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The Prevalence of Enterotoxin *SEA* and *SEB* genes in *Staphylococcus aureus* Multidrug-resistant Isolates from Clinical Specimens in Baghdad Province

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Abstract

Infections caused by *Staphylococcus aureus* are frequent in both public and medical settings. The objective of the current study was to assess the prevalence of the *SEA* and *SEB* genes in *S. aureus* clinical isolates. A hundred-sixty samples were obtained from several sources including wounds, urine samples, burns and respiratory tract, during period between October 2022 - January 2023, from Kadhimiya Hospital, Karkh General Hospital and Yarmouk Hospital. The samples were then subjected to several examinations including microscopic examination (gram staining), cultural characteristics on mannitol salt agar, hemolysis characteristic on blood agar and biochemical tests (oxidase, catalase, and coagulase), to confirm *S. aureus* isolates. These isolates were also subjected to several antibiotics to select the multi-drug resistant (MDR) isolates. Based on the results, high percentages of *S. aureus* isolates were found to be sensitive to antibiotics, All the isolates (100%) were sensitive to vancomycin (VA), 95.5% were sensitive to both chloramphenicol (C) and rifampin (RA), 93.2% were sensitive to clindamycin (DA), trimethoprim (TR), levofloxacin (LE), followed by 90.9, 88.6 and 75% were sensitive to oxacillin (OX), gentamicin (CN) and doxycycline (DO) respectively. However, 47.75 and 38.6% of isolates were resistant to azithromycin (AZM) and cefoxitin (CX) respectively. The frequency of *SEA* and *SEB* genes was estimated by polymerase chain reaction (PCR). The results revealed that, out of 27 *S. aureus* isolates tested, 8 had a single band for the *SEA* gene (102 bp), while 9 had a band for the *SEB* gene (164 bp). The findings indicated that the majority of *S. aureus* harboured both *Sea* and *Seb* genes.

Keywords: Enterotoxin, Antibiotics, MDR, *Sea*, *Seb.*

انتشار جينات السموم المعوية ***SEA*** و ***SEB*** في عزلات *المكورات العنقودية الذهبية* المقاومة للأدوية المتعددة من العينات السريرية في محافظة بغداد

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الخلاصة

العدوى التي تسببها المكورات العنقودية الذهبية متكررة في كل من الأماكن العامة والطبية. كان الهدف من الدراسة الحالية هو تقييم انتشار جينات *SEA* و *SEB* في العزلات السريرية للمكورات العنقودية الذهبية. تم الحصول على 160 عينة من عدة مصادر، شملت الجروح،عينات الادرار،الحروق والجهاز التنفسي، خلال الفترة ما بين (تشرين الأول 2022 – كانون الثاني 2023) من مستشفى الكاظمية ،مستشفى الكرخ العام ومستشفى اليرموك، وخضعت لعدة فحوصات، بما في ذلك الفحص المجهري (تصبيغ الجرام) ، والخصائص الزرعية على أجار ملح المانيتول، وخصائص انحلال الدم على أجار الدم والاختبارات الكيميائية الحيوية (أوكسيديز ، كاتلاز ، وتخثر الدم) ، لتأكيد ان العزلات هي مكورات عنقودية ذهبية. ثم خضعت هذه العزلات لعدة مضادات حيوية لاختيار العزلات المقاومة للأدوية المتعددة (MDR). استنادًا إلى النتائج ، فإن النسب المئوية العالية كانت للعزلات الحساسة للمضادات الحيوية، وكانت جميع العزلات (100٪) حساسة للفانكومايسين (VA) ، و 95.5٪ كانت حساسة لكل من الكلورامفينيكول (C) وريفامبين (RA) ، و 93.2٪ كانت حساسة للكليندامايسين (DA) ، تريميثوبريم (TR) ، ليفوفلوكساسين (LE) ، تليها 90.9 و 88.6 و 75٪ كانت حساسة للأوكساسيلين (OX) والجنتاميسين (CN) والدوكسيسيكلين (DO) على التوالي. ومع ذلك ، كانت 47.75 و 38.6٪ من العزلات مقاومة للأزيثروميسين (AZM) والسيفوكسيتين (CX) على التوالي. تم تقدير تواتر جينات *sea* و *seb* بواسطة تفاعل البوليميراز المتسلسل (PCR) ، وكشفت النتائج أنه من أصل 27 عزلة،كانت ثمان عزلات حاملة لجين (102 bp) *SEA*، بينما كانت 9 عزلات اخرى حاملة لجين (164 bp) *SEB*. أشارت النتائج إلى أن غالبية المكورات العنقودية الذهبية تحتوي على جينات *SEA* و *SEB*.

