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Comparative Molecular Analysis of Meca, Sea and Seb Genes in Methicillin-Resistant Staphylococcus Aureus (MRSA)

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ABSTRACT

Methicillin resistant Staphylococcus aureus (MRSA), one of the most common pathogens associated with an increase of antimicrobial-resistance. Infections caused by Staphylococcus aureus may be demographically focused to specific parts of the world. This study will give critical bits of knowledge into the severity of the problem of antibiotic resistant organisms in Al-Basrah and give us valuable inputs regarding the possibility of a particular source being a contaminant that could lead to infection in this geographical area and study the profile of virulence genes including: (MecA, Sea, Seb).The study showed a high percentage of Staphylococcus aureus resistant to Methicillin, 39/46 (84.8%). that Nasal swab from H.C.Ws obtained the highest rate of MRSA 7/7 (100%) followed by wound swab 10/11 (90.9%) from patient most of them were already on antibiotic, Nasal swab from patient 5/6 (83%), swab taken from H.C.Ws hand and hospital wards 4/5 (80%) for each Methicillin Resistant Staphylococcus aureus obtained from Hospital theaters before sterilization and Skin swab from patient 3/4 (75%). MecA genes appeared in all MRSA isolates, Sea gene at 41% (16/39) and Seb genes, it was 10.3% (4/39).

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Introduction

Staphylococcus aureus (*Staph aureus*) is a gram positive cocci appear as blue-violet clusters under a microscope, about 0.4-1.2µm in diameter, have a yellow pigmented colonies named (*aureus*; means golden) due to their pigments (carotenoid) that form during their growth Practically everyone will have one or more *Staph aureus* infections in his or her life-time,(20-50%) of the human population are often colonized with Staph aureus in addition it is a major pathogenic bacteria responsible for community acquired and nosocomial infections, it is one of the most common human pathogenic bacteria that cause different Sequela of infections in both genders and in all age groups. And *Staph aureus* is an opportunistic pathogen that can lead to many self-limiting and even life-threatening diseases in humans *Staph aureus* found in nose, groin, axillae, perineal area in males, mucous membranes, mammary glands, intestinal, hair, and mouth, upper respiratory tracts and genitourinary of human and sometimes results in the production of pus, abscesses, sepsis and even fatal sepsis, *Staph aureus* grows well under high osmotic pressure conditions and low humidity, does not form spores but it is resistant to dry conditions and high salt concentration, 15% NaCl and readily develops at fixations up to 10% NaCl and tolerant to 4.2-9.3 of pH range, with an ideal pH of 7.0-7.5, It could grows at temperature between 15°C and 45°C , these bacteria rapidly multiply at room temperature and produce toxins that cause disease The response rates of *Staph aureus* clinical isolates to chemical pressures differ, and are relatively similar in growth rates, there were specific strains that responded to stresses by changing their lifestyles to form a biofilm and/or Small Colony Variants (SCVs) in extreme conditions (but stress levels are still under lethal), these results suggest that phenotype alteration depends on bacterial and host factors, and indicate that some specific strains may have a unique pathway that remains at stress One of the important reasons that made Staph aureus is more infectious is its ability to survive for several months on different surfaces [1-10]. *Staph aureus* cause many diseases, including minor skin and soft-tissue infections and sever disease such as pneumonia, septicemia, endocarditis, catheter-related infections through the formation of biofilm, and it is also one of the most common eye disease pathogens. Interventions such as hospitalization or episodes of immunosuppression may lead to invasive Staph aureus naturally present in the skin and mucous membranes and cause skin or respiratory diseases, or life-threatening bacteremia [11-14]. The virulence of *Staph aureus* is dependent upon the production of a number of excessive or superficial molecules, toxins, and immune evasion strategies, The exact sequence of these virulence factors varies between different isolates, and there are many factors that are unnecessary in their function such that neutralization of one molecule may not necessarily eliminate the ability of the bacteria to Exercising a specific effect on host cells. *Staph aureus* produces a wide variety of exotoxins, among the numerous toxins of including enterotoxins, the enterotoxins super antigens have already been assigned to the pyrogenic toxin super antigen family based on their biological activity and structural similarity [15-18]. Staphylococcal enterotoxins (SEs) are broadly classified as super antigens, many types of staphylococcal enterotoxins have been reported including: A associated with *Sea* gene, B associated with

