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Virulence Genes of *Staphylococcus aureus* Isolated in Daegu and Gyeongsangbuk-do Areas

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Nine types of staphylococcal enterotoxin (SE) genes (*sea~see*, *seg~sej*), 3 types of virulence genes (*eta, etb, tst*), *mecA* and 16S rRNA as internal positive control were detected from 187 clinical MRSA (methicillin resistance *Staphylococcus aureus*) strains isolated from a variety hospitalized patients in Daegu and Gyeongsangbuk-do areas using the multiplex PCR. The frequency of the *S. aureus* strains harboring recently reported SE genes (*seg~sej*) were found to be very high (65.9%) and greater than that of the strains harboring classical SE (*sea~see*) genes (47.8%) as previously established. Taking into account that the newly described pairs form SE genes (i.e., *sec+seg+sei, seg+sei*) were many, in the other hand, single form SE genes (i.e., *seg, seh, sei and sej*) were rarely detected. The *S. aureus* with pairs form enterotoxigenic genes become more potentially toxigenic strains. Furthermore, this work indicated a systematic association between the *seg* and *sei* genes and their high incidence among the *S, aureus* strains, which suggests that these two SE's could be an important phylogenetic link among the staphylococcal enterotoxins.

Key Words : *Staphylococcus aureus*, Virulence genes, Enterotoxin, PCR

I. INTRODUCTION

Staphylococcus aureus is the major pathogen of the genus of *Staphylococcus* and an important nosocomial pathogen causing a variety of clinical infections including septicemia, pneumonia, wound sepsis, septic arthritis, osteomyelitis, post-surgical toxic shock syndrome and food-borne disease (Vincent et al., 1995; Boyce, 1997).

Many *S. aureus* accessory genes encode virulence factors such as enterotoxins A through I (*sea* to *sei*, respectively), toxic shock syndrome toxin 1 (*tst*), exfoliative toxins A and B (*eta*, *etb*) (Projan and Novick, 1997; Munson et al., 1998). These genes are often carried on mobile genetic elements, such as phages and pathogenicity islands (SaPIs), which transfer horizontally between strains (*sea, tst, eta*) (Betley and Mekalanos, 1985; Lindsay et al., 1997; Yamaguchi et al., 2000; Yoshizawa et al., 2000) or which are suspected to do so (*etb, seb*) (Jackson and Iandolo, 1986; John et al., 1998). Transfer of *tst* can occur at an extremely high frequency (Lindsay et al., 1997) in vitro, although it is not known if this occurs in the clinical setting. There is also evidence that mobile accessory virulence genes are not distributed uniformly among strains. For example, *seb* and *tst* are never seen in the same strain (Bohach at al, 1990).

The purpose of the present study was to investigate the distribution of genomic differences in clinical strains of *S. aureus,* particularly those that might have an effect on pathogenicity. We studied 259 epidemiologically unrelated strains collected from 5 hospitals located in Daegu and

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II. MATERIALS AND METHODS

We characterized 187 strains of MRSA. The strains were isolated from 5 hospitals which are located at Daegu and Gyeongsangbuk-do areas in Korea. No isolates were known to be epidemiologically related, and the isolates comprised strains were isolated from different patients on different wards and taken at different times in 2005.

The reference *S. aureus* strains used for this study were ATCC13565 for *sea* and *sed* genes, ATCC14458 for *seb,* ATCC19095 for *sec,* ATCC23235 for *sed,* ATCC27664 for *see* and previously analyzed clinical isolates for *seg, seh, sei, sej, eta, etb, mecA, tst* and 16S rRNA.

1. Selection of genes and primer design

Primers for the *sea, seb, sec, sed, see, seg, seh, sei, sej, eta, etb, tst, mecA* and 16S rRNA gene were designed to be specific for each gene and to have similar melting temperatures for multiplex PCR from published sequences. The absence of homologous regions in any other known DNA sequence available on the GenBank database was ensured by using Blast software (Altschul et al., 1990).

