Prevalence and Distribution of Superantigen Toxin Genes in Clinical Community Isolates of *Staphylococcus Aureus*  

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Introduction

*Staphylococcus aureus* is a major cause of multiple types of infections both in and outside of the hospital setting. These infections range from superficial skin infections to deeper infections of hair follicles, abscesses, and deep tissue infections, and even to systemic infections including those of the heart, lungs, bones, and blood [1].

The organism has an array of cell-surface and secreted virulence factors that allow it to cause illnesses [1]: the surface virulence factors allow *S. aureus* to colonize the host, through adhesion to mucosal surfaces and resistance to phagocytosis, the secreted factors, including exoenzymes and exotoxins, allow the organism to interfere with normal immune system function, spread into surrounding tissues, and access nutrients through cell damage. Among the secreted virulence factors that have known roles in serious human diseases, are the staphylococcal superantigens (SAgs) [2,3]. They include toxic shock syndrome toxin-1 (TSST-1) [4], staphylococcal enterotoxin (SE) serotypes A, B, Cn (in which n denotes that multiple variant forms exist), D, E, G, H, I, S. haemolyticus toxin-1 (TSST-1) [4], staphylococcal enterotoxin (SE) serotypes A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T and SE-like (SEl) serotypes J, K, L, M, N, O, P, Q, U, V [5-7]. These toxins are considered to be major virulence factors of *S. aureus* [3,5]. In addition, they exhibited superantigen activity, stimulating polyclonal T-cell proliferation through cilliation between major histocompatibility complex class II molecules on antigen-presenting cells (APC) and the variable portion of the T-cell antigen receptor β chain or α chain (TCR Vß and TCR Va, respectively), with no need for prior APC processing [2,8,9]. On the other hand, most of genes encoding these toxins are located on mobile genetic elements, such as bacteriophages, pathogenicity islands (SaPIs), genomic islands, and plasmids. This association implies a horizontal transfer of the SAg toxin genes between staphylococcal strains and an important role in the evolution of *S. aureus* as a pathogen. [10-14].

However, expression of most virulence genes in *S. aureus* is controlled by the accessory gene regulator (*agr*) locus. The *agr* locus consists of two divergent transcription units driven by promoters P2 and P3. The P2 operon encodes a two component signalling module, AgrC is the receptor and AgrA is the response regulator. It also encodes two proteins, AgrB and D, which combine to produce and secrete an autoinducing peptide (AIP) that is the ligand for AgrC. AgrA functions to activate transcription from its own promoter and from the agrP3 promoter, which drives the synthesis of RNAIII, the effector of target gene regulation [15]. Sequence variation in agrB, agrD and agrC has led to the identification of at least four *S. aureus agr* specificity groups (I to IV) [16]. Furthermore, the Agr system has been assigned a central role in *S. aureus* pathogenesis [13,17,18].

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Little information is available about the degree of superantigen genetic variability among populations of community clinical Staphylococcus aureus in Morocco. For this reason, the purpose of the present study was to investigate the presence of the staphylococcal superantigen toxin genes in a group of S. aureus isolates in the city of Casablanca (Morocco) and to correlate them with their origin, and agr groups.

Materials and Methods

Bacterial isolates

Non-duplicate community S. aureus strains from clinical specimens were collected from outpatients of the Laboratory of Microbiology, Institute Pasteur of Morocco, and from 14 clinical laboratories located in Casablanca, between January 2007 and October 2008. Patients were assessed as to whether they had previously been hospitalised for a medical condition.

Identification of S. aureus isolates

Species were identified by colony morphology, gram staining, catalase test, coagulase activity on rabbit plasma (Bio-Mérieux, Marcy l’Etoile, France), and production of clumping factor (Pastorex Plus Staph, Bio-Rad, Marnes-la-Coquette, France).

Antimicrobial resistance

Antimicrobial resistance to penicillin G, kanamycin, tobramycin, gentamicin, tetracycline, erythromycin, lincomycin, pristinamycin, chloramphenicol, pefloxacin, fosfomycin, cefoxitin, fusidic acid, rifampicin, vancomycin and trimethoprim-sulfamethoxazole was determined by the standard disc (Bio-Rad, Marnes-la-Coquette, France) diffusion technique. Results were interpreted according to the Committee for Antimicrobial Testing of the French Society of Microbiology guidelines (http://www.sfm.asso.fr) (Comité de l’Antibiogramme de la Société Française de Microbiologie, 2007). S. aureus ATCC 29213 and S. aureus ATCC 25923 were used as quality control organisms.

