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%) showed β and δ hemolysis, both ST59 and ST338 clone belong to CC59 clone. Twenty-two ST239 isolates (73.33%), 8 ST88 isolates (80%), 4 ST5 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 threonine at position 275 instead of isoleucine, then glutamic acid replaced aspartic acid at 208. Seventeen ST59 and 2 ST338 isolates had no mutation, 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 showed a hemolysis 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 β hemolysin (Hlb).

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 enterotoxigenesis [1]. Staphylococcal 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 (Hlb) 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 (*sae*) 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, SarU, 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 influenced 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 hemolytic 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, R23 Δ hla showed β and δ hemolysis, R23 Δ agr 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 (80%), 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 patterns of the MRSA isolates during 4 periods

Hemolysis	Four periods			
	2009-2011	2012-2013	2016	2017
	No(%)	No(%)	No(%)	No(%)
$\beta\delta$	26(60.47)	44(56.41)	81(77.14)	36(56.25)
$\alpha\delta$	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	43(100)	78(100)	105(100)	64(100)

Table 2
Hemolysis patterns of the MRSA isolates grouped by STs

hemolysis patterns	STs								<i>P</i>
	ST59	ST239	ST88	ST5	ST22	ST338	ST398	Others*	
$\beta\delta$	168(94.3)	0(0.00)	1(10.00)	0(0.00)	0(0)	9(100.00)	0(0)	9(23.08)	<0.0001
$\alpha\delta$	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 Hla

The amino acid sequence of Hla 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): MKTRIVSSVTTLTLLGSILMNPVANA (serial number: -26~0)ADSDINIKGTDDIGSNTTVKTGDLVTYDKENGMHKKVFYSFIDDKNHNKLLVIRTKGTIAGQYRVYSEEGANKSGLAWPSAFKVQLQLPDNEVAQISDYYPNSIDTI

We selected 43 representative isolates from 291 clinical strains in four periods for a 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 Hla amino acid sequences based on hemolytic activities

Isolates	<i>spa</i>	SCC <i>mec</i>	ST	Haemolysis	Mutations
2012-2	t437	V	338	βδ	I275T
2012-3	t437	V	59	βδ	I275T
2012-4	t7637	NT	88	αδ	D208E
2012-14	t186	□	88	αδ	I275T
2012-16	t437	□	398	αδ	-
2012-17	t437	□	398	αδ	-
2012-24	t8660	V	120	αδ	I275T
2012-28	t172	□	59	βδ	I275T
2012-32	t008	□	239	αδ	-
2012-38	t034	V	398	αδ	-
2012-43	NT	□	5	αδ	D208E
2012-44	t127	V	1	αδ	I275T
2012-48	t030	III	585	αδ	R-22H
2013-59	t114	□	1	αδ	I275T
2013-74	t4549	V	630	βδ	-
2013-82	t078	□	59	βδ	I275T
2013-90	t078	□	59	αδ	T261-
2013-92	t10555	IV	72	αδ	D208E
2012-96	t037	III	239	αδ	R-22H
2012-97	t081	NT	25	αδ	D208E
2012-9	t437	□	59	βδ	D208E
2012-12	t441	□	59	βδ	I275T
2012-26	t437	□	59	βδ	-
2013-85	t437	□	59	βδ	I275T
2013-86	t437	□	59	βδ	I275T
2012-49	t437	□	59	βδ	I275T
2012-53	t437	□	59	βδ	I275T
2010-25	t127	□	88	βδ	-
2011-32	NT	□	965	βδ	I275T
2011-46	NT	□	59	αδ	L157-
2012-52	t437	□	59	αδ	-
2016-005	t437	□	59	βδ	I275T
2016-045	t437	□	338	βδ	I275T
2016-047	t437	□	1224	βδ	I275T
2016-065	t3515	□	59	αδ	I275T
2016-078	t2049	NT	769	αδ	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	βδ	I275T
2017-033	t437	□	59	βδ	-
2017-110	t2755	□	1295	βδ	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	<i>spa</i>	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	SNP8	SNP9	SNP10	SNP11	SNP12
2013-59	αδ (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	t895	A3G		C18T	T26A	T44C	C93G		G171A				
2012-97		25	t081	A3G		C18T		T44C	C93G				C381T		
2011-46		59	NT	-											
2013-90		59	t078	-											
2016-065		59	t3515	-											
2012-52	β (n=3)	59	t437	-											
2017-039		59	t437	-											
2013-92		72	t10555	A3G		C18T		T44C	C93G			A227G	C381T		
2012-14		88	t186	A3G		C18T		T44C	C93G		G171A				
2012-04		88	t7637	A3G		C18T	T26A	T44C	C93G		G171A		C381T		
2012-24		120	t8660	A3G	A17T			T44C	C93G		G171A				
2012-32		239	t008	A3G		C18T		T44C			G171A				
2012-96		239	t037	A3G		C18T		T44C							
2012-38		398	t034	A3G					C93G						
2012-16		398	t437	A3G		C18T		T44C	C93G		G171A				
2012-17		398	t437	A3G		C18T	T26A	T44C	C93G		G171A				
2012-48		585	t030	A3G		C18T		T44C			G171A				
2016-078		769	t2049	A3G		C18T		T44C	C93G		G171A				T477
2016-122		59	t163											C448G	
2016-090		59	t172											C448G	
2016-085	βδ (n=19)	59	t437	-											
2013-82		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	-											

