**RESEARCH ARTICLE**

# Phylogeny Characterization of Seb Gene Encoding Enterotoxins in *Staphylococcus aureus* Isolated from Raw Milk and Cheese

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***Received: 09th July, 19; Revised: 18th August, 19, Accepted: 30th August , 19; Available Online: 02th September, 2019***

**ABSTRACT**

Food poisoning due to the bacteria is a big global problem in economically and human's health. This problem refers to an illness that is due to infection or the toxin that exists in nature and the food that use. Milk is considered a nutritious food because it contains proteins and vitamins. The aim of this study is to detect and phylogeny characterization of staphylococcal enterotoxin B gene (Seb). A total of 200 milk and cheese samples were screened. One hundred ten isolates of *Staphylococcus aureus* pre-confirmed using selective and differential media with biochemical tests. Genomic DNA was extracted from the isolates, and the SEB gene detects using conventional polymerase chain reaction (PCR) with specific primers. Three *Staphylococcus aureus* isolates were found to be positive for the Seb gene using PCR and confirmed by sequencing. Sequence homology showed a variety range of identity, starting from (100% to 38%). Phylogenetic tree analyses show that samples (6 and 5) are correlated with *S. epidermidis*. This study discovered that isolates (A6-RLQ and A5-RLQ) are significantly clustered in a group with non- human pathogen *Staphylococcus agnetis*.

**Keywords**: Enterotoxin, Homology, PCR, Phylogenetic, Poisoning, Raw milk, SEB, *Stahylococcus aureus*.

International Journal of Drug Delivery Technology (2019); DOI: 10.25258/ijddt.v9i3.13

**How to cite this article:** Aziz, F.N. and Mohammed-Jawad, L.A.H. (2019). Phylogeny Characterization of Seb Gene Encoding Enterotoxins in Staphylococcus aureus Isolated from Raw Milk and Cheese. International Journal of Drug Delivery Technology, 39(3): 396-402.

**Source of support:** Nil.

**Conflict of interest:** None

## INTRODUCTION

*Staphylpococcus aureus* is a major global public health problem causing serious, often life-threatening infections in the community and hospital settings that are becoming more difficult to manage with current antibiotics therapy regimens.[1] *S. aureus* is the main pathogen associated with nosocomial and community-acquired infection and cause important public health problem as a result of wide range of infection from mild infection of soft tissue and skin to severe disease in humans and animals including life-threatening pneumonia, sepsis, bacteremia, toxic shock syndrome, endocarditis and osteomyelitis.[2] Foodborne diseases have a significant public health impact. It is estimated that in the United States alone foodborne illnesses affect 6–80 million people each year, causing up to 9000 deaths and cost about 5 billion US dollars.[3] Staphylococcal food poisoning (SFP) is one of the most common foodborne illnesses resulting from the ingestion of staphylococcal enterotoxins produced in food by enterotoxigenic strains of *S. aureus*.[4] There were five major classical SEs types, named; SEA, SEB, SEC, SED, and SEE. But now, new genes encoding enterotoxin such as SEG to SEU are identified. One or more of these genes are thought to be involved in 95% of staphylococcal food poisoning.[5]

These SE proteins have a remarkable ability to resist heat and acid. Therefore, they may not be entirely denatured by the mild cooking of contaminated food. They are pyrogenic and share some other important properties that include the ability to induce emesis and gastroenteritis as well as their noted super antigenicity. They are resistant to inactivation by gastrointestinal proteases, including pepsin, trypsin, rennin, and papain.[6] Thus, they can easily outlast the bacteria that produce them.

The distribution of genes encoding for enterotoxins in *S. aureus* strains is highly variable, with some carried on stable regions of the chromosome (e.g., enterotoxigenic gene cluster—EGC) associated with particular lineages and others carried on mobile genetic elements (MGEs). MGEs are segments of DNA that encode enzymes and other proteins that confer their ability to move horizontally between bacterial cells.[7] *SEB* is the only known Staphylococcus enterotoxin that has been examined as a biological warfare weapon. There was a particular interest in weaponizing *SEB* in the Cold War Era because of its stability and potential simplicity in production and dispersal. SEB was studied in an aerosolized form for use as a weapon. It may be purified from culture supernatants in the laboratory, and therefore would be easy to produce.