2. DNA extraction

Chromosomal DNA was isolated as described by Hookey (Hookey et al, 1998) with some modification. All *S. aureus* isolates were incubated in brain heart infusion broth (Becton & Dickinson Company, Sparks, MD., USA) for 15~18h at 37℃. One mL of each cultured broth was pelleted by centrifugation at 12,000× g for 5 min, and washed two times with 700 µL of TE buffer (Bioneer Corporation, Deajeon, Korea). The bacterial pellet was resuspended in 565 µL of TE buffer, 30 µL of 20% sodium dodecyl sulfate (Sigma Chemical Company, St Louis, Mo., USA), 50 µL of 200 µg/mL lysostaphin (Sigma Chemical Company, St Louis, Mo., USA), 5 µL of 20 mg/mL proteinase K (Bioneer Corporation, Deajeon, Korea) and then incubated for 90 min at 37℃. Addition of 5 M NaCl 100 µL and 80 µL of CTAB/NaCl (Sigma Chemical Company, St Louis, Mo., USA) was followed and incubated for 20 min. at 65℃, and then centrifuged at 12,000× g for 15 min at 4℃. The lystate was extracted with equal volume of phenol- chloroform-isoamylalcohol (Bioneer Corporation, Deajeon, Korea) and centrifuged at 12,000× g for 15 min at 4℃. The supernatent was mixed with two or three times more absolute ethanol by volume and then fixed for over night at -20℃ to precipitate DNA, centrifuged at 12,000× g for 15 min at 4℃. The pellet was washed with 70% ethanol and dried in vacuum condition. The DNA pellet was resuspended in 30 µL of TE buffer and stored at -20℃ until PCR amplification.

3. Mutiplex PCR

Three sets of multiplex PCR were performed to investigation of the endotoxin genes. The first primer set contained *sea, seb, sec, sed*, and *see* primers. The second set contained *seg, seh, sei*, and *sej* primers. The third set contained *eta, etb, tst, mecA* and 16S rRNA primers. Each PCR content was made up of 2 µL of templete DNA, 2 µL of primer pools (for 1st primer set, 0.2 µL each; 2nd primer set, 0.25 µL each; 3rd primer set, 0.25 µL each), 10 µL of PCR PreMIX (Bioneer Corporation, Deajeon, Korea) contained 1 U of *Taq* DNA polymerase, 250 µM dNTP, 10 mM Tris-HCl, 40 mM KCl, 1.5 mM $MgCl_2$, stabilizer and tracking dye, and DW upto 20 µL. Each mixture was heated at 95℃ for 2 min before 28 cycles of amplification (denaturation at 95℃ for 1 min, annealing at 53℃ for 1 min, extension at 72℃ for 2 min) and final extension was performed at 72℃ for 5 min in a Mygene 96 Thermal Block (Bioneer Corporation, Deajeon, Korea).

Table 1. The distribution of *S. aureus* strains harboring the genes coding for the virulence factors collected by 5 hospitals and 1 public research institution

Gene	A-hospital* n=39	B-hospital n=35	C-hospital n=26	D-hospital n=48	E-hospital n=38	H-institute n=1	Total n=187
sea	20 (51.3)	20 (57.1)	5 (19.2)	8 (16.7)	23 (60.5)	0 (0.0)	76 (40.6)
seb	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.6)	0 (0.0)	1 (0.5)
sec	11 (28.2)	10 (28.6)	19 (73.1)	40 (83.3)	15 (39.5)	0 (0.0)	95 (50.8)
sed	1 (2.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)
see	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
seg	19 (48.7)	16 (45.7)	24 (92.3)	40 (83.3)	27 (71.1)	1 (100.0)	127 (67.9)
seh	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
sei	18 (46.2)	12 (34.3)	22 (84.6)	40 (83.3)	21 (55.3)	0 (0.0)	113 (60.4)
sej	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
eta	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
etb	1 (2.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)
tst	11 (28.2)	16 (45.7)	20 (76.9)	39 (81.3)	17 (44.7)	0 (0.0)	103 (55.1)
mecA	36 (92.3)	34 (97.1)	26 (100.0)	48 (100.0)	37 (97.4)	0 (0.0)	181 (96.8)
16S rRNA	39 (100.0)	35 (100.0)	26 (100.0)	48 (100.0)	38 (100.0)	1 (100.0)	187 (100.0)

* The percentile of investigated isolates was presented in ( ).