2013-85		59	t437	-												
2013-86		59	t437	-												
2012-49		59	t437	-												
2012-53		59	t437	-												
2016-005		59	t437	-												
2012-12		59	t441	-												
2010-25		88	t126	A3G		C18T		T44C	C93G	T170C				C381T		
2012-02		338	t437	-												
2016-045		338	t437	-												
2013-74		630	t4549	A3G		C18T		T44C								
2011-32		965	NT	-												
2016-047		1224	t437	-												
2017-110		1295	t2755	-												
2016-114	δ (n=1)	59	t437	A3G		C18T			C93G	T170C		A227G	C381T			
total	43				19/43	1/43	17/43	3/43	17/43	14/43	4/43	10/43	4/43	7/43	4/43	1/43

The detection of genes regulating *hla*

In this study, we detected eight genes regulating *hla*, including *SarZ*, *RNA III*, *Rot*, *agrA*, *SarA*, *SarR*, *SarU* and *sigB*. 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. The 8 regulatory genes were not related to the hemolytic phenotype of the isolates (Table 5).

Table 5

Distribution of genes regulating <i>hla</i> grouped by hemolysis of MRSA									
hemolysis	<i>SarZ</i>	<i>RNAIII</i>	<i>Rot</i>	<i>agrA</i>	<i>SarA</i>	<i>SarR</i>	<i>SarU</i>	<i>sigB</i>	<i>P</i>
	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	
α and δ (n=20)	9 (45%)	20 (100%)	18 (90%)	20 (100%)	8 (40%)	20 (100%)	20 (100%)	20 (100%)	>0.05
β (n=3)	1/3	3/3	3/3	3/3	3/3	2/3	3/3	3/3	
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	1/1	
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 Hla among isolates with different hemolysis phenotypes

Isolate 2016R23 showed β and δ hemolysis, we knocked out its *hla* gene to form R23Δ*hla*. Isolates with β and δ hemolysis, α and δ hemolysis and R23Δ*hla* were selected for qRT-PCR to determine a differential expression of *hla* gene, the result was shown in Fig 4. Isolates with β and δ hemolysis did not change the transcription of *hla* gene (*P*=0.84). The expression of R23Δ*hla* was 0. Transcription level of Hla wasn't related to α hemolysis in clinical isolates.