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As mentioned earlier, *SEB* is quite stable to heat, proteolytic digestion, and a wide pH range.[8]

Experiments have shown that for aerosol exposure, that minimal amount (0.004 μg/kg) is effective at inducing symptoms, and a dose of 0.02 μg/kg could be lethal.[9] The fact that a low dose of SEB is sufficient to incapacitate people is another factor that makes it a potential weapon. Inhalation of SEB leads to shortness of breath and chest pain for several hours after exposure. With heavy exposure, more serious symptoms could occur such as high fever, pulmonary edema, possible acute respiratory distress syndrome, or septic shock.[10] Many authors used PCR for the detection of staphylococcal enterotoxin genes all of them found high variability in the presence of enterotoxin genes and explain their role in food poisoning.[11-14] This study aimed to molecular detection of the Seb gene from *S. aureus* and estimates the evolutionary relationship and the convergence ratio (sequence similarity) among the sequenced/local strain in comparison with the reference strains of the GenBank by the phylogenetic tree and the similarity matrix method.

## MATERIALS AND METHODS

### Samples Collection

A total of 200 milk and cheese samples were collected from different places of Al Muthanna province. The samples were collected for the period of August 2017 to April 2018. The samples were prepared for bacteriological and molecular conformation at the Biology department/ College of Science/ AL Muthanna University.

### Isolation and identification of S. aureus

Different media (Brain Heart Infusion broth, Mannitol salt agar, STAPHYLOCOCCUS AGAR Nº110, and human blood agar) were used for culturing of *S.aureus* samples. The cultures incubated at 37°C for 24 hours. The bacterial colonies were identified by visual inspection for golden-yellow colonies on Mannitol salt agar and grape-like cluster using light microscopically. Subsequent biochemical tests including (coagulase, catalase, and oxidase and API-STAP system) were performed. For antibiotic susceptibility test, three types of antibiotic (Methicillin (10 μg/disk), Tetracyclin (30 μg/disk), Vancomycin (30 μg/disk)) were applied to test the antibiotic susceptibility of positive isolates using disk diffusion method on Mueller–Hinton agar (Oxoid).

### Bacterial genomic DNA extraction and molecular detection

Bacterial genomic DNA was extracted from 110 *S. aureus* isolates using quick bacteria genomic DNA Extraction Kit (N1151). The extracted DNA analyzed by agarose gel 1%. The *seb* gene was amplified by PCR. The gene primers listed in Table 1.

### Gene amplification and sequencing

The PCR Mixed was prepared in a volume of 50μL, containing 25μL master mix of Taq DNA polymerase (Bio Lab, England), 1μL of each primer, and 4 μL of DNA template the volume completed with nuclease free water. PCR program included: Initial denaturation at 94°C for 30 seconds, 35 cycles of 94°C for 30 second, 57.5°C for 1 minute, 68°C for 1 minute, and a final extension at 68°C for 5 minutes. Results were analyzed on 1.5 % agarose gel. PCR amplicons were purified using the PCR Purification Kit (WIZARD), and sequencing was outsourced (CD-Genomics, USA). The acquired sequences were analyzed using NCBI and MEGA software (Version 6) and were compared to reference nucleotide sequences imported from GenBank (NCBI). Novel variants of enterotoxin promoter or gene sequences were subsequently submitted to GenBank.

### Statistical analysis

The results of this work were analyzed for significant using mean ± SD.

## RESULTS

The collected milk and cheese samples were cultured, after activation, on Mannitol salt agar which considered selective and differential medium for the genus *Staphylococcus*[16] and turned the color of the medium from pink to yellow, others mannitol non-fermentor which appeared as small pink colonies as *S. epidermidis* or large deep yellow to deep orange colonies as *S. chromogenes* and in both cases no color change was observed in this medium.[17] To preformed results, the positive isolates were cultured on the agar medium Staph No.110, which consider selective medium for *S. aureus* and also cultured on CHROM agar medium. The results showed that 110 (55%) milk and cheese samples were contaminated with *Staphylococcus aureus.*

The results of antibiotic susceptibility test of *S. aureus* isolates to 3 antibiotics (Methicillin, Tetracycline, and Vancomycin) was determined using disc diffusion method; showed that all *S. aureus* isolates (100%) found to be methicillin-resistant (MRSA) and 20% were resistant to vancomycin (VRSA), and 15% were resistant tetracycline Figure 1.