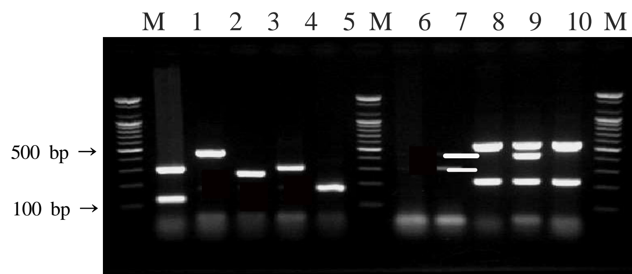

Fig. 1. Multiplex PCR amplification products of reference strains. Lane 1~5 are ATCC control strains, lane 7~10 are clinical isolated strains. Lane 1 is ATCC13565 (*sea* and *sed*), lane 2 is ATCC14458 (*seb*), lane 3 is ATCC19095 (*sec*), lane 4 is ATCC23235 (*sed*), lane 5 is ATCC27664 (*see*), lane 6 is negative control, lane 7 is D-hospital strain-4 (*seg, sei*), lane 8 and 10 are A-hospital strain-1 and D-hospital strain-31 (*mecA* and, 16S rRNA). Lane 9 is E-hospital strains-7 (*mecA, tst* and 16S rRNA genes). M is a 100 bp DNA ladder (Bioneer Corporation, Deajeon, Korea).

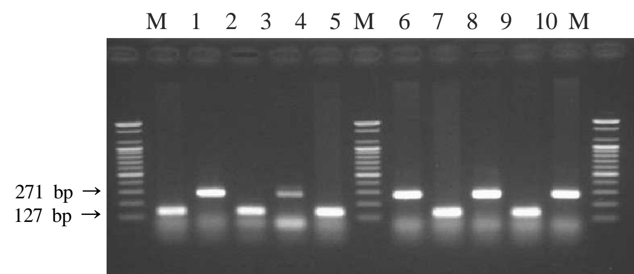

Fig. 2. Results of multiplex PCR with the 1st primer set. Amplificaton products for *sea* presented at 127 bp size are lane 1, 3, 5, 7 and 9 (obtained from A-hospital strain-12, B-hospital strain-7, C-hospital strain-20, E-hospital strain-12, respectively). Amplification products for *sec* presented at 271 bp size are lane 2, 4, 6, 8 and 10 (obtained from A-hospital strain-37, B-hospital strain-30, C-hospital strain-35, D-hospital strain-18, E-hospital strain-35, respectively). Lane M is a 100 bp DNA ladder (Bioneer Corporation, Deajeon, Korea).

4. Electrophoresis

The amplified DNA products (7 μL) were electrophoretically separated in 1.5% agarose gel (Bioneer Corporation, Deajeon, Korea) in 1× TAE buffer (Bioneer Corporation, Deajeon, Korea) by Mupid-eX (Advance electronics Inc. Tokyo, Japan), stained with ethidium bromide, visualized on a UV transilluminator, and photographed with Polaroid.

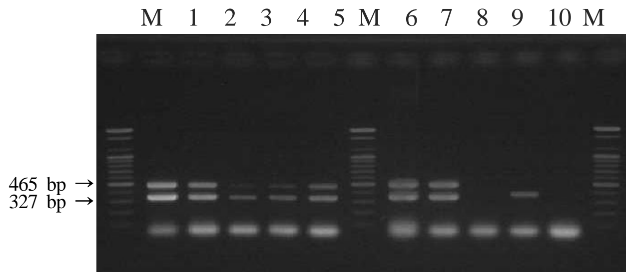

Fig. 3. Multiplex PCR results with the 2nd primer set are presented. Amplification products for *seg* (327 bp) and *sei* (465 bp) are expressed together on lane 1, 2, 3, 4, 5, 6 and 7 (obtained from A-hospital strain-6, A-hospital strain-17, B-hospital strain-9, B-hospital strain-26, C-hospital strain-13, C-hospital strain-36, D-hospital strain-3, respectively). Lane 9 is E-hospital strain-6, presented the only *seg* product. On lane 8 (D-hospital strain-18) and 10 (E-hospital strain-33), there are no products of any genes. M is a 100 bp DNA Ladder (Bioneer Corporation, Deajeon, Korea).

