

Studies on Enterotoxins and Antimicrobial Resistance in *Staphylococcus aureus* Isolated from Various Sources

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Received 29 February 2016; accepted 10 April 2016; published 13 April 2016

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Abstract

Staphylococcus aureus represents a public health challenge all over the world. Therefore, this study aims to analyze the prevalence of five genes (*sea*, *seb*, *sec*, *see* and *seg*) encoding the staphylococcal enterotoxins in *S. aureus* isolated from different sources and to evaluate the association of these toxins in comparison to susceptibility towards 12 antimicrobials; antimicrobial susceptibility was conducted by disc diffusion method. Detection of staphylococcal enterotoxins was performed by PCR and the ability to express these genes was assessed among isolates by RT-PCR. The most common enterotoxin gene was *sea* gene (66%), followed by *seb*, *sec*, *see* and *seg* (38%, 23%, 19% and 5%) respectively. Expression of *sea*, *seb* and *seg* genes was variable. However, *sec* and *see* genes were not expressed by any of the tested isolates. No statistically significant association exists between (*seb*, *sec* and *see*) and isolation sources, while the *sea* was significantly associated with clinical isolates. High significant correlation was found between elevated *sea* expression and multidrug-resistance. Our findings indicate that the pathogenic potential of *S. aureus* may be greater than previously thought. This emphasizes the utmost need to implement proactive measures and more emphasis will be placed on the application of hygiene practices in hospitals to control *S. aureus* infection and enterotoxins production.

Keywords

S. aureus, Antimicrobial Susceptibility, Enterotoxins, Expression

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1. Introduction

Staphylococcus aureus (*S. aureus*) is a major pathogen of increasing importance due to the rise in antibiotic resistance [1]. It is often found as a commensal associated with skin, skin glands, and mucous membranes, mainly in the nose of healthy individuals [2]. Approximately 20% - 30% of the general populations are *S. aureus* carriers [3].

S. aureus is associated with severe community and hospital acquired diseases and has for a long time been considered as a major problem of public health [4]. Many extracellular proteins and toxins are produced which probably contribute to the virulence of this organism [5]. *S. aureus* can produce exotoxins including exfoliative toxins, leukocidins, toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxins (SEs) [6]. The spectrum of staphylococcal diseases ranges from pimples and furuncles to toxic shock syndrome and sepsis, most of which depend on many virulence factors. In contrast, some diseases, such as staphylococcal food poisoning, rely on only one type of virulence factors: the staphylococcal enterotoxins (SEs) [4].

Staphylococcal food poisoning (SFP) is a very common, however, a rarely reported, type of food poisoning. Symptoms of SFP include rapid onset (within 1 - 6 h) of nausea, vomiting, diarrhea and abdominal cramps [7]. These symptoms subside within 24 - 48 h but the disease may remain for 7 - 10 days [8]. The staphylococcal enterotoxins (SEs), produced by *S. aureus*, are known as etiologic agents of food poisoning in man, and as potent immunologic superantigens [9] that stimulate non-specific T cell proliferation [4]. The staphylococcal enterotoxins are emetic toxins belonging to a large family of staphylococcal and streptococcal pyrogenic exotoxins (PT), having common structure, function phylogenetic relationships and sequence homology [10].

Nowadays, there are 24 types of staphylococcal enterotoxins including staphylococcal enterotoxins (SEs) with demonstrated emetic activity in a primate model (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, SEK, SEL, SEM, SEN, SEO, SEP, SEQ, SER, SES and SET) and staphylococcal-like (SEI) proteins, which are not emetic in a primate model or have yet to be tested (SEIJ, SEIU, SEIU2, SEIV, SEIX and SEIY) [11] [12], yet, SEA-SEE are the most common enterotoxins implicated in SFP [13]. SEA and SEB were found to be the most common staphylococcal enterotoxins. SEA is the most implicated toxin in staphylococcal food poisoning (SFP). For SEB, it has also been associated with food poisoning and was studied for the potential risk of being used as an inhaled bioweapon. SED is the second most common staphylococcal toxin associated with SFP in the world, and minute amounts of this toxin were found enough to induce illness. SEC was the most predominant enterotoxin detected in *S. aureus* strains isolated from mastitis milk in sheep, goats, and cattle. SEE has been reported in few cases of food poisoning [14]. SEG is an example of the non-classical enterotoxins which also show emetic properties [15]. The SEs are resistant to inactivation by gastrointestinal proteases such as pepsin and trypsin and these toxins also show thermal stability [10] making their elimination difficult to achieve [6]. Therefore, detection of SEs in foods is necessary for risk management and surveillance in food safety. Many reports have described the development of molecular methods for the detection of most of the SE genes [16].