DNA extraction

All isolates of S. aureus were grown in brain heart infusion media at 37°C overnight. Their genomic DNA used for polymerase chain reaction (PCR) was extracted by using a standard phenol-chloroform procedure as described by Sambrook et al., [19]. Amplification of nuc gene which encodes an extracellular thermo stable nuclease of S. aureus was used, as described by Brakstad et al., [20], for identification and to confirm the quality of each DNA extract. Strains with phenotypic resistance to methicillin were confirmed by polymerase chain reaction detection of the mecA gene as described by Vannuffel et al. [21]. Two reference strains: U2A1593 for methicillin-resistant S. aureus (MRSA) and U2A1594 for methicillin-sensitive S. aureus for (MSSA) from the Antibiological Agents Unit of Pasteur Institute, Paris, France, were used as controls.

Detection of staphylococcal toxin genes

Several Multiplex PCRs for the parallel detection of the presence of the following genes were performed: the classical staphylococcal enterotoxins (sea, seb, sec and sed), SEs and SEs (seh, selk, sel, sem, selo, selp, selq and ser) and the toxic shock syndrome toxin-1 (tst) ([16,22]. S. aureus ATCC19095 (sec, seh, sel, seq, sei, sem, selo and sei); FR913 (sec, sei, see, selk, selq and tst); ATCC14458 (seb); were used as positive control strains. Control chromosomal DNA samples for sed, ser and selp genes were obtained from our standard laboratory controls.

Determination of agr groups

A multiplex PCR of the agr was used to determine the agr group [I-IV] [23]. S. aureus strains RN6390 (agr group I), RN6607 (agr group II), RN4845 (agr group III), and RN4850 (agr group IV) from the National Research Center of Lyon (CNR-Lyon, France) were used for agr group identification.

Statistical analysis

Chi-square test was used to study the correlation between the prevalence of the SAg toxin genes and the agr group of S. aureus isolates on one hand, and the prevalence of these SAg toxin genes and the origin of S. aureus isolates on the other hand. Statistical analysis of the data was performed on SPSS. P < 0.05 was considered statistically significant.

Table 1: Combination of SAg genes in clinical isolates of S. aureus according to their agr group (n=140).
isolates were collected from clinical sources: (n=140).

<table>
<thead>
<tr>
<th>Genes</th>
<th>No. (%) of isolates from:</th>
<th>Total (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>genital-urinary tract</td>
<td>(n=97)</td>
<td>pus/wound (n=52)</td>
<td>sputum (n=16)</td>
</tr>
<tr>
<td>sea</td>
<td>2 (3.5)</td>
<td>1 (1.9)</td>
<td>-</td>
</tr>
<tr>
<td>seb</td>
<td>4 (7.0)</td>
<td>3 (5.8)</td>
<td>1 (6.3)</td>
</tr>
<tr>
<td>sec</td>
<td>8 (14.0)</td>
<td>9 (17.3)</td>
<td>3 (18.9)</td>
</tr>
<tr>
<td>sed</td>
<td>-</td>
<td>2 (3.8)</td>
<td>-</td>
</tr>
<tr>
<td>seh</td>
<td>7 (12.3)</td>
<td>10 (19.2)</td>
<td>2 (12.6)</td>
</tr>
<tr>
<td>sek</td>
<td>2 (3.5)</td>
<td>9 (17.3)*</td>
<td>2 (12.6)</td>
</tr>
<tr>
<td>sel</td>
<td>8 (10.5)</td>
<td>6 (11.5)</td>
<td>2 (12.6)</td>
</tr>
<tr>
<td>sem</td>
<td>22 (38.6)</td>
<td>23 (44.2)</td>
<td>9 (56.3)</td>
</tr>
<tr>
<td>seo</td>
<td>27 (47.7)</td>
<td>32 (61.5)</td>
<td>9 (56.3)</td>
</tr>
<tr>
<td>sep</td>
<td>8 (14.0)</td>
<td>3 (5.8)</td>
<td>1 (6.3)</td>
</tr>
<tr>
<td>seq</td>
<td>2 (3.5)</td>
<td>8 (15.4)*</td>
<td>1 (6.3)</td>
</tr>
<tr>
<td>ser</td>
<td>1 (1.75)</td>
<td>3 (5.8)</td>
<td>-</td>
</tr>
<tr>
<td>tst</td>
<td>6 (10.5)</td>
<td>5 (9.6)</td>
<td>2 (12.6)</td>
</tr>
</tbody>
</table>

N/B: (*) P<0.05
(1) urine (n = 30), vaginal and high vaginal swabs (n = 10), urethral swabs (n =8), sperm (n =9)
(2): nose (n = 5); pharynx (n = 8)

Table 2: Incidence of SAg genes in S. aureus isolates from various sources.

