RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2015.41.162

ScienceAsia 41 (2015): **162–169**

Virulence genes and genotypes of *Staphylococcus aureus* from blood of Thai patients

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Received 9 Nov 2014
Accepted 7 Jul 2015

ABSTRACT: *Staphylococcus aureus* is the most common cause of nosocomial fevers. We investigated the virulence genes and genotypes of *S. aureus* strains isolated from bloodstream infections of patients in a Thai teaching hospital. Thirty-three methicillin-resistant *S. aureus* (MRSA) and 52 methicillin-susceptible *S. aureus* (MSSA) collected during 1997–1998, and 29 MRSA and 52 MSSA strains collected during 2010–2011 were studied. Susceptibility to 8 antimicrobials was determined using an agar dilution method. Twelve virulence genes were detected by polymerase chain reaction. The bacterial strains were typed by SCC*mec*, *agr*, *spa*, and multilocus sequence typing. The majority of the MSSA isolates were susceptible to almost all antimicrobials tested, whereas the MRSA isolates were resistant to more than 3 of the antimicrobials tested. The *hla-sea* was the most common virulence gene profile in the MRSA isolates from both periods (46% in 1997–1998, 31% in 2010–2011), and *hla* alone was the most common pattern in the MSSA isolates (56% in 1997–1998, 35% in 2010–2011). The *S. aureus* isolates in 2010–2011 contained more virulence gene profiles than those in 1997–1998. All MRSA isolates from 1997–1998 carried SCC*mec* III-*agr* I, whereas those in 2010–2011 carried SCC*mec* III-*agr* I (48%) and SCC*mec* type II-*agr* II (31%). No specific virulence genes or genotypes of the isolates related to a poor clinical outcome were found.

KEYWORDS: bacteraemia, SCC*mec*, *spa* type, MLST

INTRODUCTION

Blood stream infection is a crucial life threatening condition. *Staphylococcus aureus* was the most common cause of a fever among patients admitted in the hospital[1]. Persistent *S. aureus* bacteraemia often results in longer hospital stays, excessive medical treatment costs, and ineffective chemotherapy with potential side effects, poor outcomes, and even death[2,3]. *S. aureus* produces several virulence factors, including surface associated adhesins, enzymes and exotoxins, which may contribute to its invasive potential and pathogenicity[4]. Clinical outcomes can vary depending on the *S. aureus* strain responsible for the infection. For example, the USA300 genotype has been associated with an increase in hospital mortality[3]. Despite the availability of effective antimicrobial therapy, the mortality rate among patients with *S. aureus* bacteraemia can be as high as 50%[5]. The mortality rate of methicillin-resistant *S. aureus* (MRSA) bacteraemia was previously shown to be higher than that of methicillin-susceptible *S. aureus* (MSSA) (67% versus 46% in Thailand[6] and 50% versus 28% in Argentina[1]). Various clones of *S. aureus* strains, especially of MRSA, have been reported in many countries[2,7]. The majority of *S. aureus* infections in Thailand were related to hospitals, and most of them were clonal lineages of ST239-SCC*mec* III, with a minority of the strains belonging to the ST5-SCC*mec* II lineage[8]. However, the genotypes of *S. aureus* strains from blood infections in Thailand have not yet been

reported. The molecular characteristics of *S. aureus* strains isolated from bacteraemia patients in a teaching hospital in North-eastern Thailand during 1997 and 1998 and during 2010 and 2011 were investigated. Their virulence gene profiles, specific genotypes and clinical outcomes were analysed to elucidate the epidemiological features present in both periods of times. The understanding of bacterial properties and their genetics may be helpful in the management of *S. aureus* bacteraemia patients and infection control.

MATERIALS AND METHODS

Bacterial strains

A total of 166 *S. aureus* samples were isolated from the blood of individual patients in Srinagarind Hospital, Khon Kaen University, Thailand. The samples consisted of 85 isolates (33 MRSA and 52 MSSA) collected during July 1997 and October 1998 and 81 isolates (29 MRSA and 52 MSSA) during October 2010 and September 2011. All the isolates were identified by Gram staining and biochemical testing (catalase, coagulase, DNase, and mannitol fermentation), and were confirmed by the PCR detection of either *femA* or *nuc* gene[9]. The MRSA isolates were identified also by PCR to detect the *mecA* gene[10]. The demographic data of the patients and the clinical information for the isolates collected in 2010–2011 were reviewed retrospectively from patient charts. This study was conducted in accordance with the declaration of Helsinki. It was approved by the Ethics Committee of Khon Kaen University (project number HE542113).