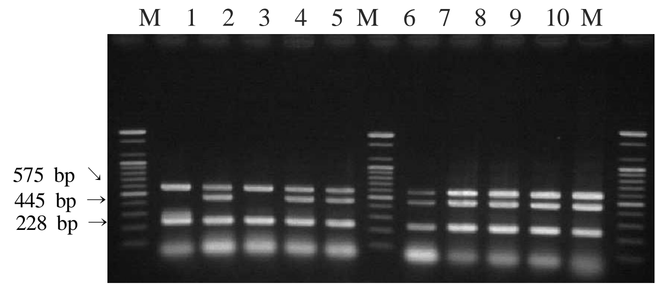

Fig. 4. Multiplex PCR results with the 3rd primer set are presented. Lane 2, 4, 5, 6, 7, 8, 9 and 10 (obtained from A-hospital strain-27, B-hospital strain-12, C-hospital strain-14, C-hospital strain-15, D-hospital strain-6, D-hospital strain-7, E-hospital strain-7, E-hospital strain-11, respectively) are PCR positive against *mecA, tst* and 16S rRNA genes. Lane 1 (A-hospital strain-16) and lane 3 (B-hospital strain-11) are amplified *mecA* and 16S rRNA genes. M is a 100 bp DNA ladder (Bioneer Corporation, Deajeon, Korea).

III. RESULTS

Of all 187 clinically identified MRSA isolated in Daegu and Gyeongsangbuk-do areas, 181 strains were *mecA* positive (Table 1). All *S. aureus* strains carried *mecA* presented at least one enterotoxin gene of classical SE or recently described SE genes (Table 1). Frequencies of those genes in MRSA were presented in the order of *seg, sei, sec, sea* at 67.9%, 60.4%, 50.8%, 40.6%, respectively (Table 1). There were no *S. aureus* carring *see or seh or sej* gene. The *seb* and *sed* genes were rarely presented in only one strain respectively.

Among other virulence factor encoding gene, toxic shock syndrome toxin 1 gene (*tst*) was expressed at 55.1%, but exfoliative toxins A and B genes (*eta*, *etb*) were not or rarely presented at 0% and 0.5% respectively (Table 1).

Of the total 181 MRSA strains, 125 strains carried two or more enterotoxin genes as described as Table 2. There were 12 kinds of multiple enterotoxin gene carrying type in this study. The most frequent multiple enterotoxin genes carrying type were in the order of [*sec*+*seg*+*sei*] type and

Table 2. Distribution of enterotoxin gene in specimens

Specimens	None	*sea*	*sec*	*seg*	*sea sec*	*sea seg*	*sec seg*	*seg sei*	*sea seg sei*	*sec seg sei*	*sea sec seg sei*	*sea sed seg sei*	*seb sec seg sei*	Total
Urine	0	1	0	0	0	0	2	4	0	2	1	0	0	10
Blood	2	3	0	0	0	1	0	1	2	5	0	1	0	15
Wound	2	16	1	1	1	1	0	2	2	4	0	1	0	31
Pus	0	5	3	2	0	1	1	1	5	7	0	0	0	25
Sputum	1	9	6	1	1	1	2	5	10	38	4	0	0	78
Other	1	3	5	0	1	0	0	1	6	10	0	0	1	28
Total	6	37	15	4	3	4	5	14	25	66	5	2	1	187

[*sea+seg+sei*] type, which respectively accounted for 35.3%, 13.4%. Especially, *seg* and *sei* genes were concurrently presented in 113 strains (62.4%) of the total 181 MRSA (Table 2, Fig. 3). It is full of significance for closely relation as enterotoxin gene rearrangement or propagation between *S. aureus* strains.

In distribution analysis of enterotoxin gene according to specimens, there were no significant differences between enterotoxin gene types or multiple enterotoxin gene carrying types and the isolated group from the specimens (Table 2).