Seb gene, C associated with *Sec* gene, D associated with Sed gene, E associated with See gene, F associated with Sef gene, G associated with *Seg* gene, H associated with *Seh* gene, I associated with *Sei* gene, J associated with *Sej* gene, K associated with Sek gene, L associated with *Sel* gene, M associated with Sem gene, N associated with Sen gene, O associated with Seo gene, P associated with *Sep* gene, Q associated with *Seq* gene and R associated with Ser, most SEs genes are portable on (MGE) , the enterotoxins are similar to each other in terms of activity and biological structure, but differ in antigen characteristics *Sea*, *Sed*, and *See* share 70–90% sequence homology [19-21].

Materials and Methods

Sampling

A total of 484 samples collected from patients (Skin swab, Nasal swab and Wound swab), Health Care Workers (Skin swab and Nasal swab), hospital words (Orthopedic and Surgical words) and Operation Theater (various places of Operation Theater before and after sterilization). Samples were collected in the period Between November 2018 and August 2019, from two locations, Al-Basrah Teaching Hospital and Al-Saddr Teaching Hospital. Each swab was transferred in to enrichment medium (brain heart infusion broth (BHIB)) for 2-4 hour at 37°C [22].

Culturing and Identification

Staphylococcus grows easily on most routine media at aerobic or micro-aerophilic conditions. It was quickly grows at (37°C), and the ideal temperature in which the pigment is formed is 20-25°C *Staph aureus* usually forms grey to golden yellow colonies due to carotenoids, Produces β-haemolysis on horse, sheep or human blood agar plates The bacterial morphology was observed microscopically as Gram-positive cocci arranged in grape-like irregular clusters All Staph aureus strains produce coagulase enzyme [13,23]. *Staph aureus* are catalase positive and oxidase negative *Staph aureus* express a clumping factor (fibrinogen affinity factor) *Staphylococcus* can grow in a medium with a high salt concentration, so they can grow easily in MSA [13,24]. The acidity of the medium changes as the bacteria ferments mannitol and turn phenol red pH-indicator; *Staph aureus* changes color of MSA from the alkaline (red)to the acidic(yellow), while the rest of the *Staphylococcus* will grow without changing the color of the medium [23].

Detection of MRSA

- **Cefoxitin disc diffusion**, Significant method to detect MRSA, by testing MRSA resistance to the cefoxitin disc, culture was done on MHA plate , incubation temperatures at 35-36°C and times of 18-24 hour strains of *Staph aureus* having zone of inhibition less than 19mm defined as MRSA.
- **PCR Methods**, Strains of *Staph aureus* harboring MecA gene defined as MRSA [25].

Genomic DNA extraction

As the instruction of manufacturer (promega company) Polymerase Chain Reaction (PCR) according to the manufacturer's instructions, the DNA were detected by gel electrophoresis, the samples were loaded in 0.8% agarose gel 1×TBE (54g Tris-base, 0.5M EDTA, 1-l distilled water, pH=8 and diluted with 400 ml of distilled water) and electrophoresed at 60 V for 30 min.

Polymerase chain reaction technique

PCR is a very effective method to amplify a particular DNA as many copies of a specific DNA, all MRSA isolates were assayed for the presence of the *MecA*, *Sea* and *Seb* genes by PCR using previously described primers, for PCR used diluted forward and reverse primers to reach (100pmol/µl) concentration as stock solution, distilled water was used as the negative control [26].

Table 1: PCR Master Mix Volume

PCR mix		Volume
Promega Green Master Mix		12.5µL
DNA template		2.5µL
Primer	Forward Primer	1.5µL
	Reverse Primer	1.5µL
nuclease free water		7µL
Total		25µL

Table 2: Oligonucleotide Sequences and Amplicon Size of Each Gene Used In This Study

Gene	Oligonucleotide Sequence (5'-3')	Amplicon Size, bp	Reference
MecA		147	Funaki, et. al.,2019
MECA-1	GTGAAGATAT ACCAAGTGATT		
Sea		102	Rossato, et. al., 2018
GSEAR-1	GGTTATCAATG TGCGGGTGG		
GSEAR-2	CGGCACTTTTT TCTCTTCGG		
Seb		164	Curry, et. al., 2016
GSEBR-1	GTATGGTGGTG TAACTGAGC		
GSEBR-2	CCAAATAGTGA CGAGTTAGG		

