Detection of toxin-associated genes in seven *spa*-types of *Staphylococcus aureus* in Iraq

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Abstract

Background: Staphylococci frequently cause opportunistic infections in patients with underlying disease, such as those with prosthetic devices, surgical patients, and patients with diabetes or individuals undergoing dialysis. Consequently, detecting the toxin-associated genes in different *S. aureus* strains, isolated from patients with wound and burn injuries and their environments, is essential to monitor and prevent the complication in such cases.

Methods: Identification of *S. aureus* that isolated from burn and wound injuries has been done according to the biochemical tests, then *Spa*-typing method was used to diagnose at the strain level. Toxin-associated genes, enterotoxin gene(*sea*, *seb*),exfoliative gene(*eta*), toxic shock syndrome gene(*tst*), and pantone-valentine leukocidin (*pvl*), were detected by polymerase chain reaction (PCR) method.

Results: Two isolates were harboring *tst* and a single isolate harbored *pvl*, at the time that no isolates were found *eta* and *seb* positive among 19 *S.aureus*. While *sea* was the predominant toxin-associate gene in the studied *S. aureus* strains, it was detected in 13 isolates.

Conclusion: Distribution of specific *spa*-type/*sea*-positive in diabetic foot ulcers at hospitals, indicating the poor sanitization conditions at Iraqi wound wards, and it is recommended to take swabs from the patients and their environments as a routine procedure.

Keywords: *sea, pvl, tst, spa-type, Iraq, wound*

Introduction

S. aureus is a pathogen that can cause various infections which can be divided into three types: (i) toxinoses such as scalded skin syndrome, food poisoning and toxic shock syndrome (ii) surface lesions like wound infection, and (iii) systemic as well as cases with life-threatening such as endocarditis, pneumonia, osteomyelitis, brain abscesses, bacteremia, and meningitis [1, 2].

S.aureus pathogenicity is associated with a variety of virulence factors that allow the organism adherence, prevent the immune system and cause harm to the host. *S. aureus* has a wealth of pathogenic determinants that facilitate colonization of tissues, tissue damage and distant diseases [3]. Notably, there is no immunity defense against *S. aureus*, therefore, previous infections by *S. aureus* does not protect a person from subsequent infections [4].

Several important secreted toxins were produced by *S. aureus* usually during the post-exponential and early-stationary phase of growth[5].

PVL is a pore-forming cytolytictoxin; it is encoded by two co-transcribing genes, *lukS-PV* and *lukF-PV* which are components of the phage genome inserted into bacterial chromosome. The majority of the *pvl* positive was obtained from pus specimens whereas, a lower

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percentage of remaining clinical sample types carried *pvl* gene. However, among the hospital environment samples, none of the methicillin-resistant *S. aureus* isolates was found *pvl* positive [3]. The production of PVL is considered as the most intensive way to distinguish community-associated *S. aureus* from hospital-associated *S. aureus*, it is present in the majority of community-associated *S. aureus*isolates and rarely present in hospital-associated *S. aureus* (*pvl* genes are found in approximately 2% of clinical *S. aureus* isolates) hence, it is recognized as a marker of the community strains [3, 6-8].

About 30% to 50% of *S. aureus* isolates are producing Enterotoxins[9]. Different enterotoxins have been described and traditionally subdivided, and they are arranged chronologically from SEA to SEE (classical types) and SEG to SEU (new types) [10] except SEF which is later re-named as toxic shock syndrome toxin 1 (TSST-1) [11]. SEs are encoded by mobile genetic elements, they are considered the major cause of food poisoning, they predominantly induce enteritis characterized by emesis, while diarrhea is not always shown in SE-mediated intoxications. Furthermore, these toxins possess superantigenic activity [12, 13].

TSST-1 is a chromosomal-mediated toxin, causes nearly all cases of menstruating-associated Toxic shock syndrome (TSS), also it has been associated with 50% of the non-menstruating associated TSS cases such as surgical wound complications and localized infection at the skin or respiratory tract [9, 14, 15].

At least four *S. aureus* exfoliative toxins (ETA through ETD) were identified. ETA involved in staphylococcal scalded skin syndrome, and it is encoded by prophage ETA, ETA2, and ETA3[16, 17].

This study aimed to monitor the spreading of *S. aureus* carrying toxin associated genes at Iraqi hospitals Therefore, *S. aureus* was isolated and genotyped through *spa*-typing method then characterized each *spa*-type according to their genes (*tst*,*pvl*, *eta*, *sea*, and *seb*) that encoded extracellular toxins.