### Detection of S. aureus enterotoxin gene

Staphylococcus is a gram-positive bacteria; its thick peptidoglycan layer makes DNA extraction difficult work because of the requirement of lysozyme, genomic DNA then analyzed using 1%agarose gel electrophoresis. The PCR analysis was applied to DNA extracted from pre-conventional microbiological confirmed of *S. aureus* isolates from milk and cheese samples. Out of 110 isolates were analyzed by PCR technique for *SEB* genes, only Two isolates of *S. aureus* (1.8%) found to possess this gene (50% from raw milk and 50% from traditional cheese) Figure 2.

**Table 1:** Primers used in this study

| *Primer sequence* | | *Accession numbers* | *Size (bp)* | *Source* |
|---|---|---|---|---|
| Forward | CCTTAAACCAGATGAGTTGCACA | M11118 | 405 | 15 |
| Reverse | ACCATCTTCAAATACCCGAACA | AY852244 | | |

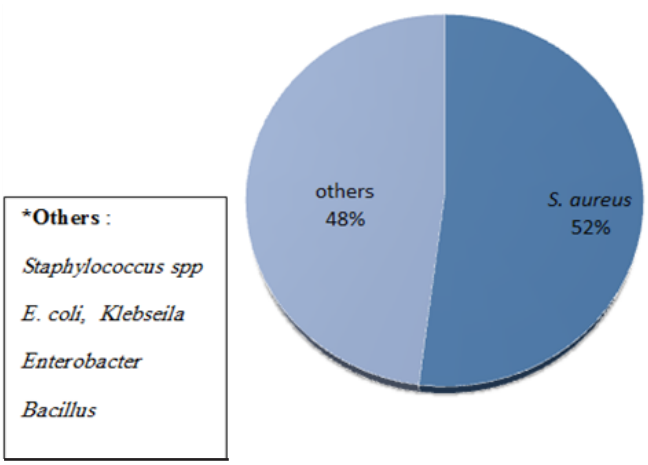


**Figure 1:** The percentage of *S aureus* isolated from milk and cheese samples.

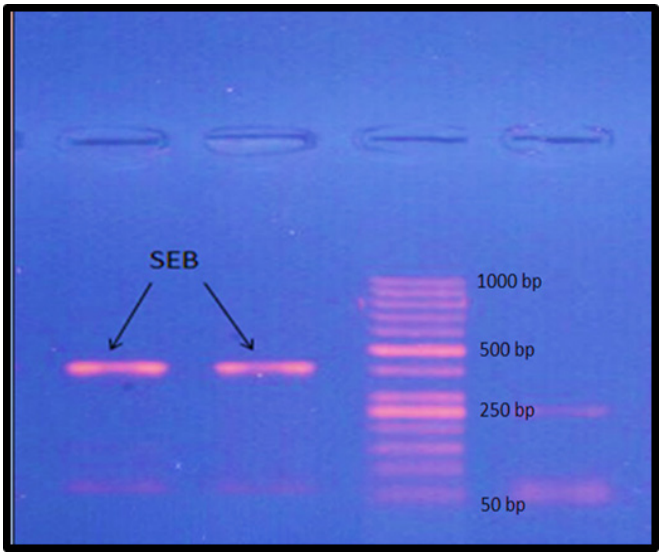


**Figure 2:** 1.5 % Agarose gel electrophoresis showing amplification of *seb* gene

## Sequencing and phylogenetic Analysis

The analysis of sequence data done by using the national center for biotechnology information (NCBI) tools, the position of mutations detected by used NCBI blast by alignment Query with the specific reference sequence (RefSeq). For sequence homology analysis, SEB gene was deposited at the NCBI database under the GeneBank accession number KM450112, and a total of 12 homologous *S. aureus* strains sequences were selected based on the degree of sequence similarity, taxonomical group, and gene as shown in (Table 2). Two samples (A6 and b4) give 100% identity to staphylococcal enterotoxin type B (SEB) isolated from India, while the third one (A5) gives 94%. Also, the same two samples give 99% identity to *S. aureus* enterotoxin gene in USA, whereas A5 gives 94% identity. Also, for SEB in USA, B4 and A6 showed 87% similarity, and A5 give 85% identity. The similarity with RefSeq of SEB showed to be 86% identity in B4 and A5, and 67% in A6.