*1= Forward 2=Reverse

Table 3: Thermal Cycler Programs Used In This Study

Gene	Temperature (˚C)/Time					Cycle No.
	Initial denaturation	Cycling condition			Final extension	
		denaturation	annealing	extension		
MecA	94/5 min	95/30 sec	50/45 sec	72/1 min	72/7min	30
Sea	94/2 min	94/15sec	61/30 sec	72/30Sec	72/5min	30
Seb	94/3 min	94/15 sec	57/2 min	72/30Sec	72/7 min	35

Statistical analysis

Statistical analysis was done using SPSS (Statistical Package for Social Science) program V. 20, Experimental data were presented in terms of observed numbers and percentage frequencies, and then analyzed by using Chi-square ($\chi 2$) test to determine the relationship between the variables, P value ≤ 0.05 was considered statistically significant.

Results

Identification of bacterial isolates

Samples were collected in the period between November 2018 and August 2019 from two locations, Al-Basrah Teaching Hospital and Al-Sadder Teaching Hospital. Out of 485 samples only 46 (9.48%) were identified as coagulase positive staphylococci, as shown in table (3-1) PCR product was electrophoresed in 1.5 % agarose gel, Stained with ethidium bromide, 7µL of PCR products and promega DNA ladder (50-1000bp) carefully loaded in the wells and electric current was matched (65 volt for 45h). The gel was then observed under a UV light and compare with ladder (50-1000bp).

Detection of MRSA Isolates

Cefoxitin resistance staph aureus isolate harboring mecA gene (MRSA) was detected in 39from 46(73.4%) S. aureus isolates. 10(25.6%) isolates of MRSA were from wound samples, 6 (15.4%) from patient skin swab and 5 (12.8%) from patient noses, 4(10.3%) from hospital words, 4(10.3%) from health care workers hands, 7(17.9%) from health care workers noses and 3 (7.7%) from hospitals theaters samples.

MecA gene Detactions

Staphylococcus MecA gene presence in all MRSA isolates, to detect *Staph aureus* isolates with *MecA* gene, it was subjected to PCR technique, *MecA* gene band detected at 147bp region. Figure 1

Table4: Number of *Staph aureus* isolates from various studied samples

Type of specimens	Staph aureus				Total	
	MRSA		MSSA			
	No.	%	No.	%	No.	%
Hospital wards	4	10.3	1	14.3	5	10.9
Hospital theatre's before sterilization	3	7.7	1	14.3	4	8.7
H.C.Ws hands	4	10.3	1	14.3	5	10.9
H.C.Ws nasal swab	7	17.9	0	0	7	15.2
Wound swab	10	25.6	1	9.1	11	23.9
Patient skin swab	6	15.4	2	28.6	8	17.4
Patient nasal swab	5	12.8	1	14.3	6	13.0
Total	39	100	7	100	46	100

df=6, P Value =0.851, ^{2} =2.654

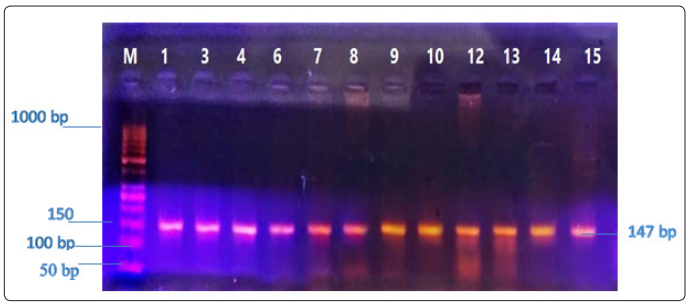

Figure 1: Amplified *MecA* gene (147bp) of *Staph aureus* isolates in PCR technique Agarose gel (1.5%), (95voltage for 45minutes)

M: DNA Ladder (50-1000bp). All MRSA was positive for *MecA* gene.