For classical staphylococcal enterotoxin detection, the reversed phase latex agglutination (SET-RPLA) and staphylococcal enterotoxin enzyme immuno-assay (SET-EIA) kits, were used to be employed. However, due to the unavailability of immunoassay kits for detecting new SEs, other methods as polymerase chain reaction (PCR) primers, DNA probes and reverse-transcription (RT)-PCR were used for the detection of these toxin genes and their activity in *S. aureus* strains [17].

This study has been undertaken to detect enterotoxin genes in *S. aureus* by PCR, assess their distribution among various clinical and environmental sources and measure their expression levels by RT-PCR. Moreover, as the relationship between resistance and virulence is poorly understood, we tried to find any possible correlation between prevalence and expression of enterotoxins on one hand and antimicrobial susceptibility on the other hand.

2. Materials and Methods

2.1. Study Design

During the period from June 2013 and January 2014, 250 samples were collected from different hospitals. These isolates were obtained from different clinical and environmental sources. One hundred isolates were identified as *S. aureus* using standard microbiological techniques according to [18]. The experimental protocol conducted in the study was approved by the Ethical Committee of faculty of Pharmacy, Mansoura University, Egypt, with code (2013-28) in May 2013.

2.2. Antimicrobial Susceptibility Testing

The antimicrobial susceptibility testing was carried out by disk diffusion method using Mueller-Hinton agar plates (oxoid) according to [19] where 12 antimicrobial agents belonging to different classes were selected including: ampicillin-sulbactam (SAM, 10/10 µg), amoxicillin-clavulanic acid (AMC, 20/10 µg), oxacillin (OX, 1 µg), imipenem (IPM, 10 µg), cephalothin (KF, 30 µg), cefoxitin (FOX, 30 µg), ceftazidime (CAZ, 30 µg), cefepime (FEB, 30 µg), amikacin (AK, 30 µg), tobramycin (TOB, 10 µg), erythromycin (E, 15 µg) and ciprofloxacin (CIP, 5 µg).

Cefoxitin (30 µg) disk was used for detection of methicillin resistant *Staphylococcus aureus* (MRSA), where strains with Inhibition zone diameter ≤ 21 were identified as MRSA [19] [20].

2.3. PCR Detection of Staphylococcal Enterotoxins Genes

PCR amplification of staphylococcal enterotoxins (SE) genes, namely (*sea*, *seb*, *sec*, *see* and *seg*) was performed using a single plex PCR reaction (FPROGO2D, Tche LTD, Oxford Cambridge, UK) and specific primers listed in **Table 1**. Genomic DNA was prepared by picking up one - two colonies grown overnight on nutrient agar medium and resuspended in 100 µl of nuclease free water then heated to 95°C for 10 mins. The toxin genes were detected and amplified using the following reaction: 12.5 µl DreamTaq Green PCR Master Mix (2X) (Thermo scientific), 3 µl of bacterial DNA, 1 µl of each primer (10 µM), and 7.5 µl nuclease free water (Thermo scientific) for a total of 25 µl per reaction.

The PCR program started with an initial denaturation of DNA at 95°C for 5 mins, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at temperatures specified for each primer as listed in **Table 1** for 30 s and extension at 72°C for 1 min. This was followed by a final extension step at 72°C for 5 mins. The amplified genes were visualized on 2% agarose gel electrophoresis stained with ethidium bromide and compared with a 100 base pair (bp) DNA ladder (Thermo scientific).

2.4. RNA Isolation

RNA was isolated from 20 isolates. We selected isolates that harbored 3 or more of the tested toxin genes as detected by PCR (11 isolates), and isolates shared the same resistance and toxin gene pattern (9 isolates). RNA isolation was done according to [22] using glass beads and TRI reagent (Sigma Aldrich).

The concentration and the purity of RNA for each isolate were determined spectrophotometrically using 260 and 260/280 nm ratio respectively, using NanoDrop (ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, Delaware, USA). The purity of RNA was determined by calculating the ratio of A260/A280. Pure RNA has a ratio of 1.8:2.1.

Table 1. Specific amplification primer sets of staphylococcal enterotoxin genes and reference gene (*nuc*).