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of 8 antimicrobials (Sigma Chemicals); vancomycin (VA), ofloxacin (OF), sulphamethoxazole/trimethoprim, (SXT), tetracycline (TE), erythromycin (ER), oxacillin (OX), cefoxitin (FOX), and gentamicin (GN), were determined by the agar dilution method and the results were interpreted according to the CLSI breakpoints[11].

Virulence gene detection

Twelve virulence-associated genes, including staphylococcal enterotoxin (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*), α-haemolysin (*hla*), toxic shock syndrome toxin-1 (*tst*), and Panton-Valentine leukocidin toxin (*lukSF-PV*), were detected according to the methods described previously[12–14].

Strain typing

All the isolates were subjected to *agr* typing by multiplex PCR[15]. In addition, the SCC*mec* typing was performed for the MRSA isolates by multiplex PCR[10]. Nine representatives of the isolates in each group were randomly selected to process further for *spa* typing which was designated by using the Ridom StaphType program (www.ridom.de) according to a previous report[16] and multilocus sequence typing (MLST)[17]. The nucleotide sequences of the 7 house keeping gene loci in MLST were concatenated, and a phylogenetic tree was produced using the PhyML program (v3.0 aLRT).

Statistical analysis

The data were analysed with SPSS STATISTICS 19. Categorical variables were compared using the Chi squared test or Fisher's exact test and the Mann-Whitney U test for non-normally distributed variables; $p < 0.05$ was considered to be statistically significant.

RESULTS

Antimicrobial susceptibility

The majority of the MSSA isolates were susceptible to OX, FOX, ER, GN, OF, SXT, TE, and VA (96, 87, 96, 81, 83, 38, 60, and 100%, respectively, for isolates in 1997–1998 and 100, 75, 92, 100, 98, 94, 54, and 100% were susceptible in 2010–2011). All the MRSA isolates in 1997–1998 were however resistant to OX, FOX, ER, and GN whereas 3, 3, 3, and 100% were susceptible to OF, SXT, TE, and VA, respectively. Similarly, all the MRSA isolates in 2010–2011 were resistant to OX and FOX, while 3, 14, 3, 56, 7, and 100% were susceptible to ER, GN, OF, SXT, TE, and VA, respectively (Table 1). All the oxacillin and cefoxitin non-susceptible MSSA strains

Table 1 Antimicrobial susceptibility of *S. aureus* from blood infection in Northeast Thailand.

Samples (*n*)	Antimicrobials (% susceptible)[†]							
	OX	FOX	ER	GN	OF	SXT	TE	VA
MSSA 1997–98 (52)	96[*]	87[*]	96	81	83	38	60	100
MSSA 2010–11 (52)	100	75[*]	92	100	98	94	54	100
MRSA 1997–98 (33)	0	0	0	0	3	3	3	100
MRSA 2010–11 (29)	0	0	3	14	3	56	7	100

[†] OX, oxacillin; FOX, cefoxitin; ER, erythromycin; GN, gentamicin; OF, ofloxacin; SXT, sulphamethoxazole/trimethoprim; TE, tetracycline; VA, vancomycin.
[*] the resistant isolates had border-line MICs to OX and/or FOX.

Table 2 Virulence associated gene profiles, genotypes and clinical outcomes of *S. aureus* bacteraemia patients in 1997–1998 and 2010–2011.