The Similarity with RefSeq of enterotoxin gene in *S. delphini* showed to be 68–69% and 60–61% for RefSeq of enterotoxin in *S. epidermidis.* The identity with *streptococcus pyogenes* enterotoxin protein in USA was 61%. The results show different values of similarity between the present samples and the other types of staphylococcal enterotoxin, for SEA (USA) was 37–40%. The similarity with bovine-SEC was 65–66% and 39–40% to SEG (Jaban). The similarity with SEE (USA) into isolates was 38–48.

Multiple Sequence Alignments (MSA) showed highly conserved area in all studied sequences, by performing individual multiple sequence alignments we were able to confirm that the selected region to demonstrated high homology between same-toxin sequences and species obtained from different strains (via GenBank), which indicates that this region plays an important role in the function of gene. Clearly, change in amino acid was observed at the positions 165(K) in the first sample (B4-R), 194 (E) in both the first and second samples (B4-R and A6-R), and 197(R) in the second sample (A6-R). As shown in MSA Figure 3, there are some differences in amino acid type, which may result from a change in the nucleotides sequence. This change may affect the biological activity of the enterotoxin and its properties. Amino acid composition for the studied sample showed a visible increase

**Table 2:** Sequence homology between three isolates with other strains used in this study

| | | | *Identities* | | |
|---|---|---|---|---|---|
| *Organism* | *Sequence ID* | *Length* | *Isolate B4-RLQ* | *Isolate A6-RLQ (KM450112)* | *Isolate A5-RLQ* |
| *S. aureus* | ABF93356.1 | 239 aa | 100% | 100% | 95% |
| *S. aureus* | EFB50676.1 | 202 aa | 99% | 99% | 94% |
| *S. aureus* | EHT21119.1 | 144 aa | 87% | 87% | 85% |
| *S. agnetis* | WP_107401511.1 | 215 aa | 68% | 67% | 68% |
| *S. delphini* | WP_096543689.1 | 266 aa | 68% | 69% | 68% |
| *Streptococcus pyogenes* | PZO96497.1 | 260 aa | 61% | 61% | 61% |
| *S. aureus* | AAG29599.1 | 271 aa | 65% | 66% | 65% |
| *S. epidermidis* | WP_002457255.1 | 266 aa | 60% | 61% | 60% |
| *S. aureus* | P0A0L6.1 | 258 aa | 40% | 39% | 40% |
| *S. aureus* | P0A0L1.1 | 257 aa | 40% | 37% | 37% |
| *S. aureus* | P0A0L9.1 | 241 aa | 39% | 39% | 48% |
| *S. aureus* | P12993.1 | 257 aa | 39% | 38% | _ |