Gene name	Sequence	Temp.	Amplicon Length (bp)	Reference
<i>sea</i>	F TGCCGATCAATTTATGGCTAGA	62	207	This study
	R CCGAAGTTCTGTAGAAGTATGAA			
<i>seb</i>	F GGACACTAAGTTAGGGAATTATGA	60	200	This study
	R GCTCAGTTACACCACCATAC			
<i>sec</i>	F GGTATGATATGATGCCTGCAC	61	111	This study
	R GGTGGACTTCTATCTTCACACT			
<i>see</i>	F GATCTTCAGGCAAGGCATTATT	61	122	This study
	R TAACTTACCGTGGACCCTTC			
<i>seg</i>	F TGTATGGTGGTGTAACTGAGCA	64	272	This study
	R TGGTGCAGGCATCATGTCATA			
<i>nuc</i>	F GCGATTGATGGTGATACGGTI	55	267	[21]
	R AGCCAAGCCTTGACGAACTAAAGC			

F: forward; R: reverse; Temp: annealing temperature; bp: base pair.

2.5. Expression of SE Genes

Isolated RNA was used for preparation of complementary DNA using Quanti-Tect Reverse Transcription kit (QIAGEN, Germany). RT-PCR was done using 5× FIREPol Eva Green, qPCR Mix, plus no ROX dye (Solis Bio-Dyne, Tartu, Estonia) using primers listed in **Table 1**.

RT-PCR was performed using a Rotor Gene Q thermocycler (QIAGEN, Hilden, Germany) programmed as follows: 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at temperatures specified for each primer as listed in **Table 1** for 30 s and extension at 72°C for 1 min. Expression of the target genes was normalized to the expression of reference gene (nuc) encoding nuclease enzyme. The gene expression level in samples was calculated relative to the housekeeping gene (endogenous reference gene) using a calibrator sample by the comparative ($\Delta\Delta\text{ct}$) method [23]. All measurements were performed in triplicate.

2.6. Statistical Analysis

Correlations between data were statistically analyzed using the Graphpad Instat 3. Fisher's exact test was used to evaluate these correlations where P value < 0.05 was considered statistically significant.

3. Results

3.1. Bacterial Isolates

A total of 250 samples were collected from different hospitals. One hundred were identified as *S. aureus*. These isolates were obtained from various sources (93 clinical isolates and 7 environmental isolates).

Clinical sources included diabetic foot cases (D.F, 19 isolates), wound (W, 19 isolates), blood (Bl, 12 isolates), urine (U, 11 isolates), abscess (A, 9 isolates), burn (B, 10 isolates), sputum (Sp, 8 isolates) and throat swab (T.S, 5 isolates), where environmental (En, 7 isolates) were collected from hospital walls and floors to detect any abnormal increase in level of antimicrobial resistance or toxin prevalence in these hospitals environments.

3.2. Antimicrobial Susceptibility Testing

As determined by disc-diffusion antimicrobial susceptibility testing, all isolates showed resistance to at least one antimicrobial agent. A high resistance percentage was observed for ceftazidime (100%), ceftazidime (88%) and amoxicillin-clavulanic (77%). A moderate activity was noticed for tobramycin, erythromycin, ampicillin-sulbactam, cefepime and oxacillin where (32% - 47%) of isolates were resistant. On the other hand a lower resistance was observed for imipenem, ciprofloxacin, cephalothin and amikacin (**Figure 1**).

Among isolates, 88 were identified as methicillin resistant *Staph. aureus* (MRSA) where they showed resistance to ceftazidime. In addition, 33 were considered multidrug-resistant (MDR) (resistant to at least three different antimicrobial classes).

3.3. Polymerase Chain Reaction (PCR)

PCR analysis of the five toxin genes revealed that 88% of isolates harbored one or more of the tested

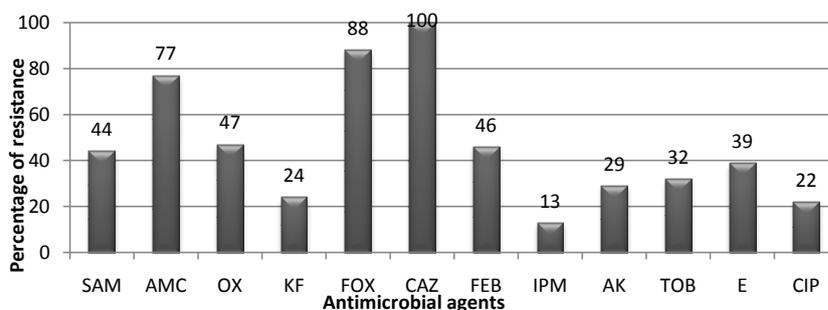


Figure 1. Percentage of resistant isolates to each antimicrobial agent. **SAM:** ampicillin-sulbactam, **AMC:** amoxicillin-clavulanic acid, **OX:** oxacillin, **KF:** cephalothin, **FOX:** ceftazidime, **CAZ:** ceftazidime, **FEB:** cefepime, **IPM:** imipenem, **AK:** amikacin, **TOB:** tobramycin, **E:** erythromycin, **CIP:** ciprofloxacin.