الكلمات المفتاحية: السموم المعوية، مضادات حيوية، مقاومة الادوية المتعدد، *SEA* ، *SEB*

1. Introduction

The family of staphylococcal enterotoxins (SEs) includes more than twenty various streptococcal and staphylococcal exotoxins. There is structural, functional and sequence homology between these toxins. Staphylococcal enterotoxins B and A (SEB and SEA) are the most prevalent enterotoxins generated by *S. aureus* [1]. Several different kinds of staphylococcal enterotoxins (SEs) have been described, with some belonging to the super antigen category and others like SEs A and B have been associated with the sea and seb genes respectively [2]. *S. aureus* is a common Gram-positive bacterium that exist as a natural flora on the mucous membranes and the skin of human being [3][4]. It is one of the most popular and prevalent bacterial infections responsible for hundreds of thousands to millions of cases of more serious and invasive infections every year, in addition to an unknown number of cases of basic skin infections. It causes pneumonia, as well as various respiratory, surgical site, prosthetic joint, cardiovascular and nosocomial bacteremia infections [5]. Community and nosocomial infections caused by this organism can range from moderate soft tissue and skin infections to life-threatening such as osteomyelitis, bacteremia, infective endocarditis and pneumonia [6]. When compared to other infectious diseases like HIV, tuberculosis and malaria, *S. aureus* causes an overabundance of mortality and morbidity in poor countries [7]. One of the most important pathogens is *S. aureus* [8]. It needs several virulence factors, including enzymes and proteins, in order to survive in various environmental conditions [9]. This bacterium grows best at 37 degrees Celsius, pH 7.4, and in the presence of oxygen. Genetically, it has been reported that all *S. aureus* isolates harboured *nuc* gene [10]. Their β-hemolysis characteristic showed up as a distinct border around their colonies on blood agar [11]. *S. aureus* is clinically significant due to its rapid evolution of drug resistance and its important virulence factors, surface proteins, metabolites and enzymes [12]. Its isolates show high levels of resistance to the lactam group as they express *mecA* and its regulatory

determinant, encoding for the mutant PBP called PBP2A and/or lactamase encoded by *blaZ* and the *AbcA* efflux pumps expressed by *abcA. S. aureus* develops resistance to beta-lactam antibiotics in part through a process known as the efflux system [13]. The emergence of resistance may be due to increased consumption of a particular class of antibiotics, resulting in resistance due to mutation(s) at drug target sites or the disruption of drug accumulation in the cytoplasm caused by cell wall rearrangement [14]. The goal of this work was to evaluate the frequency of both *SEB* and *SEA* genes among *S. aureus* clinical isolates.

2. Materials and Methods

2.1 Collection, Isolation and Identification of Clinical Specimens

A hundred sixty samples were obtained from several sources including wounds (45 isolates), urine (24 isolates), burns (59 isolates) and respiratory tract (32 isolates) during the period between October 2022 and January 2023 from Kadhimiya Hospital, Karkh General Hospital and Yarmouk Hospital in Baghdad province with approval of the University of Baghdad, College of Science, Researeh Ethics Committe(CSEC/1222/0147) e based on. All samples were kept in sterilized containers and transferred into the laboratory where these samples were subjected to various examinations including microscopic examination (gram staining), cultural characteristics on mannitol salt agar, hemolysis characteristic on blood agar (Himedia/India) at 37^{o}C for 24 hours and biochemical tests (oxidase, catalase, and coagulase examinations) that were positive as a result [15] for the isolation and identification of *S. aureus* isolates [16].