Prevalence of SAg toxin genes

All of 140 S. aureus isolates under study harboured the nuc gene. The all 13 SAg toxin genes analysed in this study were detected; the prevalence were variable from 53.6% to 1.4% (selo 53.6%, selm 43.6%, sec 17.1%, seh 14.3%, sell 12.9%, tst 12.1%, seko 10.7%, seko 9.3%, selp 8.6%, selp 5.7%, ser 2.9%, sea 2.1% and sed 1.4%). However no virulence toxin gene was diagnosed as positive in 33 (23.6%) isolates. Whereas 1 gene was detected in 18 (12.8%) strains, in all remaining isolates (63.6%) at least two SAg genes were present in the same isolate: Forty isolates carried two genes simultaneously, twenty-six isolates contained three genes, nineteen isolates were found with four genes and in four isolates five SAg genes were detected (Table 1). A total of 43 superantigen toxin genotypes were found, the toxin genotype with the highest incidence was selm+selo (20%). This pair was detected in 57 of all isolates.

Distribution of SAg genes in S. aureus isolates from various sources

Regarding the origin of S. aureus isolates, two major locations could be distinguished: genital-urinary tract (40.7%), most of isolates from this location were from urine (n = 30), the others were from vaginal and high vaginal swabs (n = 10), urethral swabs (n =8) and sperm (n =9), the second major location was pus/wound (37.1%). In addition, two minor locations were noted: sputum (11.4%) and naso-pharynx (9.3%), strains isolated from blood were scarcely represented (3.1%) (Table 2). The most abundant genes (selo and selm) were found in 61.5% and 44.2% of pus isolates, respectively; 47.7% and 38.6% of genital-urinary tract isolates, respectively; 56.3% of respiratory tract isolates, 46.2% of naso-pharynx isolates and 50 % of blood isolates (Table 2).
Distribution of SAg genes by agr group

All of S. aureus isolates were classified according to the four agr groups. 47.2% strains were found to belong to agr group I, 20.7% were agr group II, 30.0% were agr group III and only 2.1% of isolates were found to belong to agr group IV. In contrast to agr group I or II S. aureus isolates, all agr group III strains, were positive for at least one of the tested genes (Table 1 and Table 3). A total of 22 genotypes were detected among agr group III S. aureus isolates and 16.7%, 33.3%, 28.6%, 19.0% and 2.4% of these strains contained one, two, three, four and five SAg toxin genes, respectively. Nineteen SAg genotypes were observed among agr group I strains, against 10 found among agr group II isolates. On the other hand, 13.7%, 22.7%, 12.1%, 13.6% and 3% of agr group I isolates carried one, two, three, four and five SAg toxin genes, respectively. The most abundant SAg toxin gene (selo) was carried by 50.0%, 55.2% and 54.8% of isolates belonging to agr group I, II and III, respectively. The second abundant SAg toxin gene, namely sem, was carried by agr group I strains with 43.9%, agr group II with 55.2% and agr group III with 31.0%.

Distribution of agr group S. aureus isolates by origin of samples

The relationship between agr group S. aureus strains and source of isolates is summarized in (Table 4).

Discussion

Staphylococcus aureus isolates collected during the present study were originated from ambulatory patients, they were collected from 15 clinical laboratories located in Casablanca; therefore, they should represent randomly selected strains. In fact, a significant number were recovered from vaginal swabs, high vaginal swabs and urine. Isolates from high vaginal swabs are usually associated with puerperal sepsis or neglected foreign bodies, and less frequently with bacterial vaginosis, and significant numbers of MRSA organisms have been found among these isolates [24,25]. While it is likely that many S. aureus organisms might be passive colonizers or due to contamination from the skin, some are associated with complications of pregnancy [24]. Although S. aureus is a rare cause of urinary tract infections, accounting for only 0.5% to 6% of all positive urine cultures [26,27], their finding is increasingly being recognized as significant, especially in patients with urinary tract catheterisation, as under-treatment or delayed treatment could lead to development of staphylococcal bacteremia [26,28,29].