Table 2. Distribution of enterotoxin gene patterns among isolation sources and resistance categories of *S. aureus* isolates.

Toxin pattern number	Pattern					Percentage of isolates	Resistance category		Distribution among sources								
	<i>sea</i>	<i>seb</i>	<i>sec</i>	<i>see</i>	<i>seg</i>		MDR (n)	NMDR (n)	Clinical							Non clinical En	
									D.F	W	BI	U	B	A	Sp		T.S
T1	+					25%	7	18	3	5	7	2	1	3	3	1	0
T2		+				9%	2	7	1	3	0	1	0	1	0	0	3
T3			+			2%	1	1	1	0	0	0	1	0	0	0	0
T4				+		3%	1	2	0	1	1	1	0	0	0	0	0
T5	+	+				11%	7	4	3	3	0	1	3	1	0	0	0
T6	+		+			10%	4	6	4	3	0	0	1	0	2	0	0
T7	+			+		9%	3	6	0	1	3	2	2	0	1	0	0
T8		+	+			2%	0	2	0	0	0	1	0	1	0	0	0
T9		+		+		4%	1	3	0	1	1	0	0	1	1	0	0
T10		+			+	2%	1	1	0	0	0	0	0	0	0	1	1
T11	+	+	+			5%	1	4	4	0	0	1	0	0	0	0	0
T12	+	+		+		1%	0	1	0	1	0	0	0	0	0	0	0
T13	+	+			+	1%	0	1	0	0	0	0	0	0	0	0	1
T14	+		+	+		1%	1	0	0	0	0	0	0	1	0	0	0
T15	+	+	+		+	2%	0	2	0	0	0	0	0	1	0	1	0
T16	+	+	+	+		1%	1	0	0	0	0	0	1	0	0	0	0
T17						12%	3	9	3	1	0	2	1	0	1	2	2

D.F: Diabetic foot, **W:** Wound, **BI:** Blood, **U:** Urine, **B:** Burn, **A:** Abscess, **Sp:** Sputum, **T.S:** Throat swab, **En:** Environmental, **MDR:** Multi-drug-resistant, **NMDR:** Non-multidrug-resistant, **n:** number of isolates.

staphylococcal enterotoxin genes. The most frequent gene was *sea* (66% of isolates), followed by *seb* (38%), *sec* (23%), *see* (19%) and *seg* (5%).

The tested *S. aureus* isolates were distributed into 17 different patterns (T1-T17) according to **Table 2**. The most predominant pattern was T1 involving 25 isolates harboring *sea* gene only. The second most common pattern was T5 representing 11 isolates harboring both *sea* and *seb* genes. However, patterns T12, T13, T14 and T16 were the least common, where only one isolate represented each pattern.

In addition, 39% of isolates harbored only one toxin gene (*sea*, *seb*, *sec*, *see*), while 49% carried toxin gene combinations (38% harbored 2 genes, 8% harbored 3 genes and only 3% harbored 4 genes).

Among the 88 MRSA isolates, 76 harbored at least one enterotoxin gene and among them 56 isolates harbored the *sea* gene, 33 harbored *seb* gene, 17 harbored *sec*, 17 harbored *see* gene and *seg* gene was harbored by 4 isolates. The most common enterotoxin pattern in the MRSA toxigenic isolates was T1 coding for *sea* gene only (21 isolates) followed by T5 coding for *sea* and *seb* gene combination (11 isolates).

3.4. Real-Time PCR Analysis of *S. aureus* Enterotoxins

In order to study the relative expression of the tested genes, RNA was isolated from the selected 20 *S. aureus* isolates. The isolated RNA had an A260/A280 ratio that ranged between 1.766 and 2.12, while the concentration range was (0.352 - 12.95) µg/µl. From each isolate 1 µg of RNA was used in c-DNA synthesis. The standard curve of the housekeeping gene *nuc* and of all expressed enterotoxins genes showed R² values of 0.97 - 0.99. They showed the same melting profile and formation of pure amplicons indicated the specificity of the assay. The relative expression levels of SE were analyzed using the comparative method (2^{-ΔΔCt}) method.