2.2 Antimicrobial Susceptibility Test

To estimate clinical *S. aureus* isolates that are resistant to different antibiotics, the standard disc diffusion method on Muller-Hinton agar was used based on recommendations of the Clinical and Laboratory Standard Institute's (CLSI, 2022). The susceptibility patterns of the examined isolates to eleven antibiotic discs included oxacillin (OX; 1µg), vancomycin (VA; 30µg), doxycycline (DO; 30µg), clindamycin (DA; 2µg), trimethoprim (TR; 5µg), rifampin (RA, 5µg), gentamycin (CN; 10µg), azithromycin (AZM; 15µg), levofloxin (LE; 5µg) and chloramphenicol (C; 30µg) [17].

2.3 DNA Extraction

According to the manufacturer's claims the entire genomic DNA was isolated from isolates of *S. aureus* using an extraction kit for genomic DNA of bacteria (Promega, USA) supplemented with 30 µg/ml the enzyme lysozyme. Nanodrop instrument (ACT Gene/ Korea) was used for determination of the concentration and purity of the DNA samples.

2.4 Oligonucleotides and PCR Amplification

The reaction of PCR was carried out for *S. aureus* identification by *16SrRNA* gene, *Sea* and *Seb*. The primer sequence and size of product are mentioned below in Table 1.

Table 1: The targeted genes and PCR primers used in study

Gene	Primer	5'-3'Sequence	Size (bp)	References
16S rRNA	F	GGCCGTGTTGAACGTGGTCAAATCA	370	[18]
	R	TIACCATTTCAGTACCTTCTGGTAA		
Sea	F	GGTTATCAATGTGCGGGTGG	102	[18]
	R	CGGCACTTTTTTCTTCGG		
Seb	F	GTATGGTGGTGTAACTGAGC	164	[18]
	R	CCAAATAGTGACGAGTTAGG		

The PCR reaction amplification was used to carried out the experimentin a thermal cycler (labnet- USA). The compositions were utilized as follows: Co Taq®Green Master Mix (Promega /USA) (12.5µl) which contained 1 µl of each primer (10pmol), buffer, deoxynucleosides (dNTP), $MgCl_2$ and *Taq* DNA polymerase). In addition, the template of DNA (1µl of 100ng) was utilized and deionized water (9.5µl) was utilized into reaching the total volume of 25µl. The final volume of the PCR solution which included the deionized water, was 25 µl and comprises 1X master mix buffer, 10 pmol/ µl of both forward and reverse primers, 200 ng of DNA, and 1X master mix buffer. Thermocycler was applied under the following conditions: denaturation for 1 min at 95ºC; 30 cycles of 30 s at 95ºC, 30 s at 57ºC and 1 min at 72ºC; and a final extension step for 5 min at 72ºC. An agarose gel (2%) (w/v) electrophoresis was used for 1.5 hours at 70 volts to test the PCR results. DNA bands and ladders of 1000 bp were photographed under a UV transilluminator.

3. Results and Discussion

3.1 Identification of S. aureus

The 160 samples were obtained from several sources including wounds (n= 43; 26.8%), urine samples (n= 22; 13.75%), burns (n=55; 34.35%) and respiratory tract samples (n= 30; 18.75%). In this investigation, the vast majority of isolates were obtained from burn infections by 36.66 % of the total number of bacterial isolates, followed by wound infections, respiratory tract infections and urine samples by 26.8, 18.75 and 13.75% respectively. The common source of these bacteria are wounds, as reported previously [16], [19]. Whereas anither study [20] reported that methicillin drug resistant *S. aureus* is common in burn infections. *S. aureus* may colonize in tissue, multiply in the respiratory system and cause several illnesses.

Firstly, the microscopic field of *S. aureus* showed that these bacterial cells were gram-positive purple cells arranged in grape-like cluster. On mannitol salt agar (MSA), these bacteria appeared as golden colonies surrounded by yellow zones as a result of the fermentation of mannitol. Results on blood agar indicated the presence of β- hemolysis type by *S. aureus* isolates. In addition, different biochemical tests were performed and 150 bacterial isolates were recognized as *S. aureus* and the results indicated that the isolates were oxidase-negative, coagulase-positive and catalase-positive.