IV. DISCUSSION

Many articles used the PCR method to deteet staphylococcal enterotoxin genes (Tornadijo et al., 1996; Holeckova et al., 2002). All of them found a high variability (75~80%) in the presence of enterotoxin genes. Our experiments confirmed this fact as well. We detected the genes for nine types of enterotoxin (*sea~see, seg~sej*), 3 types of virulence genes (*eta, ,etb, tst*), *mecA* and 16S rRNA as the internal positive control in our set of 187 *S. aureus* strains. The majority of enterotoxin positive staphylococci (97.3%) carry genes for enterotoxin. Some data published by other authors show that the staphylococcal enterotoxin type [*seg+sei*] was the most frequent type (Rosec et al., 1998) but our most frequent type was [*sec+seg+sei*], without the production any of the *sea*, *seb*, *sec*, *sed* or *see*, which harbored one or more of the *seg*, *seh, sei*, or *sej* genes (Mac Launchlin et al., 2000). Thus, it is likely that the corresponding SE's should be the causative agent of these outbreaks. A similar observation was made with clinical strains implicated in staphylococcal toxic shock syndrome and scarlet fever without producing classical SE's or toxic shock syndrome toxin 1, leading to the suggestion that *seg* or *sei* caused these diseases (Jarraud et al., 1999)

When taking into account the *seg, seh, sei* and *sej,* the incidence of potentially enterotoxogenic *S. aureus* considerably increases among the strains, which were isolated from the clinical environment. The *seg* gene was systematically detected in association with *sei*, as was *sed* with *sej*. The latter association has been described (Zhang et al., 1998) and the former was also observed in clinical strains (Jarraud et at., 1999) where *seg* and *sei* were in tandem orientation and separated by 1.9 kb length intergenic DNA. Nevertheless, this systematic *seg* and *sei* association is surprising since these two genes were initially cloned from two different strains (Munson et al., 1998) and Mac Lauchlin et al. (2000) found 8 strains which harbored *seg* alone, and over 109 ones which were *seg* and/or *sei*.

Furthermore, *seg* and *sei* were the sole SE gene which could be detected in association with each other (Table 2). The apparent systematic association between *seg* and *sei* and the horizontal spread of these two genes among our *S. aureus* strains could suggest that they are either a reservoir or a part of a reservoir for an enterotoxin gene rearrangement in *S. aureus*, as has been hypothesized for the region where *sei* was identified and sequenced (Munson et al., 1998)

The result conclude that the incidence of the recently described SE genes (*seg~sej*), and particularly *seg* and *sei*, among the clinical *S. aureus* isolated in Korea is very high. Thus, the role of *seg*, *seh*, *sei* and *sej* in the staphylococcal disease is probably underestimated, unless their corresponding genes are rarely or weakly expressed. Given the results of this work, there is a need to improve or develop detection methods for these SE's.

Further, this work indicates a systematic association between *seg* and *sei*, and a wide distribution of these two genes among the *S. aureus* strains. Thus, extensive studies about the *seg* and *sei* genes could be of great interest to investigate the existence of regions for the enterotoxin gene rearrangement in *S. aureus* and the phylogenetic aspects of the staphylococcal enterotoxins.

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대구광역시와 경상북도 지역에서 분리한 *Staphylococcus aureus* 병독소 유전자의 분자적 연구

Multiplex-PCR을 이용하여 대구와 경상북도 내의 다양한 입원 환자들로부터 분리된 187주의 MRSA 균주를 재료로 9가지 종류의 내독소(*sea* ~*see, seg* ~*sej*), 3종류의 병독소(*eta, etb, tst*) 그리고 내부 양성 지표로써 16S rRNA와 *MecA* 유전자를 검출하였다. *S. aureus* 균주에서 새로운 형태의 내독소 유전자(*seg~sej*)의 빈도가 65.9%로 매우 높게 잠복하고 있었으며, 고전적 형태의 내독소 유전자(*sea~see*)도 47.8%로 선행 연구에서 검출된 것만큼 높게 잠복하고 있었다. 새로운 형태의 내독소 중 쌍을 이룬 형태(즉, *sec+seg+sei*, *seg+sei*)는 많이 검출된 반면 단일 형태의 내독소(즉, *seg*, *seh*, *sei*, *sej*)는 거의 검출되지 않았거나 없었으며, 쌍을 이룬 내독소 유전자를 가진 *S. aureus*는 잠재적으로 보다 더 독성균주가 될 것으로 판단된다. 더 나아가 *S. aureus* 균주들 사이의 높은 보유율을 보이는 *seg*와 *sei* 사이의 체계적인 관련성은 staphylococcal enterotoxin들 사이에 중요한 계통발생적 연계가 있을 수 있다는 것은 암시한다.