DNA Sequence for MecA gene

Figure 2 Showed DNA Sequence and Figure 3 Showed the Alignment of *MecA* Gen

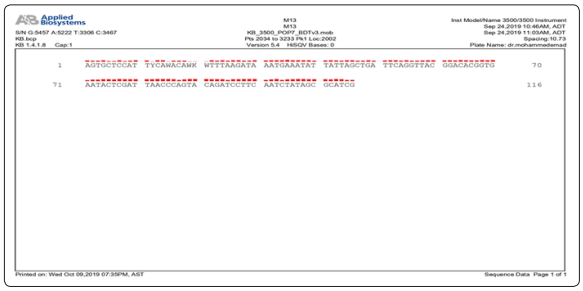

Figure 2: DNA Sequence for *MecA* gene

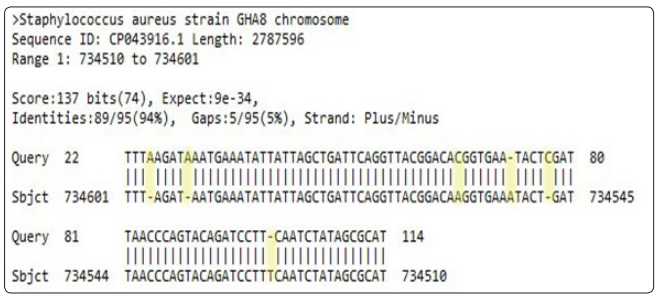

Figure 3: Alignment of *MecA* gen

Query isolate (our isolate) begin from (22-114) bp when compared with subject isolate (Stander isolate) begin from (734601-734510) bp, where the compatibility occur between the two isolates, for identification Query isolate identities was 94%.

There is a mismatch in six places; addition in nitrogen base Adenine (A) in two different locations, Substitution in nitrogen base replacement Cytosine (C) in Query isolate in instead of Adenine (A) in subject isolate, deletion in Adenine (A), Insertion in Cytosine (C) and deletion in Thymine (T), This difference may be due to point mutation.

Sea gene detactions

Staphylococcus enterotoxin type A (Sea) presence in 41% of *staph aureus* isolates. After PCR amplification Sea gene band detected at 102bp. Figure 4

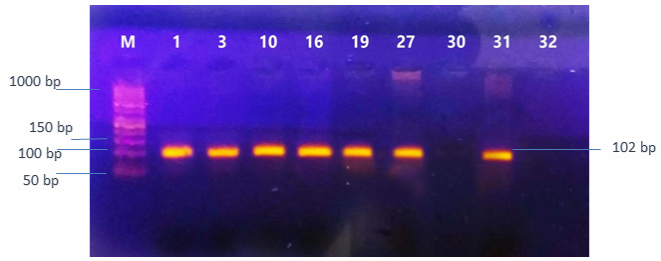

Figure 4: Amplified Sea Gene (102bp) Of *Staph Aureus* Isolates In PCR Technique

Agarose Gel (1.5%), (95voltage For 45minutes), M: DNA Ladder (50-1000bp), 1, 3, 10, 16,19,27,31 Samples Harboring Sea Gene, 30,32,36,37 Are Negative Samples.

DNA Sequence for Sea gene

Figure 5 showed DNA sequence and Figure 6 showed the Alignment of *Sea* gen

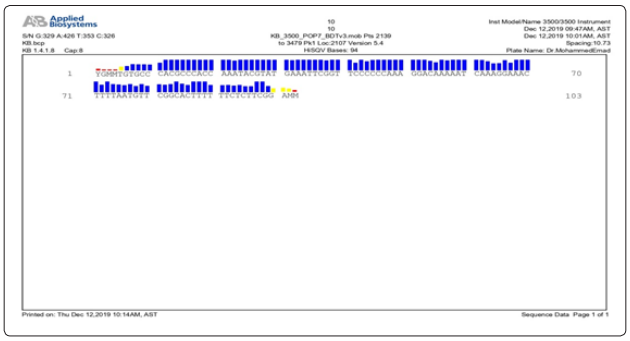

Figure 5: DNA Sequence for *Sea* gene

Alignment Optimize for Somewhat similar sequences (blastn)

Staphylococcus aureus subsp. aureus NCTC 8325 chromosome, complete genome

Sequence ID: NC_007795.1 Length: 2821361

Range 1: 289751 to 289770

Score:	Expect:	Identities:	Gaps:	Strand:
32.8 bits (35)	0.056	19/20(95%)	0/20(0%)	Plus/Minus

```
Query  58      AATCAAAGGAAACTTTTAAT  77
               ||||||| ||||||||||||
Sbjct  289770  AATCAAATGAAACTTTTAAT  289751
```

Figure 6: Alignment of *Sea* gene

Query isolate (our isolate) begin from (58-77) bp when compared with subject isolate (Stander isolate) begin from (289770-289751) bp, where the compatibility occur between the two isolates for identification Query isolate, identities was 95%; There was a mismatch in one place substitution occurs with nitrogen base replacement Guanine (G) in Query isolate in instead of Thymine (T) in subject isolate (Stander isolate), This difference may be due to point mutation.