Virulence gene profiles	No. (%)	MRSA						Clinical outcomes	
		SCC*mec* II		SCC*mec* III			SCC*mec* IX	Septic shock	Poor
		agr I	*agr* II	*agr* I	*agr* II	*agr* III	*agr* II		
MRSA 2010–2011: ($n = 29$)									
- *hla, sea*	10 (34)	1 (3)	7 (24)	9 (31)[a]				3 (10)	6 (21)
- *hla, seb, seg, sei, tst*	8 (28)				1 (3)			2 (7)	6 (21)
- *hla*	6 (21)			6 (21)[b]				3 (10)	2 (7)
- *sea*	1 (3)			1 (3)					
- *hla, seg, sei, tst*	2 (7)			1 (3)[c]			1 (3)[d]		1 (3)
- *hla, seb*	1 (3)		1 (3)						
- *hla, seb, sec, seg, sei, tst*	1 (3)		1 (3)					1 (3)	1 (3)
MSSA 2010–2011: ($n = 52$)									
- *hla*	18 (35)			16 (31)	2 (4)			1 (2)	4 (8)
- *hla, tst*	5 (10)			1 (2)	1 (2)	3 (6)		1 (2)	
- *hla, sea*	4 (8)			2 (4)		2 (4)		1 (2)	1 (2)
- *hla, seb, seg, sei, tst*	1 (2)				1 (2)				
- *hla, seb, seg, sei*	1 (2)				1 (2)				1 (2)
- *hla, tst, lukSF-PV*	1 (2)			1 (2)					
- *hla, seb, seg, sei, tst, lukSF-PV*	1 (2)				1 (2)[e]				
- *hla, seb, tst*	1 (2)					1 (2)			1 (2)
- *hla, seb, sec, seh, tst*	1 (2)					1 (2)[f]			
- *hla, seg, sei lukSF-PV*	1 (2)				1 (2)[e]				
- *seb, seg, sei*	1 (2)					1 (2)[f]			1 (2)
- *seg, sei*	1 (2)					1 (2)			
- *hla, seb, sec, seh, tst*	1 (2)					1 (2)		1 (2)	
other various types (1 each)	10 (19)				6 (12)	4 (8)		1 (2)	2 (4)
not found	5 (10)			5 (10)				1 (2)	
MRSA 1997–1998: ($n = 33$)									
- *hla*, *sea*	15 (45)			15 (45)[g]					
- *hla*	14 (42)			14 (42)[h]					
not found	4 (12)			4 (12)					
MSSA 1997–1998: ($n = 52$)									
- *hla*	29 (56)			29 (56)					
- *hla, sea*	14 (27)			14 (27)[i]					
- *hla, sea, lukSF-PV*	5 (10)			5 (10)					
- *hla*, *lukSF-PV*	4 (8)			4 (8)[j]					

[a] Two representatives were ST239-t037; [b] ST239-t037; [c] ST72-t324; [d] ST9-t337; [e] ST121; [f] ST1-t5445; [g] Three representatives were ST239-t037; [h] Two representatives were ST239-t037; [i] Two representatives were ST88; [j] Two were ST121.

yielded border-line MIC values which may be due to the hyper-production of penicillinase.

Virulence genes

The distribution of virulence genes in *S. aureus* isolates and genotypes are summarized in Table 2. Of the 12 virulence-associated genes, *hla* was the most common virulence gene among the isolates from both periods at a frequency of 88% in MRSA and 100% in MSSA in 1997–1998 and 93% in MRSA and 81% in MSSA in 2010–2011. The next most common virulence gene was *sea* (45%/37% of MRSA/MSSA isolates in 1997–1998 and 38%/11% of MRSA/MSSA isolates in 2010–2011). The MRSA and MSSA isolates from 1997–1998 had 2 and 4 virulence-gene profiles, respectively, whereas those in 2010–2011 carried 7 and 24 profiles in the MRSA and MSSA groups, respectively. Among the MRSA isolates, the *sea-hla* profile was the most common profile (45% in 1997–1998 and 34% in 2010–2011). In contrast, among the MSSA isolates, the *hla* singleton was the most common profile (56%

in 1997–1998 and 35% in 2010–2011). The *tst* gene was found only among the isolates from 2010–2011 (38% and 22% of MRSA and MSSA isolates, respectively). The *lukSF-PV* gene was detected only in MSSA isolates (17% in 1997–1998 and 11% in 2010–2011).

Strain typing

***agr* typing:** All the isolates from 1997–1998 were *agr* I, while the isolates in 2010–2011 showed various *agr* types. Of the 29 MRSA isolates from 2010–2011, 18 (62%) were *agr* I, and 11 (38%) were *agr* II. Among the 52 MSSA isolates from 2010–2011, *agr* I (48%) was the most prevalent followed by *agr* III (27%), and *agr* II (25%).