It is important to discuss, even briefly, the susceptibility to the tested antibiotics of all S. aureus isolates. This study provides important data on current antimicrobial resistance, including methicillin-resistance, for a collection of 140 recent clinical isolates of S. aureus from community source in Casablanca, Morocco. We found that 90% of isolates possessed resistance to penicillin, followed by strains with resistance to one or two more antimicrobial substances. Resistance to methicillin conferred by carriage of the mecA gene is rather low; it is found with only two agr group I isolates (1.43%). Epidemiological data of community-acquired methicillin-resistant S. aureus (CA-MRSA) in the countries of the Maghreb, i.e. Morocco, Algeria, Tunisia and Libya are scarce; although, recent reports suggest that Hospital-acquired MRSA epidemiology is changing, with a dramatic increase of incidences [30,31].

On the other hand, it is hypothesized that the evolution of CA-MRSA is a recent event due to the acquisition of mec DNA by previously methicillin-susceptible strains that circulated in the community [32]. Then, considering the relationship that might exist between MRSA and MSSA isolates, and the limited data on the prevalence and distribution of SAg toxin genes among S. aureus isolates in Morocco; we need more specific knowledge about the circulating S. aureus isolates, especially MSSA strains.

In a first step, we found that the overall rate of SAg toxin gene-positive isolates reached 76.4%. This is in agreement with results published by Hu et al., [33], they have reported that 75.7 % of the MSSA isolates tested carried a number of toxin genes, ranging from 1 to 11, with extensive variation between individual strains. In a second step, we found that differences in the occurrence of genes between pus/wound versus the genital-urinary tract, sputum or naso-pharynx isolates were not significant as demonstrated by the Chi-square test (P>0.05); with exception for selk and selq genes, which were slightly frequent in pus/wound S. aureus isolates (P<0.05). In a third step, we found that 47.2% of all isolates were agr group I, this finding concurs with results of other studies [16,34]. Thereafter, we have analysed the agr group specificity of all strains, and we found that there are many SAg toxin genes in S. aureus isolates belonging to agr group I, II, III and/or IV from many clinical specimens. However this distribution was not uniform among all agr groups isolates; we found that agr group III S. aureus isolates (n=42) carried more SAg toxin genes (mean 2.55 gene/isolate), compared to 66 S. aureus isolates in agr I and 29 S. aureus isolates in agr II (mean 1.65 gene/isolate). Secondly, we found that all agr group III isolates were positive for at least one of the tested virulence genes, whereas 23 and 10 strains among agr group I and agr group II isolates, respectively, had none SAg toxin genes. Thirdly, our finding showed that 50% of agr group III isolates were found with 3 to 5 virulence toxin genes, versus, only 28.9% and 27.6% of agr group I and II isolates, respectively. From these data, we can deduct that agr group III isolates were more prevalent for the presence of enterotoxin and/or tst genes than agr groups I and II isolates.

However, agr group I was prevalent in all clinical specimens whatever their origin, nevertheless it was slightly higher in genital-urinary tract samples than others. Whereas, both agr groups II and III were dominant in S. aureus isolates from pus/wound specimen. These differences were not statistically significant.

Most of virulence toxin genes under study are associated with mobile genetic elements. However, the repertoire of toxin genes encoded by SaPIs seems to be specified. These elements, as well as the majority of genetic elements encoding enterotoxins, can be horizontally transferred among S. aureus strains. But, unlike plasmids, they cannot spread autonomously. It was shown that in the presence of certain staphylococcal phages, SaPIs are excised from the genome and encapsidated. This mechanism in thought to be responsible for the transfer of pathogenicity islands [13]. For this reason the prophage ϕM u50A and ϕN315, shown to be integrated in close proximity to the TSS1-1 pathogenicity island family of S. aureus Mu50 and N315, respectively, are considered to be involved in the horizontal transfer of these SaPIs [35]. These data led us to define the prevalence of each of the mobile genetic elements among all 140 S. aureus isolates under study.

In the current work, in agreement with other [22], selm and selo genes were more frequently detected in all isolates, whatever their origin and their agr group. Both genes belong to the recently described enterotoxin gene cluster (egt) that harbours 5 to 6 genes (seg, sei, selm, selo, and sometimes selh), which cluster on a staphylococcal pathogenicity island type I γSAβ [33,36,37], this cluster will be found without all of the

each component [33,38-40]. Furthermore, many studies reported that some isolates contained selo gene with sed, selj and ser genes instead of the egc genes [40-42]; in fact, Thomas et al. [42] suggested that this phenomenon is caused when a part of egc is expressed or inserted in the chromosome of other S. aureus strains intermediated by insertion sequences.