Seb gene Detection

Staphylococcus enterotoxin type b (Seb) lowest MRSA gene prevalence among MRSA genes in our study it was found only in 10.3%. Seb gene band detected at 164bp. Picture (7)

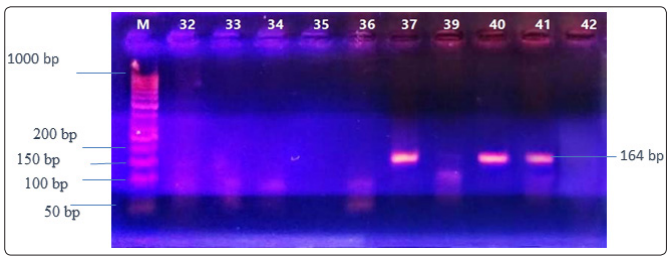

Figure 7: Amplified Sebgene (164bp) Of *Staph Aureus* Isolates In PCR Technique.

Agarose Gel (1.5%), (95voltage For 45minutes), M: Dna Ladder (50-1000bp), 37, 40, 41 Samples Harboring Seb Gene, 32, 33, 34, 35, 36, 39, 42, 44 Are Negative Samples.

DNA Sequence for Seb gene

Figure 8 showed DNA sequence and Figure 9 showed the Alignment of Seb gen

Figure 8: DNA Sequence for *Seb* gene

Alignment Optimize for Somewhat similar sequences (blastn)

Staphylococcus aureus subsp. aureus NCTC 8325 chromosome, complete genome

Sequence ID: NC_007795.1 Length: 2821361

Range 1: 626408 to 626421

Score:	Expect:	Identities:	Gaps:	Strand:
26.5 bits (28)	4.4	14/14(100%)	0/14(0%)	Plus/Minus

```
Query  40      TATGAGTGTCACTC  53
               ||||||||||||||
Sbjct  626421  TATGAGTGTCACTC  626408
```

Figure 9: Alignment of *Seb* gene

Query isolate (our isolate) begin from (40-53) bp when compared with subject isolate (Stander isolate) begin from (626421-626408) bp, where the compatibility occur between the two isolates for identification Query isolate, identities was 100%.

Table 5: Prevalence of various genes of MRSA isolated

Source of isolates	MRSA genes		
	MecA	Sea	Seb
Wound swab	10 (100%)	5(50%)	1(10%)
Patients skin swab	6 (100%)	3(33.3%)	1 (16.7%)
Patients nasal swab	5 (100%)	3(60%)	0
Hospital wards	4 (100%)	2(50%)	0
H.C.Ws hand	4 (100%)	0	0
H.C.Ws Nasal swab	7 (100%)	2 (28.6%)	0
Operating Theaters	3 (100%)	2 (66.7%)	2(66.7%)
Total of positive result	36 (100%)	16 (41%)	4 (10.3%)
Negative result	0	23)59%)	35(89.7%)
P value	-	0.493	0.044
□2	-	5.403	12.926