Among the tested twenty isolates, 20 harbored *sea* gene, 12 harbored *seb* gene, 12 harbored *sec* gene, 7 har-

bored *sea* gene and 3 harbored *seg* gene as detected by PCR.

Sea gene expression was observed in all of the 20 isolates by variable degrees (Figure 2). Regarding *seb* gene, it was expressed in 11 out of 12 isolates (Figure 3). In contrast, *sec* and *see* genes were not expressed in any of the tested isolates, while the new (non-classical) enterotoxin gene (*seg*) was expressed in the 3 harboring isolates (Figure 4).

It was found that 70% of MDR isolates of the tested isolates expressed *sea* gene with high degree (P value = 0.0031) (Figure 2), *seb* and *seg* genes were highly expressed in 2 isolates that were non-multidrug-resistant (NMDR) and showed low *sea* gene expression (Figure 3, Figure 4) but no significant association was found.

4. Discussion

S. aureus is a major human pathogen found in hospitals and the community [24]. This bacterium is an opportunistic pathogen that can result in a variety of self-limiting and also life-threatening diseases in humans [25].

The clinical importance of *S. aureus* is attributed to remarkable virulence factors, toxins and enzymes, surface proteins, as well as the rapid development of antimicrobial agents resistance [26]. Among the most associated virulence factors with this bacterium, are the heat-stable staphylococcal enterotoxins (SEs) [27]. These toxins are the causative agents of staphylococcal food poisoning syndrome in man and they may be involved in other types of infections [28].

Emergence of antibiotic resistant strains of *S. aureus* is a major public health problem since the bacteria can be easily spread in the environment. During the last two decades, the rate of infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) has been notably increased world-wide [29]. Multidrug-resistant *S. aureus* have been frequently isolated from different origins, including food, water, human nasal mucosa and clinical cases [30]. Accordingly, it was extremely important in this study to detect the enterotoxigenicity as well as the antimicrobial susceptibility of *S. aureus* isolates belonging to various sources and try to find correlations between different variables.

In the present study, 100 *S. aureus* isolates were investigated for susceptibility against 12 antimicrobial agents belonging to four different classes (Figure 1). All of the isolates were resistant to ceftazidime. Also, 77% of isolates were resistant to amoxicillin/clavulanic acid. In a study of Manoharan *et al.* 2008, the percentage of resistance to this combination was 84.38% of isolates. These high levels of resistance may be attributed to misuse of antibiotics [31].

Intermediate frequencies of resistance towards oxacillin, cefepime, ampicillin/sulbactam, erythromycin and tobramycin antibiotics (47%, 46%, 44%, 39% and 32% respectively) were recorded. The level of oxacillin resistance (47%) was higher than those reported by Sina *et al.* 2013 and Tokajian *et al.* 2011 (32%, 25%) respectively [32] [33]. Perveen *et al.* 2013 demonstrated that 12% of isolates were resistant to cefepime [34] which come in contrast with our finding. Opere *et al.* 2013 reported that erythromycin level of resistance was 32%, which was consistent with our results [35]. Resistance towards tobramycin in this study was coherent with Schmitz *et al.* 1999 who demonstrated that 29% of *S. aureus* isolates were resistant to tobramycin [36], while Elazhari *et al.* 2011 showed that only 1.43% of isolates were resistant [37].

For ciprofloxacin, 22% of isolates are resistant antimicrobial agent, Shittu and Lin, 2006 showed a lower level of resistance to ciprofloxacin (<10%) [38], while Opere *et al.* 2013 showed higher level of ciprofloxacin resistance (39%) [35].

The majority of our isolates were susceptible to amikacin and cephalothin (71%, 76%) respectively. This was also reported by Hauschild *et al.* 2008, Gade and Qazi, 2014 who demonstrated that 75.6% and 74% of *S. aureus* isolates were susceptible to amikacin respectively [39] [40]. Since cephalothin is a first generation cephalosporin so, it has good activity against *S. aureus* infections [41]. Imipenem antibiotic showed the highest activity towards isolates, where 87% of them were susceptible. This result was in accordance with the study of Asadollahi *et al.* 2014, in which 98% of isolates were susceptible [42].

In this study, 88% of isolates were identified as MRSA depending on cefoxitin (30 µg) disk diffusion results [19]. Methicillin resistance was detected in *S. aureus* using cefoxitin with 100% specificity and sensitivity [43]. Also Alfatemi *et al.* 2014 demonstrated that cefoxitin had 100% sensitivity and 99% specificity for MRSA detection [20]. Our findings agree with the study of Yaseen *et al.* 2013, who reported that (88%) of isolates were MRSA [44]. Multidrug-resistance (MDR) was defined as resistance to at least 3 antimicrobial classes [45] [46]. 33% of our isolates were MDR, Seas *et al.* 2006 demonstrated a lower percentage, where 25% of isolates were MDR [45].