3.2 Antibiotic Susceptibility Test

In this investigation, the resistance of all *S. aureus* isolates was evaluated against a panel of 11 different antibiotic discs. The findings in Figure 1 show that high percentages of *S. aureus* isolates were sensitive to nine antibiotics, including DO, CN, LE, RA, TR, C, DA, VA and OX by 75, 88.6, 93.2, 95.5, 93.2, 100 and 90.9%, while 47.75 and 38.6% of isolates were resistant to AZM and CX respectively. The appearance of resistance in pathogenic bacteria to many antimicrobial medications has become a threat to public health [21]. *S. aureus* develops resistance to beta-lactam antibiotics in part through a process known as the efflux system [13]. In agreement with Kader *et al.* [22], who found that 88.24% of the isolates were resistant to oxacillin and methicillin discs, the current results also indicated that the sensitivity to oxacillin was 90.9%. In contrast, vancomycin resistance was found to be low among *S. aureus* isolates (10.0%) [23]. Multiple factors contribute to bacterial antimicrobial resistance including as enzyme-mediated drug breakdown, protein modifications that reduce susceptibility to antibiotics and alterations in membrane permeability. Plasmids, integrons and transposons are all potential mediators of antibiotic resistance. Beta-lactamase, an enzyme produced by bacteria, catalyzes the hydrolysis process and is the primary resistance

mechanism to cephalosporins and penicillins. By developing a genomic island called staphylococcal chromosomal cassette (*mec*), *S. aureus* develops resistance to methicillin and other beta-lactam antibiotics [18].

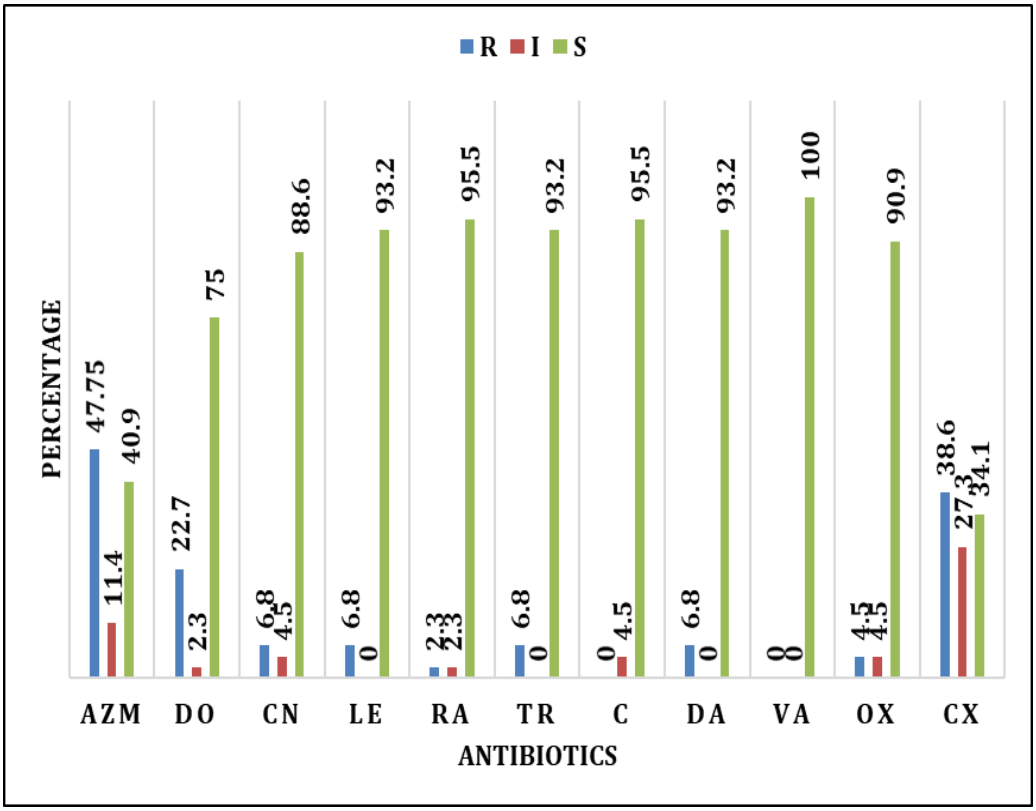

Figure 1: Antibiotic sensitivity frequency in *S. aureus* isolates: Azithromycin (AZM), Doxycycline (DO) , Gentamicine (CN), Levofloxacin (LE), Rifampin (RA), Trimethoprime (TR), Chloramphenicol (C), Clindamycin (DA),Vancomycin (VA), Oxacillin (OX), and Cefoxitin (CX)

As a result, only 27 *S. aureus* isolates were identified as a multi-drug resistant bacterium which were then subjected into further experiments.