Discussion

Findings obtained by this study showed that these bacteria isolated from the hospital environment (Operating rooms and patients' rooms) and hands and noses of workers and patients, may be deposited in the wound and causes wound infection. Bacteria can be transmitted from a patient's wound in the hospital to another, and bacteria may be able to enter the wound while changing the bandages, especially when the patient stays in the hospital for a long time Wounds infection may be initial in the operating room before wound closure or after the patient has been returned to the ward. The current study is designed to investigate the possible cause of sepsis in wounds. *Staphs aureus* is a common bacterium colonizing the human skin and mucous membranes that can transmitted from hands and nose to wounds, H.C.Ws were a major source of MRSA transmission to hospitals patient In the current study, the percentage of *Staph aureus* in H.C.Ws hand were 13.8% and their noses were 22%, which is considerably lower than reported elsewhere showed that the nasal courage rate of *Staph aureus* were 47.6% ; among H.C.Ws sample and lower than other study conducted in Baghdad, the percentage of *Staph aureus* appeared in nasal swab for H.C.Ws 34% [27-30]. The rate of carriage of *Staph aureus* in the H.C.Ws hand was almost similar to that presented in other study Bauer et al.1990 recovered *Staph aureus* from the hands of 20.5% of the personnel in a medical ICU much higher than we found in our study. Pessoa-Silva, et, al, recovered 2.5% of *Staph aureus* from H.C.Ws hand in neonatal unit lower than we found in our study Detection of Methicillin resistant *Staph aureus* (MRSA) was carried out using Cefoxitin disc Plates were incubated at 350C for 18–24 hours and inhibition zone diameters (mm) were measured There is increasing concern about MRSA contamination and infections in the hospital words meanly in post-operative wound, in our study isolation showed high prevalence range of MRSA strains 85% (39/46) of the total *staph aureus* isolated from various samples, higher rate of MRSA isolations from H.C.W nasal swabs 100% (7/7), followed by wound swabs 90.9% (10/11), Nasal swab from patient 83% (5/6), H.C.Ws hand swabs and hospital words 80% (4/5) for each, lowest rate were recorded for the patient Skin swab (6/8) and operative rooms (3/4) 75% each. The finding about high prevalence of MRSA is not surprising and is also in line with several studies carried out in Iraq [31-34]. The ratio of MRSA was relatively low in a study conducted in Kurdistan region of Iraq, in 2015 where the MRSA prevalence was 53% In another study in Iran was 69% while in a study conducted in India, the percentage was much lower 16.6% Prevalence of MRSA 51.4% in in a Korean hospital from staph aureus collected from blood and nasal colonizers [35-40]. In general MRSA was highly prevalent in Asian countries [12]. The prevalence of MRSA in wounds was high, and this is consistent with previous studies, 76.9%, 44%, 60.1%, 34.8% [41-44]. The isolation of MRSA from surgical wounds has been shown to be related to lower chances of primary healing and delayed healing. In other study the average of MRSA rate for Wound Infections After Cardiothoracic Surgery was 54% in a three-year period from 2007 to 2010 [45,46].

During the present study, MRSA isolate from HCWs noses was (19.4%) Which corresponds to previous studies 12.7%, 12% in; 14.3%, MRSA rate was low in other studies; only 5.3% in Iran study; and it was 0% in Kenya [47-54]. Asymptomatic carriers represent as a risk factor for the acquisition and transmission of MRSA to others Identification of the *MecA* gene is the most reliable method for detecting MRSA isolates; Methods based on disc diffusion are often not entirely reliable at detecting some strains that harbor the *MecA* gene. Methicillin resistance in Staphylococci is mediated by the MecA gene (lies in the *SCC*mec resistance island) which encodes for a penicillin-binding protein (PBP2a) resulting in reduced affinity for β-lactam drugs [55-59]. All MRSA isolate in our study harboring MecA gene. This result agreed with many other studies that showed all MRSA isolate harboring *MecA* gene other studies considered that Methicillin resistance in Staphylococci can happen in the absence of MecA gene, MRSA could have other resistance mechanism(s) e.g., altered target site or reduced drug accumulation. Another possibility is that they could not detect the MecA gene by the used methodology [60-65]. *Staph aureus* is a very common organism capable of producing several enterotoxins (SEs) that cause intoxication symptoms of varying intensity in humans when ingested through contaminated food. *Staph aureus* enterotoxin also may be implicated as virulence factors in some cases of toxic shock-like syndromes, Prevalent of enterotoxin a and b in our study was 41% and 10.3% respectively. In a study from the United States, positivity range 3-30% of Seb gene In Tehran of Iran, positivity range of Sea and Seb was 23% and 2%, respectively In a study from Turkey, Sea gene was the most common (40.1%) in hospital and in community-acquired *Staph aureus* isolates Arabestani,et,al reported that the most prevalent genes were Sea 45.0%, Seb34%. In a local study of MRSA, the detection of enterotoxins genes that encoding for Sea, Seb showed the prevalence in percentage (24.78%, 0%) respectively In general, the *Sea* gene was the most common compared to the *Seb* gene, and this corresponds to what was stated in our results [66-73].

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