Material and Methods

Bacterial Isolation and Identification

One hundred Burn, wound, and environmental samples had been swabbed then cultured on a selective medium (Mannitol salt agar) that differentiate *Staphylococcus aureus* through the fermentation of mannitol and formation of acidic products which reduce the pH of the medium, and this will turn the phenol red indicator to yellow [18]. The microscopic examination by Gram stain was performed for the isolates that ferment mannitol of Mannitol salt agar. Only the Gram-positive grape-like clusters were preserved at Nutrient agar slants at the refrigerator (4℃) to complete the tests of *S. aureus* identification.

A biochemical catalase test was then performed, to indicate catalase enzyme through adding one drop of (3%) H_2O_2 on a glass slide then adding bacterial colony on it, bubbles will then appear as a positive result [18].Another biochemical test was performed to detect another Staphylococcal enzyme through a tube coagulase test, using human plasma at the dilution (1:5) with autoclaved distilled-water; the plasma will be added to an equal volume of cultured Nutrient broth. Coagulase enzyme will clot plasma after 2 to 4 hours of incubation at 35℃ and sometimes the clot will appear after overnight incubation [19, 20].

Extraction of *S. aureus* DNA and preparation of PCR mixture

Bacterial DNA was isolated by using Wizard Genomic DNA Purification Kit/Promega. The concentration value of DNA was detected using Quantus Fluorometer by mixing 199 µl of Quanty Flour diluted dye with 1 µl of extracted DNA, then incubated at room temperature for 5min, to investigatethe goodness of samples and perform the downstream applications.

PCR amplification was done through preparing the primers in final concentration (100 pmol/µl)as a stock solution by adding nuclease-free water to the primer, according to the manufacturing company information then stored in the deep freeze.

In order to use this diluted stock solution in PCR mixture, it must be diluted to get 10 pmol/ µl as a final concentration by adding 10 µl from the original stock solution to 90 µl of deionized distal water and stored in the deep freeze until its usage in the PCR mixture.Final

volume 20 µl of PCR mixture was prepared (Master mix 12.5 µl, forward and reverse 1µl for each, nuclease-free water 7.5µl, and DNA 3µl), then short spin by microcentrifuge.

Spa- typing of *S. aureus*

Spa protein in the cell wall of staphylococcus is considered an important virulence factor because it binds to the Fc portion of IgG through a region that consisted of four to five tandem repeats (E, D, A, B, and C) which are followed by a polymorphic region (X region). The X region is consisting of X_R (a region of repeats), and another region called X_C that have LPXTG-binding motif to anchor the cell wall [21].

Detection of *spa* gene by PCR was the first step in genotyping,by amplifying the X region, and a single amplicon will yield for each isolate.*Spa*-typingwas done by using the primer *spa*-1095F and *spa*-1517R [22, 23].

The DNA have been amplified, by using Thermal Cycler System,according to the following program: Initial denaturation for 4 min at 95℃ for 1 cycle, Denaturation for 30 Sec at 95℃ for 30 cycles, Annealing for 45 Sec at 50℃ for 30 cycles, Extension for 45 Sec at 72℃ for 30 cycles, Final extension for 7 min at 72℃ for 1 cycle, and then Hold for 10 min at 10℃ for 1 cycle. To confirm the presence of amplified DNA by PCR, agarose gel electrophoresis was adopted.

The PCR products of the reverse primer 1517R were sent to Macrogen Corporation in Korea, and the results of sequencing *spa*amplicons were received through E-mail, and then analyzed by BioNumerics software in order to genotype *S. aureus* isolates and identify their *spa*-types.

Detection of *S. aureus* toxin-genes by PCR

All*S. aureus* isolates were tested for the presence of TSST, ETA, SEA, SEB and PVL toxins by using the primers in (table 2) according to PCR programs given in (table 1). The PCR product will finally be loaded into Agarose gel.

Table 1: PCR programs for *pvl*, *tst*, *eta*, *sea* and *seb* Staphylococcal genes [24, 25]

Steps	˚C	Min:Sec	Cycle
Initial denaturation	95	05:00	1
Denaturation	95	00:30	35
Annealing	56	00:45	
Extension	72	0045	
Final extension	72	07:00	1
Hold	10	10:00	

Table (2): Primers

Primers	Sequence (5′- 3′)	Size of amplified product(bp)	Referance
sea-F sea-R	GGTTATCAATGTGCGGGTGG CGGCACTTTTTTCTCTTCGG	102	(24)
seb-F seb-R	GTATGGTGGTGTAACTGAGC CCAAATAGTGACGAGTTAGG	164	
eta-F eta-R	GCAGGTGTTGATTTAGCATT AGATGTCCCTATTTTTGCTG	93	
tst-F tst-R	ACCCCTGTTCCCTTATCATC TTTTCAGTATTTGTAACGCC	326	
Luk-pvl-F Luk-pvl-R	ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A GCA TCA AGT GTA TTG GAT AGC AAA AGC	433	(3, 26)

Results and Discussion

Identification of *spa*-types

Five different sized ampliconshave been observed in the present study (200, 240, 300, 380, 440 bp), as shown in figure 1.