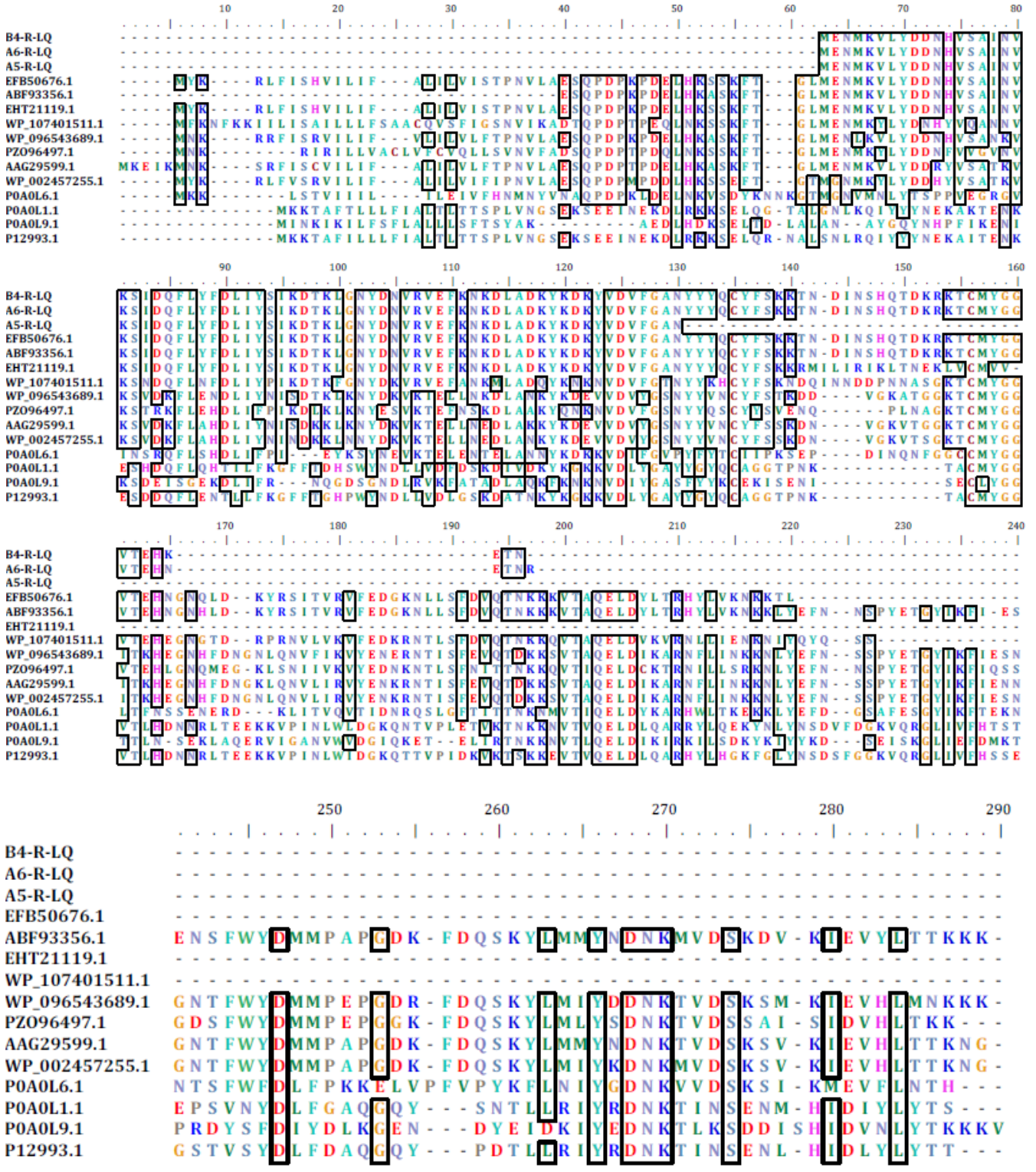


**Figure 3:** Multiple sequence alignment of three isolates (A6-RLQ, A5-RLQ, and B4-RLQ) with other studied species. The arrows indicate nucleotides variations.

in four amino acids (Ala, Cys, Gly, Thr) in compare with its level in RefSeq. Phylogenetic relatedness was determined by analyzing the nucleotide sequences of the gene for the synthesis of staphylococcal enterotoxin B. The MR Bayesian system used to perform the phylogeny of SEB gene. The Bayesian tree presented in Figure 4. This tree is broadly consistent with one