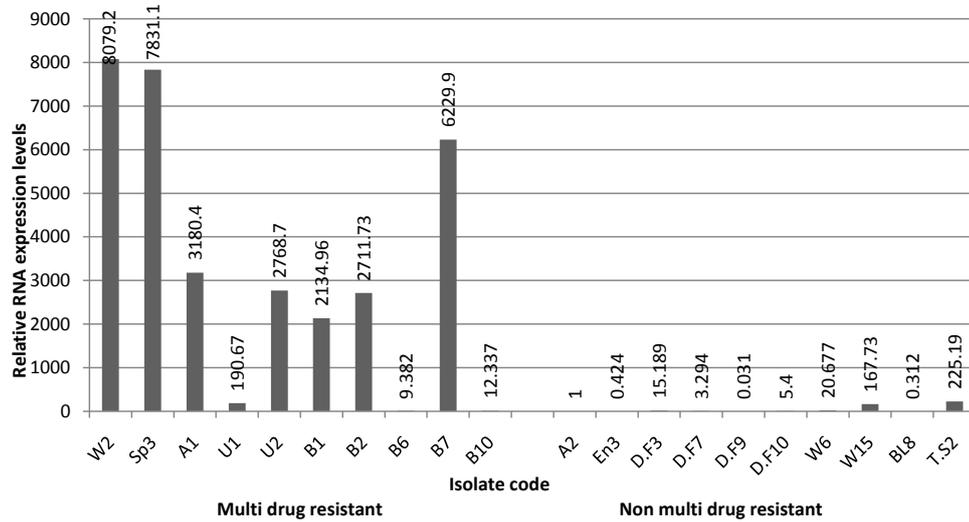


Figure 2. Relative *sea* gene expression levels in 20 isolates calculated by the comparative method ($\Delta\Delta Ct$) method.

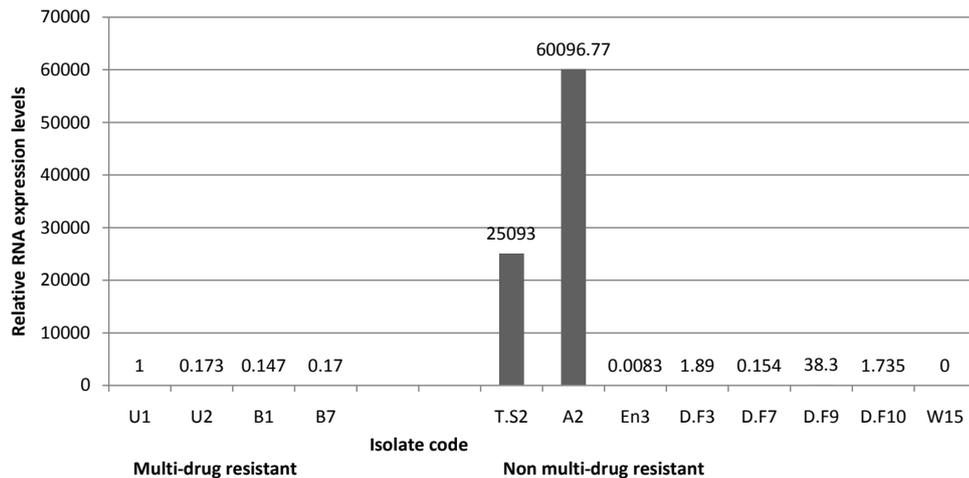


Figure 3. Relative *seb* gene expression levels in 12 isolates calculated by the comparative method ($\Delta\Delta Ct$) method.

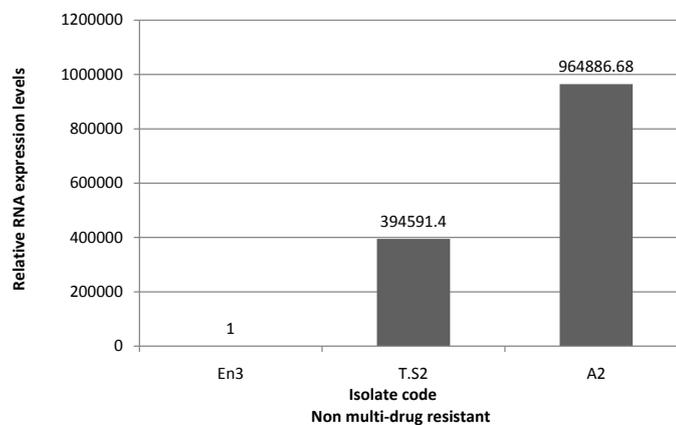


Figure 4. The relative *seg* gene expression levels in 3 isolates calculated by the comparative method ($\Delta\Delta Ct$) method.