Production of Hla in USA300, R23 and R23Δ*hla*

We selected USA300 (α and δ hemolysis) R23 (β and δ hemolysis) and R23 Δ hla (β and δ hemolysis) for Western blot to detect Hla. R23 Δ hla didn't produce Hla. USA300 and R23 produced Hla, indicating that even though R23 didn't show a hemolysis phenotype, Hla protein was still produced.

Overexpression of Hla restores α hemolysis of the R23 Δ hla isolate. We considered that α hemolysis could be restored by augmenting the expression of Hla. As the R23 Δ hla 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 R23 Δ hla mutant. R23 Δ hla contained multicopy plasmid displaying a 5-fold level of overexpression of the toxin relative to the endogenous expression in the R23 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 (*agr A*, *agr C*, *agr D*, *agr B* and *hld*), 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 μ mol/L) they can also bind nonspecifically to phosphocholine headgroups of phospholipids like sphingomyelin or phosphatidylcholine of the plasma membrane [18]. The Hl β could hydrolyze the sphingomyelin, which is the bander of Hla. Our ST59 isolates harbored the two hemolysins together. These results brought forward a hypothesis that in CC59 isolates the Hl β inhibits α 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 Hl β 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, Hl β and sphingomyelin on membrane.

Methods

Bacterial isolates

The Key Laboratory routinely received MRSA-positive samples that the bacteriology room isolated from patients ($\leq$ 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
<i>hla</i>	TTAGCCGAAAAACATCATTTTC	TTATTCCCAGCAAATTCCAA	2014 [13]
<i>hla-pro</i>	CACTATATTAATAACATAC	GTTGTTACTGAGCTGAC	2014
<i>AgrA</i>	GAATCATATGATGAAAATTTTCATTTGCGAAGACGATCC	GAACCCTCGAGTTATATTTTTTTAACGTTTCTCA CCGATGCATAGCA	sunjing [16]
<i>RNA III</i>	CACAGAGATGTGATGGAAAATAG	CATGACTAAACATAGATTTATGAG	sunjing
<i>SarA</i>	ACGCGTCGACGTCGAAAGCGTTGATTTGGGTAGTA	CGCGGATCCGCGAGTGCCATTAGTGCAAAACCT	This study
<i>SarR</i>	ATGAGTAAATTAATGATATTAAT	TCGTTCAATGTTATTAAACG	This study
<i>SarU</i>	TGGGACATCAGAAAATAGTAGGAG	TCTGCAAGGGATCGCTCTTT	This study
<i>SarZ</i>	CGCGGATCCCCAAGAAAATGCTACTGCAAC	CCCAAGCTTCAAAGAAGTGAAGCATCGAGATT	This study
<i>SigB</i>	GCGAAGGTGGCCTAGGTTTA	GCCGTTCTCTGAAGTCGTGA	This study
<i>Rot</i>	TGGGATTGTTGGGATGTTTGT	TCTTAAGCATTGCTGCGCT	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 μ g 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 Taq™ (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 *hla*-F (5'-AATAACTGTAGCGAAGTCTGGTGAAA-3') and *hla*-R (5'-GCAGCAGATAACTTCCTTGATCCT-3'). As an endogenous control, primers were used to amplify a 91 bp

fragment of the DNA gyrase (*gyrB*): the primer pair *gyrB*-F (5'-CAAATGATCACAGCATTGGTACAG-3') and *gyrB*-R (5'-CGGCATCAGTCATAATGACGAT-3'). Relative quantification was calculated using the $2^{-\Delta\Delta CT}$ 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

Figure 1

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

Figure 2

The hemolysis of methicillin-resistant *Staphylococcus aureus* isolated from patients of Beijing Children's Hospital over four periods: 2009-2011, 2012-2013, 2016, 2017.

Figure 3

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.

Figure 4

Expression levels of Hla in isolates showed α and δ hemolysis, β and δ hemolysis and R23Δhla. The results are the means of every group.

Figure 5

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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 a hemolysis of the R23 Δ hla isolate, there were α , β and δ hemolysis in chla isolate.