3.3 Molecular Diagnosis of S. aureus Isolates

The biochemical results of 27 *S. aureus* isolates were validated by performing a molecular diagnostic of the bacteria using particular primers for the *16S rRNA* gene as an indication for the identification of isolates of *S. aureus*. Using a nano drop equipment, this current study determined that the DNA extraction yielded between 100 and 1500 ng/µl, with a purity of between 1.8 and 2.1. Fifty of the *S. aureus* isolates tested positive for 16S rRNA as a single band of DNA product of PCR with a 370 bp molecular base Figure (2). This was demonstrated using traditional PCR at an annealing temperature of 55°C which was defined for *S. aureus* and is regarded as a crucial trait for distinction from other species of *Staphylococci* [24]. PCR has been reported in previous research as a faster and more accurate way to identify 16S rRNA. The current study's results are consistent with those of Heidari *et al.* [25], who found that 27 Staphylococcal isolates were correctly identified as *S. aureus* after being amplified for 16S rRNA. Using 16S rDNA, researchers were able to positively identify all *S. aureus* isolates in several experiments [26].

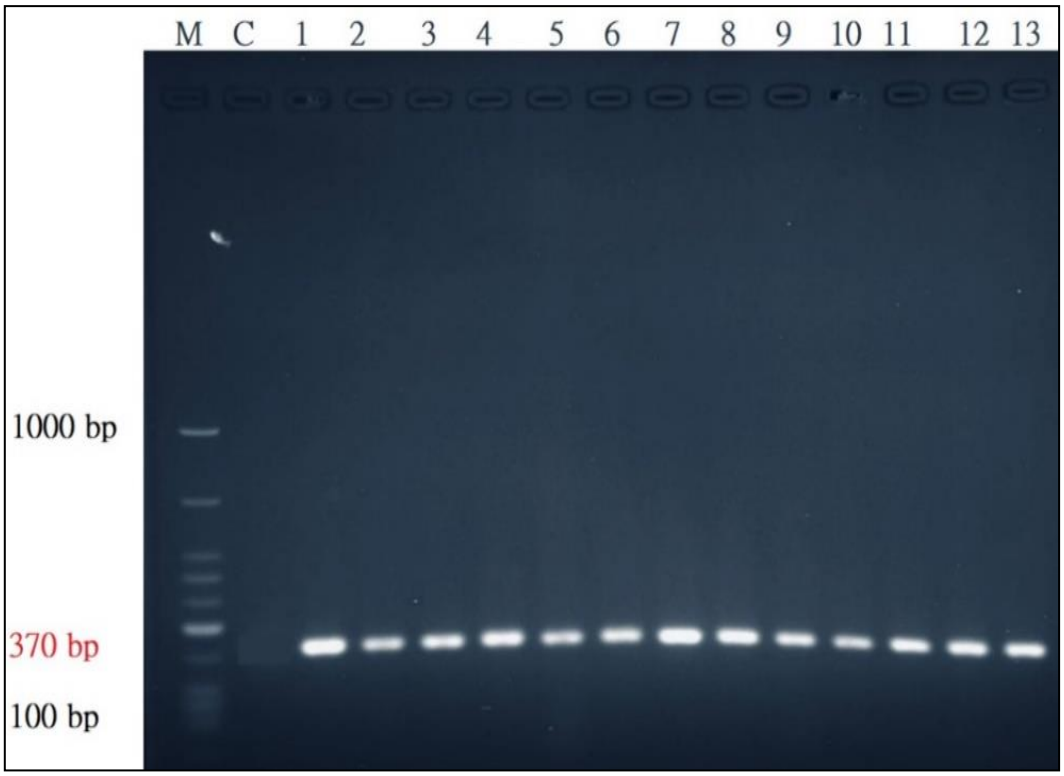

Figure 2: Under UV illumination, gel electrophoresis (2% agarose, TBE buffer 1X, 90 volt) was used to separate the products of amplification of *Staphylococcus* isolates using a particular gene of primer (370bp) for one hour. M: Molecular size marker = (100-1000bp), (1-13) = *Staphylococcus* isolates, C = Negative control.