A chromatogram had been displayed and read by MEGA-X software then manually edited the misinterpreted nucleotide base, in order to prepare these sequences for typing by Bionumeric software.

Typing and recognizing *spa*-repeats was done by Bionumerics software. The following *spa*-types were identified: (n=10; t037, n=3; t304, n=1; t386; t223; t005; t13157, n=2; t14870). Genotypes of *S. aureus* are displayed in table 3.

Table 3: distribution of *spa*-types at hospitals and clinical communities according to Ridom SpaServer

The code of the Isolate	No. of repeats	Size of Spa-gene (bp)	Spa-type	Site of isolation	
18	7	300	t037	D.F	Outpatients at the clinics
15	12	440	t005	Out patient with burn blister containing pus	
16	9	380	t304	D.F	
17					
57	4	240	t14870		

Cont... **Table 3: distribution of *spa*-types at hospitals and clinical communities according to Ridom SpaServer**

22	4	240	t14870	Clean surface of nursing cart	Hospital environment
19	7	300	t037	Medical waste at nursing cart	
5	9	380	t304	bullet injury	Wound samples
2	7	300	t037	D.F	
3					
6					
8					
9					
4	11	440	t223	D.F	
10	3	200	t386	After stabbing wound and surgery to the bladder	
13	7	300	t13157	Burn	injuries
11	7	300	t037		
12					
14					

D.F: Diabetic foot ulcer

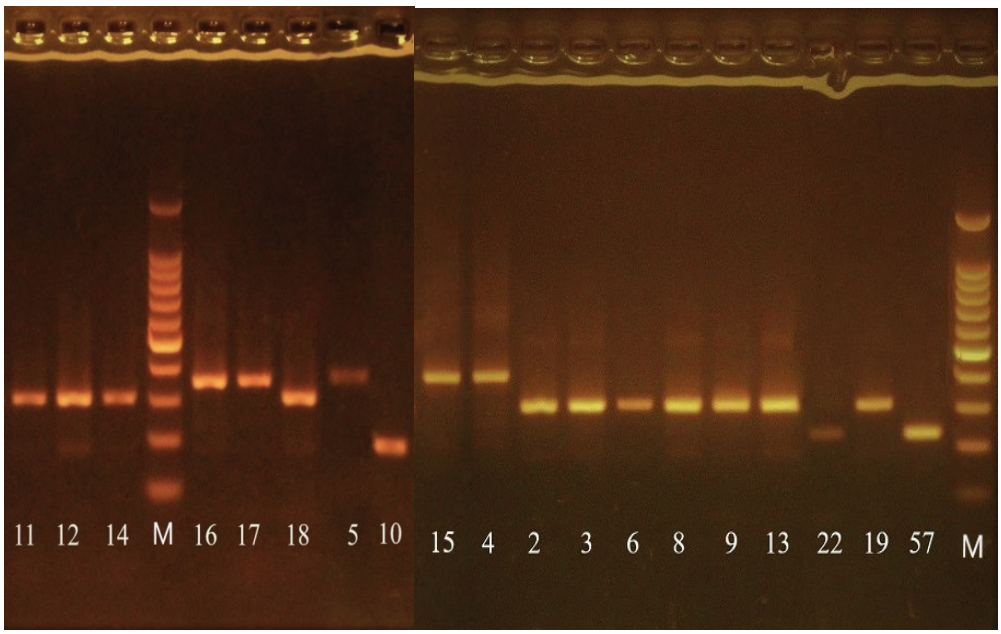

Figure 1: Agarose gel-electrophoresis showing M; marker (100bp), and lanes: 11, 12, 14, 18, 19, 13, 9, 8, 6, 3, 2 (300bp); 16, 17, 5 (380bp); 15, 4 (440bp); 22, 57 (240bp); 10 (200bp)

The presence of toxin-associated genes in different strains of *S. aureus*

In this study, the *S. aureus* in diabetic foot ulcers has been recorded as the highest percentage (n=10, 52%) among other types of injuries.All the ten isolates were carrying toxin gene. (8 isolates *sea*-positive, one with *tst*, and one with both *sea* and *tst*), 6 were *spa*-type t037, single isolate was t14870 and one was t304 which have only *sea*, while isolate that harbors *tst* was t223 and the one that harbors both *tst* and *sea* was t304 (table 4).