The prevalence of five SEs encoding genes was investigated by PCR amplification. 88% of isolates harbored one or more enterotoxin genes. This result was to some extent in accordance with the studies of Elazhari *et al.* 2011 and Cao *et al.* 2012 who found toxigenicity levels of (76.4% and 93.5%) respectively [37] [47], however, Becker *et al.* 2003 and Naffa *et al.* 2006, reported a lower level of toxigenicity 43% and 23%, respectively [48] [49].

Among 88 SE genes positive isolates, only 16 different toxin patterns were detected (Table 2); this can be explained by the horizontal-gene transfer among strains [50]. Since all SE genes are carried on accessory genetic elements, including plasmids, genomic island vSa, *S. aureus* pathogenicity islands (SaPIs) and prophages, or next to the staphylococcal cassette chromosome (SCC) elements. Most of these are mobile genetic elements, which enable them to spread among *S. aureus* isolates increasing their ability to cause disease and contribute to the emergence of pathogenic strains [51].

The *sea* gene was the most predominant toxin gene detected in 66% of isolates, this high percentage is in the line with other studies who reported *sea* gene prevalence rate of 55.1%, 65.22%, 60.6% [52]-[54]. However, a much lower frequency of *sea* gene was reported by other studies where *sea* gene prevalence was 25% and 18.86% [33] [55]. The second most prevalent toxin gene detected in this study was *seb*, where (38%) of isolates harbored this gene, this was in accordance with the study performed by Sina *et al.* 2013 who reported that 44.3% of isolates were *seb* gene carriers [32]. It was found that only one isolate (8%) out of twelve isolates from blood carried the *seb* gene, similarly only 5% of *S. aureus* isolates collected from blood stream infections harbored this gene [56].

Regarding the *sec* gene, it was detected in 23% of isolates. This was in accordance with the results of Lim *et al.*, 2012 who reported that the prevalence rate of *sec* gene was 19% [57]. However, higher prevalence rates for *sec* gene (61.3% and 41.5%) were reported [47] [55]. For *see* gene, only 19% of isolates harbored it. Likewise, *see* gene was harbored by 15.09% [55]. However, Asadollahi *et al.* 2014 reported a higher level of *see* gene (31%) [42]. In contrast, other studies did not detect *see* gene in any of the isolates [33] [47] [53].

The prevalence of the non-classical (recently described) enterotoxin G encoding gene (*seg*) was also studied; it was the least prevalent gene in our study, where it was harbored by only 5% of isolates. Among *S. aureus* isolates collected from pediatric patients in Iran [55], none of the isolates carried the *seg* gene. However, a higher level of *seg* gene was recorded in other studies, where the prevalence rate of this gene was 25% and 32.6% [33] [53]. The coexistence of two or more toxins was observed with a prevalence of 49%, higher combination rates (67.2%, 76.9%, 94.57%) were found in previous studies [47] [53] [58].

The tested *S. aureus* isolates were distributed into 17 different patterns (T1-T17) according to their toxins. Patterns T1:T4 represents only a single gene, patterns (T5:T16) represent two or more toxin genes. Pattern 17 was for isolates devoid from toxin genes. The most common combinations in this study were (*sea* and *seb*) represented by pattern T5 (11%), followed by pattern T6 (*sea* and *sec*) genes (10%) and pattern T7 (*sea* and *see*) (9%). In a study of Kamarehei *et al.* 2013, 18.8% of isolates combined *sea* and *seb* genes [54]. Our data show that the coexistence of three toxin genes was found only in 8 isolates. A lower frequency (3%) was noticed for isolates harboring four toxin genes. However, some of our findings differed from those reported in other countries. In a study carried out in the Bronx, New York up to 12 SE genes were detected per a given *S. aureus* strain [59]. In our study, we did not observe any isolate harbored more than 4 enterotoxin genes.

Variations in enterotoxin encoding genes prevalence among studies may be attributed to differences in many factors, including the origin of the isolates (food, clinical or environmental samples), study locations, levels of hygiene restrictions among countries and also assay methods.