As can be seen in Figures 3 and 4, 14 of the 27 *S. aureus* isolates showed a positive single band (102 bp) of the *sea* gene, while 15 of the isolates tested positive for the *seb* gene (164 bp). This research demonstrated that two kinds of enterotoxins are formed by *S. aureus* isolates. The seb gene was found to be less common than the type A enterotoxin gene. According to our findings, the sea gene is more common than the seb gene in *S. aureus* isolates which is consistent with the findings of Jassim and Kandala [18]. Several staphylococcal enterotoxins (SEs) that have the potential to induce gastro-enteritis have been shown to be more resistant to pasteurization and higher temperatures, establishing *S. aureus* as a key aetiological agent of food intoxications [27]. Sea, for example, preserved its biological activity after being heated to 121 degrees Celsius for 28 minutes [28], demonstrating the extreme heat resistance of SEs. According to Asao *et al.*[29], enterotoxins are a major virulence factor in food poisoning [30]. Staphylococcal enterotoxin A, either alone or in combination with another toxin, has been shown to be produced by a significant percentage of bacterial isolates from outbreaks of staphylococcal poisoning of food in France, Japan, South Korea and the United Kingdom [31], [32].

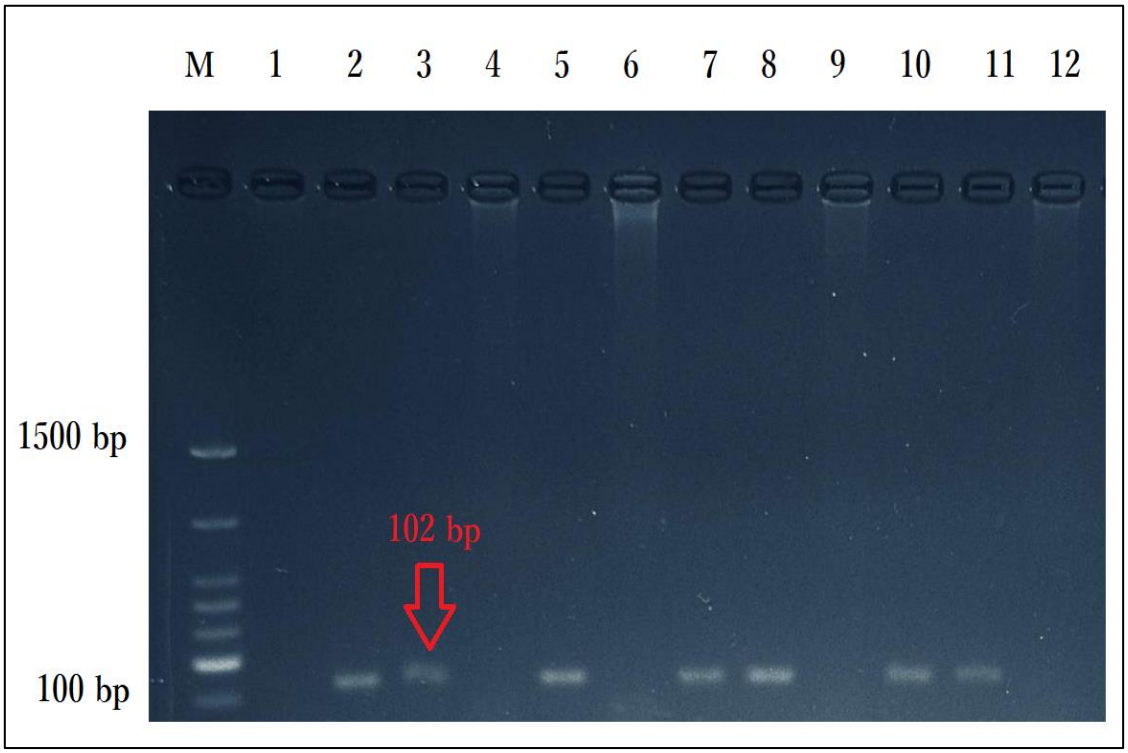