SCC*mec* typing: Using a combination of the *mec* gene complex class and the *ccr* gene complex type, all 33 MRSA isolates in 1997–1998 were SCC*mec* III, whereas the MRSA isolates in 2010–2011 carried SCC*mec* III (62%), SCC*mec* II (38%), and SCC*mec* IX (3%).

***spa* typing and MLST:** Of the 5 representative MRSA isolates in 1997–1998, all were t037-ST239-SCC*mec* III-*agr* I, 2 MSSA isolates were ST88-*agr* I, and 2 isolates were ST121-*agr* I. Among the 9 representative isolates in 2010–2011 (5 MRSA, 4 MSSA isolates), the MRSA isolates were *spa* type t037 (3 isolates), t324, and t337, whereas 2 MSSA isolates were *spa* type t5445. Combining the typing results, the MRSA isolates in 2010–2011 were t037-ST239-SCC*mec* III-*agr* I (3 isolates), t324-ST72-SCC*mec* III-*agr* I, and t337-ST9-SCC*mec* IX-*agr* II; while the MSSA isolates were ST121-*agr* II (2 isolates), and t5445-ST1-*agr* III (2 isolates). The *spa* types of 6 MSSA isolates were undetermined. The phylogenetic tree of the sequence types found in this study is shown in Fig. 1.

Clinical characteristics

The patients' information was available only for the isolates from 2010–2011 (27 of 29 cases of MRSA infection and 49 of 52 cases of MSSA infection) and is summarized in Table 3. Of the 27 patients infected with MRSA, 15 cases (56%) were females, whereas 11 cases (22%) of the MSSA infection group were females. The age of the patients ranged from newborn to 101 years (mean ± SD, 46 ± 27) in the MRSA group and from newborn to 82 years (48 ± 23) in the MSSA group. The underlying conditions of patients infected with MRSA and MSSA were comparable except that the prevalence of liver disease was significantly higher in MSSA group ($p = 0.044$). Regarding the clinical course and outcome of the patients, those infected with MRSA had significantly higher incidences of the following conditions than those infected with MSSA: hospital stay > 30 days (70% versus 29%, $p = 0.0007$, odds ratio = 5.9, 95% CI = 2.1–16.6), poor outcome (52% versus 22%, $p = 0.01$, odds ratio = 3.7, 95% CI = 1.3–10.2) and septic shock (33% versus 12%, $p = 0.03$, odds ratio = 3.5, 95% CI = 1.1–11.5). Cloxacillin only or cloxacillin combined with other antimicrobials was the most common drug used for the treatment of MSSA infection, whereas MRSA infections were most commonly treated with vancomycin. The mortality rates of patients infected with MRSA and MSSA were not significantly different ($p = 0.41$).

No specific virulence gene profile or specific genotype of the isolates was found to be related to any patient with septic shock or to a poor outcome ($p = 0.8$, data not shown).

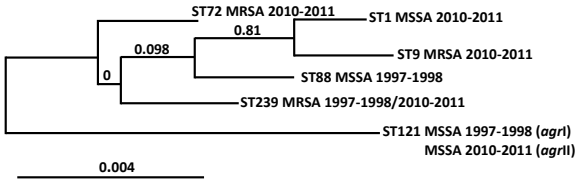

Fig. 1 Phylogenetic diversity of the sequence types detected among representative *S. aureus* strains collected in 1997–1998 and 2010–2011.

DISCUSSION

Genotypic differences were observed among *S. aureus* isolates from several countries, and particular strains were associated with poor clinical outcomes. The PVL-positive community-acquired (CA) -MRSA isolates in Taiwan caused more serious infections in patients presented with non-multidrug-resistant MRSA bacteraemia, and most of the isolates were *spa* type t437-ST59[18]. The MRSA strain USA300 was associated with increased mortality in the US[19]. In contrast, the isolates in the present study did not show specific virulence genes related to mortality or poor outcome. This may be due to the geographic differences between the strains or the different statuses of the patients. The most common virulence gene among both sample groups in this study was *hla*, similar to a previous report from Denmark[20]. The toxin promotes cell lysis and induces inflammation, leading to critical diseases such as pneumonia and sepsis[21]. However, no significant clinical outcomes were observed relating

Table 3 Clinical information of patients with *S. aureus* blood stream infection in 2010–2011.