One of the most important finding in our study is that no statistically significant association between prevalence of toxin genes (*seb*, *sec* and *see*) and isolates sources, except for *sea* gene, which was significantly associated with clinical isolates (P value = 0.0059), where the environmental isolates did not show a remarkable increase in the toxin gene prevalence.

The relationship between virulence and resistance remains unclear. Some studies suggested a negative correlation between methicillin resistance and virulence, as resistance to antibiotics is associated with loss of pathogenic fitness and decreased virulence potential [60] [61]. Others observed that, in general, there were no differences between MRSA and MSSA in SEs levels [62]. In agreement with the study of Indrawattana *et al.* 2013 who found that *sec* gene was more associated with MSSA [53]. Our study confirmed that *sec* gene was significantly associated with isolates susceptible to cefoxitin (MSSA) (P value = 0.0281). Moreover, *sea* gene was more prevalent in isolates resistant to imipenem and ciprofloxacin antimicrobials with (P value = 0.0038 and

0.0237), respectively which was in accordance with Pourmand *et al.* 2009 who found a significant association between *sea* gene prevalence and resistance to a number of antimicrobial agents including ciprofloxacin [63].

It is well known that detection of toxin genes, does not necessarily mean the ability of toxin production [58], this may be due to mutation in the gene regulatory region [64], so it was important for us to demonstrate the ability to express the studied enterotoxin genes among selected 20 isolates by real-time PCR technique. *Sea* gene was expressed in all the tested 20 isolates by variable degrees (Figure 2). In addition, *seb* gene was expressed in 11 out of 12 isolates that harbored this gene. However, only two of these eleven isolates showed relatively very high expression levels (Figure 3). For *seg* gene, it was expressed in all of the 3 tested isolates with two of them showing very high relative expression levels (Figure 4). Regarding *sec* and *see* genes, they were not expressed in any of the isolates tested by RT-PCR. The same results were obtained by Vasconcelos *et al.* 2011, where no expression of *see* gene was observed for any isolate [65]. Also, our finding was in agreement with Zigo *et al.* 2014, where *sec* gene was not produced in any of the tested isolates [28].

Analysis of toxin pattern distribution among multidrug-resistant and non multidrug-resistant isolates did not allow the determination of a clear correlation between the determined toxin pattern distribution among MDR and NMDR. This is because 64.7% of patterns were shared by both MDR and NMDR. Our results were consistent with Indrawattana *et al.* 2013 who reported that no data was available in the literature correlating the ability to produce toxins in *S. aureus* and antibiograms [53]. However, an interesting correlation was found between high *sea* gene expression and multi-drug resistance (MDR) (P value = 0.0031). In addition, the 2 isolates that showed high *seb* gene expression as well as high *seg* gene expression were NMDR strains, but no significant correlation was found.

5. Conclusion

The present study highlights the high prevalence of enterotoxin genes, especially *sea* gene. The enterotoxin genes (*seb*, *sec* and *see*) were demonstrated to be strain dependent and not associated with the isolation source. However, *sea* gene was significantly associated with clinical isolates. Since detection of toxin genes does not necessarily mean the ability of toxin production, our results suggest that measurement of expression level of those toxins at the transcript level is necessary to evaluate the potential of *S. aureus* to cause severe disease. Although the genetic association between resistance and enterotoxins is poorly understood, evidence in our study indicates that there is a significant correlation between *sea* gene and both imipenem and ciprofloxacin resistance. Moreover, *sec* gene was significantly associated with MSSA isolates. An interesting correlation was also found between high *sea* gene expression and multidrug-resistance. Although isolates collected from hospitals environment did not show the expected antimicrobial resistance and toxin prevalence, more governed health restrictions are still needed to control the spread of *S. aureus* infections in our hospitals environment. Since these isolates may provide a source of fomite transmission, which may be associated with a high degree of antibiotic resistance and virulence.

Acknowledgements

All appreciation to Mansoura University hospitals staff members and patients for providing isolates used in the study. Many thanks for workers in Seeds Unit faculty of agriculture, Mansoura University.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contributions

Shaymaa H. Abdel-Rhman, Rasha Barwa and Mohammed A. El-Sokkary conceived and designed the experiments; Enas Y. Mohammed performed the experiments; Shaymaa H. Abdel-Rhman, Rasha Barwa and Enas Y. Mohammed analyzed the data; Shaymaa H. Abdel-Rhman, Rasha Barwa and Enas Y. Mohammed contributed reagents/materials/analysis tools; all authors wrote the paper, read and approved the final manuscript.

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