It is important to detect *pvl*, since PVL toxin can cause a high mortality rate up to 75%. One case that represents 5.2% of *S.aureus* isolates was harboring *pvl* gene and it had been collected froman outpatient at a clinic, and the strain was identified as *spa*-type t005. Makgotlho *et al*. have found 4% of the collected *S. aureus* at a hospital are carrying *pvl* gene and this percentage was not far from that mentioned above [27]. In Kuwait *pvl* gene was also detected in t005 strain from clinical samples [28].Moreover, In Iran, 0.8% of the *pvl*-carrying strains was t005 isolated from wound infections[29]

No isolates in injuries or environmental samples were carrying *eta* gene, this was in agreement with that found in Algeria [30] as well as in Palestine, Hadyeh*et al*. isolated different *spa*-types and they found that strains t037, t304, t005, t223, and t386 were negative to ETA [31]. While locally in Baghdad, Kandala and her colleagues found 11.62% of *S. aureus* isolates were harboring this gene [32]this may be due to the varieties in the clinical samples collected in their study.

In this study 10.5% is a low percentage of *S. aureus* that harbored *tst* gene (2 out of 19 isolates) which are t304 and t223. This low percentage was also noticed locally at Baghdad hospitals in 2017 where *tst* was representing 18.60% in *S. aureus* isolated from different clinical samples [32]. Strain t223 that isolated from clinical samples was harboring *tst* gene according to the studies in Jordan, Kuwait, Palestine and Iran[28, 31, 33, 34]. Another study agreed with the present results, were conducted in Kuwait on *S. aureus* from different clinical samples isolated between 1992 and 2010, had detected *tst* in strain t223, and *sea* in some t304 isolates. However they didn't detect *sea* in t037 [35] unlike our study in which *sea* was noticed in all (n=10) t037 strains, this may be due to the differences in strain's virulence according to different geographic area.

Table 4: The presence of toxin-associated genes in different *spa*-types of *S. aureus*

Number of isolates	The site of isolation	Spa-type	Toxin gene
10	6 D.F	t037	sea
	1 Medical waste at nursing cart		
	3 Burn injuries		
1	Burn injuries	t13157	*ND
1	Clean surface of nursing cart	t14870	*ND
1	D.F		sea
1	Out patient with burn blister containing pus	t005	pvl
1	D.F	t223	tst
1	After stabbing wound and surgery to the bladder	t386	*ND
1	bullet injury	t304	*ND
1	D.F	t304	sea+tst
1	D.F	t304	sea

*ND: Toxin genes are not detectable, D.F: diabetic foot ulcer

Based on the data reported in a review, a research conducted in 2009 had detected *sea* with low percentage (12%) among *S.aureus* isolated from clinical samples[36]. Furthermore, in another research none of clinical samples, including wounds, were *sea* positive [30]. The disagreement of these results with the current study maybe due to the site of *S.aureus* collection, sincemost of the isolates in this study where from diabetic foot ulcers (n=9 out of 12). *Sea* is known to be the most virulence genes that predicted in the infected diabetic foot ulcers [37].

The gene of Enterotoxin-B (*seb*) was not detected in this study. Nevertheless, 3% of the isolates harbor *seb* according to a study in a review [36]. This may be due to *seb* being infrequently identified in *S. aureus* strains [38].

In Iran, several toxins in *S. aureus* strains have been studied, and by comparing the percentage of the *sea*, *seb*, *pvl*, *eta*, *tst* genes, it was clear that *sea* was the highest, then followed by *tst*, while *seb*, *pvl*, and *eta* were less than the first two toxins [39], this was in agreement with the result of the present study.

Conclusion

The *pvl* which is prevailed in the community-acquired *S. aureus* was not detected in this study among the clinical samples collected from hospitals, this indicates that there is no transmission of *S. aureus* isolates from the community to hospitals. Most of the injuries were contaminated with *S. aureus* t037-*sea* positive, and this maybe due to the widespread of this strain in the hospital environment. It is recommended to take a swab regularly from each patient and its surrounding environment to monitor and control the spreading of bacteria among the patients.

Conflict of Interest: Nil

Source of Funding: Self

Ethical Clearance: Samples were collected from Al-Kindy and Al-Yarmouk teaching hospitals, Baghdad/ Iraq, after administrative approval.

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