Clinical characteristics	*S. aureus* isolates		Odds ratio	95% CI	*p*
	MRSA $n = 29$ (%)[a]	MSSA $n = 52$ (%)[b]			
Male	12 (44)	38 (78)	0.2	0.08–0.63	0.004
Female	15 (56)	11 (22)	3.1	1.1–8.3	0.02
Age: median (range)	51 (NB–101)[*]	50 (NB–82)			
Underlying:					
- Diabetes mellitus	6 (22)	14 (29)	0.7	0.2–2.1	0. 54
- Hypertension	6 (22)	13 (27)	0.7	0.2–2.3	0.67
- Renal disease	8 (30)	14 (29)	1.0	0.3–2.9	0.92
- Heart disease	3 (11)	4 (8)	1.4	0.2–6.8	0.67
- Liver disease	0	8 (16)	0.08	0.004–1.6	0.10
- Malignancy	2 (7)	8 (16)	0.4	0.08–2.0	0.28
- Others	5 (19)	8 (16)	1.1	0.3–3.9	0.80
- No underlying	2 (7)	8 (16)	0.4	0.08–2.0	0.28
agr Type I	18 (62)	26 (50)	1.7	0.6–4.7	0.25
agr Type II	11 (38)	13 (25)	1.9	0.7–5.1	0.20
agr Type III	0	13 (25)	0.4	0.002–0.8	0.03
SCC*mec* Type II	10 (34)				
SCC*mec* Type III	18 (62)				
SCC*mec* Type IX	1 (3)				
Antibiotics:					
- Cloxacillin[c]	0	29 (59)	0.01	0.0007–0.2	0.002
- Vancomycin[d]	23 (85)	7 (14)	34.5	9.1–130.3	< 0.0001
- Others	4 (15)	13 (27)	0.4	0.1–1.6	0.24
Duration > 30 days in hospital	19 (70)	14 (29)	5.9	2.1–16.6	0.0007
Outcome:					
- Septic shock	9 (33)	6 (12)	3.5	1.1–11.5	0.03
- Good	4 (15)	26 (53)	0.1	0.04–0.5	0.002
- Poor	14 (52)	11 (22)	3.7	1.3–10.2	0.01
- Death	9 (33)	12 (24)	1.5	0.5–4.3	0.41
- Co-infect w/ Gram neg. bacilli	5 (19)	9 (18)	1.0	0.3–3.3	0.09

[a] Clinical information was not available for 2 cases (7%);
[b] Clinical information was not available for 3 cases (6%);
[c] Oxacillin or oxacillin combined with other antimicrobials;
[d] Vancomycin or vancomycin combined with other antimicrobials;
[*] NB, new born.

to the strains containing *hla* gene compared to those without this gene. The *sea* gene was primarily found in the SCC*mec* III MRSA isolates (91%), whereas *tst* was dominant in the SCC*mec* II isolates (72%). These findings are similar to those reported by Kim et al[22] and Hongsrichan et al[23], suggesting that these virulence genes may be related to a certain SCC*mec* type. The *seg* and *sei* genes were detected within the same isolate with the majority were *agr* II (58%). These results imply that the genes may be located on the same enterotoxin gene cluster (*egc*)[24].

The *lukSF-PV*, a set of bicomponent genes, encodes the PVL toxin. The role of this toxin as a virulence factor was controversial because an experimental study in mice showed that PVL did not contribute to the pathogenesis of staphylococcal infection[25]. However, it has been shown to be related to necrotizing pneumonia and skin and soft tissue infections in human[26]. This syndrome was often found in CA-MRSA infections, which usually carry the SCC*mec* IV element[27]. This gene was rarely (less than 5%) found in the hospital-acquired-MRSA strains which generally carry either the SCC*mec* I, II or III element[28]. In this study, the *lukSF-PV* gene was not found in any MRSA isolates. Most of the patients infected with *lukSF-PV*-carrying *S. aureus* isolates had good outcomes and did not develop necrotizing symptoms. Unfortunately, it was not possible to determine whether the isolates charac-

terized in the present study were from community- or hospital-acquired infections.

