacturer’s instructions. The recovered cDNA was quantitated spectrophotometrically.

**Quantitative real-time PCR**

Quantitative real-time PCRs (qRT-PCR) were performed using SYBR Premix Ex TaqTM (Takara, Japan) according to the manufacturer’s instructions. Real-time detection and relative quantitation were achieved using the Bio-rad CFX96 PCR Detection System. Selected genes were analyzed using the primer pair hlaF (5'-AAATACTTGTACGAAGGTCTGTTGA-3') and hlaR (5'-GCACGAGATAACTTCTTGTACCT-3'). As an endogenous control, primers were used to amplify a 91 bp
fragment of the DNA gyrase (gyrB): the primer pair gyrBF (5'-CAAATGATCACAGCATTTGGTACAG-3') and gyrBR (5'-CGGCATCAGTCATAATGACGAT-3'). Relative quantification was calculated using the 2-ΔΔCT method with the expression of R23 used as the reference for hla. The qRT-PCR assays were performed in triplicate.

**Detection of secreted Hla by Western blot**

Overnight cultures were diluted 1:100 in 15 mL tryptic soy broth and incubated at 37 °C with shaking at 220 rpm until grown to the stationary growth phase. Cell aliquots harvested during the stationary phase were pelleted by centrifugation at 12000 rpm for 5 min at 4 °C. 15ml supernatant was added to protein concentrate column (pore size: 3 kD) and pelleted by centrifugation at 4500 rpm for 120 min at 4 °C. The supernatant was Concentrated to about 2 ml, separated into 50ul and reserved at -80°C.

The concentration was adjusted to the same concentration and underwent electrophoresis on an SDS-PAGE gel. The proteins were then transferred to a nitrocellulose membrane and the membrane was blocked with 5% skim milk for 1 hour. Mouse anti-staphylococcal Hla polyclonal antibodies (Abcam) were added at a final concentration of 2 μg/ml. The reaction mixture was then incubated at 4°C overnight. After the membrane was washed, 1:600 diluted HRP-labeled rabbit anti-mouse IgG antibodies were added and incubated at 37°C for 2 hours. Finally, chemiluminescent substrates were added for color development. The expression of Hla was finally observed under the imager.

**Statistical analysis**

Categorical variables were analysed using the Chi-square or Fisher's exact test. Differences were considered statistically significant when \( P < 0.05 \). All analyses were performed using JMP software (version 11.0, 2013 SAS Institute Inc., USA).

**Abbreviations**

MRSA: Methicillin-resistant *Staphylococcus aureus*; CC: Clonal Complex; MLST: multilocus sequence typing; Hla: α-hemolysin; Hlb: β-hemolysin; *S. aureus*; *Staphylococcus aureus*; PCR: Polymerase Chain Reaction; SBA: sheep blood agar; qRT-PCR: quantitative real-time PCRs; agr: the accessory gene regulator; AIP: autoinducer peptide; sigB: sigma factor B; BCH: Beijing Children's Hospital; SNP: Single Nucleotide Polymorphisms;

**Declarations**

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None.

**Authors’ contributions**

WLJ performed, analyzed and interpreted all data and was a contributor in writing the manuscript. SC performed and analyzed the qRT-PCR and Western blot data. DF and SWQ collected all the isolates. LKC and SHSG performed and analyzed PCRs. QSY analyzed and interpreted all data. YKH was the adviser, contributing in analyze and interpretation besides of defining the goals of the study. All authors have read and approved the manuscript.

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**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

This study was approved by the Committee of Ethics in Beijing Children's Hospital, Capital Medical University, National Center for Children's Health.

**Consent for publication**

Not applicable.

**Competing interests**
The authors declare that they have no competing interests.

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Figures
CAMP test USA300 showed α and δ hemolysis, R23 showed β and δ hemolysis, R23Δhla showed β and δ hemolysis, R23Δagr exhibited β hemolysis.

The hemolysis of methicillin-resistant Staphylococcus aureus isolated from patients of Beijing Children's Hospital over four periods: 2009-2011, 2012-2013, 2016, 2017.
Hemolysis patterns of STs, most ST59 isolates, all ST338 showed β and δ hemolysis, twenty-two ST239 isolates, 8 ST88 isolates, 4 ST5, 13 ST22 and 6 ST398 showed α and δ hemolysis. A few isolates exhibited β or δ hemolysis alone.

Expression levels of Hlα in isolates showed α and δ hemolysis, β and δ hemolysis and R23Δhla. The results are the means of every group.
Representative image of Western blot analysis performed using concentrated supernatants derived from USA300, R23 and R23Δhla cultures for Hla.

Figure 6

Overexpression of Hla restores α hemolysis of the R23Δhla isolate, there were α, β and δ hemolysis in chla isolate.