Our data on the frequency of sec and sell genes are in agreement with those previously reported [33,43,44]. It is also notable that, in the presence work, sell gene (n = 18) was accompanied by sec gene in 14 agr group I S. aureus cases. This finding agrees with the fact that both genes were demonstrated to occur together on the staphylococcal pathogenicity islands SaPIm1 and SaPIn1 [35]. However, in one S. aureus isolate, sell gene was found together with sec and tst genes, these genes are associated with another mobile genetic element, named type I SaP14 [33]. Furthermore, we have noted that sec and sell genes were also detected, but not together, in fourteen S. aureus isolates. Our data suggests that the staphylococci may carry other, yet unknown, pathogenic island.

The tst gene is carried by a family of closely related pathogenicity islands that interact in highly specific way with certain staphylococcal phages. This gene encodes for TSST-1, which is considered to be the cause of nearly all cases of menstrual TSS, and of at least 50% of nonmenstrual cases [3,45]. About 12% of our S. aureus isolates were tst-positive, however, in contrast to what has been reported by Ji et al., [46], distribution of this virulence gene by agr groups provide evidence that tst was more frequently coupled to agr group III and was frequently found in combination with selm and/or selo genes, this finding is in agreement with a previous observation [32,39].

On one hand, it has been known that, unlike the horizontal transfer of virulence genes, such as tst, most of the toxin genes that did not demonstrate this mobility type belonged to the egc, which is restricted to the clonal complexes CC5 (characterized by agr group II) and CC30 genomic background [47]. On the other hand, since most of tst + sem and/or seo positive isolates were agr III, we assume that tst-selm and/or selo positive genes were CC30.

The prevalence of seh gene, which is rather strictly linked to CC1 [47], is highly variable. It was not detected by Sila et al., [43]; Peck et al., [48] have reported that seh was more frequently detected in isolates from nasal than from blood specimens; El-Huneidi et al., [49] have reported that this SAg toxin gene was only infrequently detected in clinical isolates of S. aureus from Jordan; Hu et al., [33] have shown that 11/140 MSSA isolates were positive for this toxin gene. Recently, in Kuwait City, Udo et al., [34] reported that staphylococcal enterotoxin gene H was found with a frequency of 21.5% among strains, but not all agr group III S. aureus isolates. Compared to other enterotoxins, seh gene is non-mobile, so that clonal complex (CC1) affiliation does matter.

Two of all genes which were found prevalent from pus/wound S. aureus isolates (selk, selq) were found without other toxin genes, whereas one S. aureus strain was found with seh+selk+selq+seh genes, and another was found with sea+selk+selq+seh genes. It was demonstrated that seh+selk+selq and sea+selk+selq genes are carried by SaP13 [50] and staphylococcal phage \( \phi 3 \) in S. aureus MW2 [14], respectively.

The selp gene that is encoded on prophage \( \phi N315 \), was shown to be linked to pathogenicity islands containing the sec and sell genes, such as SaPIm1/SaPIn1 [35]. Two out of selp-positive isolates were found to be negative for other enterotoxin genes and one carried sec without sell gene. Interestingly, all of sec-sell positive strains were found to be selp negative; thus the linkage between these genes does not seem obvious.

Other, such as sea and seb, were present significantly less frequently. The fourth classical enterotoxin gene, named sed, was detected in 2 (1.4%) pus agr group II isolates, in both cases, this virulence gene was accompanied by ser gene, with selm and selo. It was reported that the coexistence of sed, sel and ser genes are commonly found on plasmid pIB485 [51,52]. However, ser gene was also found in two other agr group III S. aureus isolates (isolated from pus and urinary tract infections), without carrying the sed gene.

Conclusions

Despite the fact that the agr group I was the most prevalent group, among 140 community S. aureus isolates, in Casablanca, Morocco, strains belonging to the agr group III harbour more virulent superantigen toxin genes. So, we can assume that agr group III isolates may carry more enterotoxin and/or tst genes than agr groups I and II isolates. Of all thirteen SAg toxin genes, two were detected more frequently in all agr group isolates, whatever their origin: selo and selm.

Statistical analysis of the comparison of the prevalence of SAg toxin genes in the four studied agr group isolates (agr group I, II, III and VI), on one hand, and the prevalence of these virulence toxin genes according to the source of isolates, on the other hand, using the chi-square test, showed that agr group III and agr group I S. aureus isolates, whatever their origin, were more prevalent for the presence of seh, selq, selk and/or tst virulence toxin genes and sec and/or sell virulence toxin genes (P<0.05%), respectively. Finally, it is also particularly noteworthy that, selk-positive and selq-positive isolates, were highly prevalent from pus/wound (P<0.05%).

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