The accessory gene regulator (*agr*) systems play a major role in controlling virulence factor production in *S. aureus* isolates and were classified into four different *agr* groups. In this study, *agr* IV was not found in any isolates, similar to what was observed in a previous report[29], suggesting that the *agr* IV *S. aureus* isolate was not common. The *agr* III and IV groups were associated with *tst* and exfoliatin toxin production, respectively[30,31]. The *tst* gene was most common in the *agr* II *S. aureus* isolates (59%), followed by the *agr* III (27%) and *agr* I (14%) isolates. Interestingly, most of the *agr* II and III isolates in this study carried more virulence genes (> 3 virulence genes) than the *agr* I isolates. In addition, the isolates that did not contain any of the assessed virulence genes were all *agr* I (Table 2). These data are similar to previous reports showing that most toxin-producing *S. aureus* strains were *agr* II[32] or III[33].

The genotype of *S. aureus* isolates between the two periods was quite different. The MSSA isolates from 2010–2011 demonstrated a wider variety of virulence gene profiles and *agr* groups than those from 1997–1998. Likewise, all the MRSA isolates from 1997–1998 carried SCC*mec* III-*agr* I, which was thought to be a major clone in this hospital[8], whereas all but one of the isolates from 2010–2011 contained either SCC*mec* II or III. The percentage of SCC*mec* II MRSA isolates from 2010–2011 was much higher than the previous report from the same hospital in 2002–2003 (34% versus 2%)[8], suggesting that the SCC*mec* III strain dominant in 1997–1998 was gradually replaced with the SCC*mec* II strain by 2010–2011. Moreover, an SCC*mec* IX-*agr* II MRSA isolate that was recently isolated from a skin infection of an outpatient of this hospital[34] was also detected in this study, suggesting that the SCC*mec* IX-*agr* II MRSA strain may be a new clone distributed in this hospital. The SCC*mec* IX MRSA strains were related to swine and products from swine[35]. The source of this strain may be related to pig. The change of bacterial strains over time may be due to the change of the environmental selective pressure[36]or to globalization. This situation was similar to one that occurred in Hungary in which ST239-SCC*mec* III was replaced by ST5- SCC*mec* II and ST228- SCC*mec* I between 1994 and 2004[37], and in Japan the clone changed from ST30-SCC*mec* I or ST30-SCC*mec* IV in 1979–1980 to ST5-SCC*mec* II in 1999[38]. As far as we know, this is the first report describing the trend in the MRSA clonal change in Thailand over the last decade.

The sequence types of isolates in this study belonged to different clonal complexes: ST1 (CC1), ST9 (CC9), ST88 (CC88), ST239 (CC8), and ST72 (CC1) suggesting that there are several *S. aureus* clones in this area. The ST121 MSSA and ST239-t037 MRSA isolates were detected in both periods, indicating that they were endemic strains in this area. However, the ST121 strains from both periods carried different virulence genes and *agr* types which may be due to the horizontal transfer of the virulence genes among different strains. The ST88-MRSA-III/IV was proposed to be a typical African clone but it was also reported sporadically around the world[39]. The ST88 MSSA isolates in this study may derive from different ancestor from that of the African clone. Comparison of the whole genome sequences or pulse field gel electrophoresis patterns of these strains would reveal their association.

Although the clinical data showed that the mortality rates of the patients infected with MRSA and MSSA were not significantly different, the MRSA-infected patients tended to stay in the hospital longer than the MSSA-infected cases ($p = 0.0007$). In summary, a temporal change was observed among the *S. aureus* strains isolated from blood stream infections of patients in a teaching hospital in Northeast Thailand between 1997 and 1998 and 2010–2011. The isolates from 2010–2011 carried more virulence genes and were more genetically diverse than those from 1997–1998.

Acknowledgements: This project was financially supported by Khon Kaen University under the Incubation Researcher Project (Fiscal year 2012). The authors thank the Centre for Research and Development of Medical Diagnostic Laboratories (CMDL), Faculty of Associated Medical Sciences, Khon Kaen University, and the Division of Research Administration, Khon Kaen University. We also wish to thank the staff of the Clinical Microbiology Laboratory at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University for collecting the clinical isolates. We thank Yukifumi Nawa for his valuable suggestions.

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