Figure 3: Toxin-producing *S. aureus* PCR products gene (102 bp) conceived by UV light after separation by electrophoresis on 2% agarose gel (1X TBE buffer, 90 V) for one hour, staining with ethidium bromide. M = Molecular weight indicator (100-1500 bp). Lane 1: Negative control. Lanes: 2, 3, 5, 7, 8, 9, 10 and 11 = *S. aureus* isolates positive for *SEA* gene. Lanes: 4, 6, 9, 12 = *S. aureus* isolates negative for *SEA* gene.

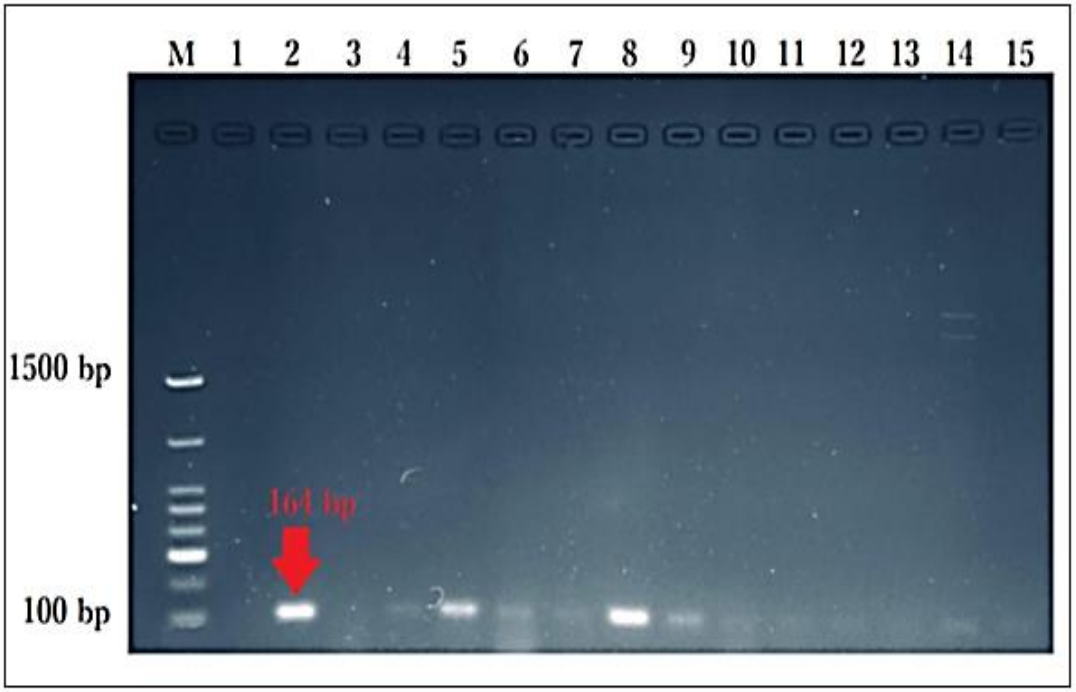

Figure 4: The amplification of products of PCR of the 164bp *S. aureus* toxin B gene were separated utilizing gel electrophoresis (1X TBE buffer, agarose 2%, 90 volt) for one hour,, stained with ethidium bromide, and seen under UV illumination. Marker for molecular size

(100-1500 base pairs; M). Lane 1 = Negative control. Lanes: 2, 5, 6 and 9 = *S. aureus* isolates positive for *Seb* gene. Lanes: 1, 3, 4, 7, 10-15= *S. aureus* isolates negative for *SEB* gene.

4. Conclusion

The findings of the current study indicated that burn infection was one of the most common sources of *S. aureus* isolation. Due to their ability to invade the host using different virulence factors, these bacteria should be considered as a threat public problem. Hence, the findings indicated that the majority of *S. aureus* harboured both *SEA* and *SEB* genes.

5. Ethical Clearance

This research was was approved by the Ethics Committee of Medical City Hospital and the University of Baghdad for Postgraduate Studies. Everyone present signed a form to show that they agreed in writing.

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