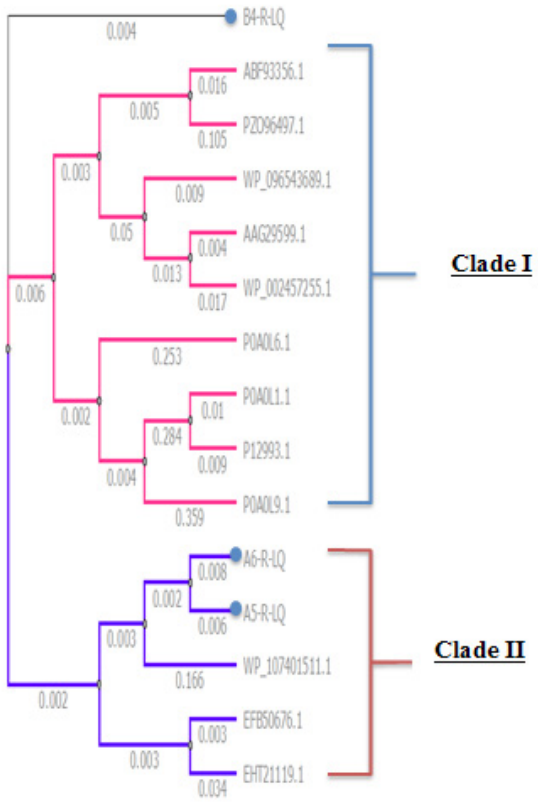


**Figure 4:** Phylogeny tree of SEB gene (A6-RLQ, A5-RLQ, and B4-RLQ) in *S. aureus*

previously published based on the concatenated sequences. The phylogenetic tree of the current study reflected some diversity resistance patterns among the isolates, which was probably generated from an ancestral gene through gene duplication and variation.

The phylogenetic tree consist of two clades (Clade I and II) cleaved from the main root. Clade I, subgroup one, includes *S. aureus* and *Streptococcus pyogenes* and supported with a bootstrap value of 0.005%. These two sequences were branched with other two branches at 0.05% bootstrap value, one of them carry *S. delphini* at 0.9% while the other contain *S. aureus* and *S. epidermidis* at 0.13%. The subgroup-2 contain four sequences belong to *S. aureus* enterotoxins (G, A, C, H) at 0.002%. Clade II includes two of the sequencing samples (A6 and A5) at 0.002% branched from *S. agnetis* at 0.166 in one subgroup with 0.003% bootstrap. Subgroup two, contain two sequences belong to *S. aureus* at 0.003%. One sample (B4-R) separated in unique branch from the root of the tree; this may be indicated special characterization for the first sample Figure 4.

## DISCUSSION

The increasing prevalence of *S. aureus* and its emerging antibiotic resistance in milk and cheese is a part of the serious problem for public health. This study demonstrated that the *seb* protein sequence was found to share similarities amongst the different staphylococcal and streptococcal exotoxin with different levels of similarity. *S. aureus* is the causative agent of mastitis in lactating animals[18]. Other possible sources that contribute to high levels of *S. aureus* in milk are improper hygiene and poor farm management.[19] Milk is a suitable substrate for *S. aureus* growth, and dairy products are familiar sources of intoxication.[20] *S. aureus* can gain access to milk either by direct excretion from udders with clinical or subclinical staphylococcal mastitis or by contamination from the environment during handling and processing raw milk.[21]

The nature of cheese is a complex and dynamic microbial ecosystem characterized by the presence of a large variety of bacteria, yeasts, and molds, some microorganisms including species of lactobacilli or lactococci, are known to contribute to the organoleptic quality of cheese, whereas the presence of other microorganisms may lead to spoilage or constitute a health risk, *S. aureus* is recognized worldwide as an important foodborne pathogen, owing to the production of enterotoxins in food matrices.[22]

The significant differences in toxicity of *S. aureus* isolates from bulk milk and mastitis milk contributed to genetic variation of enterotoxin genes with reference to geographical locations [23, 24] or might be due to differences in the reservoir in the various countries or ecological origin of strains, the sensitivity of detection methods, detected genes and number of samples, and kinds of examined samples included in these studies.[25]

Fooladi *et al.* (2010)[26] investigated the enterotoxigenic of *S. aureus* isolates and found that 9.3% had the *seb* gene. In Basrah, Mohammed *et al.* (2012),[27] found that seb gene was not detected in 57 isolates, which analyzed by the PCR technique.

Jolanta (2015)[28], found that Coagulase-positive staphylococci were found in 71 of 115 (62%) analyzed milk samples. The genes encoding SEB and SEE toxins were not identified. SEB gene was used to estimate the phylogenetic characterization of staphylococcal enterotoxin in local isolates. The results showed it belongs to several species of *staphylococcus* (*S.aureus, S.epidermidis, S. delphini* ) and *Streptococcus pyogenes* bacteria, phylogenetic tree in the present study branched in two clade, clade II hold sequences belong to *S. aureus* enterotoxin but each with different bootstrap value, this may indicate continuous evolution or genetic variation in enterotoxin coding genes in *S. aureus*. The variation in *S. aureus* SEB between these studies isolates might be due to different factors such as type of breed, lactation number, lactation period, the volume of milk produced, animal age, methodology, and or pathogens producing infections.[29] The seb gene resides in one of seven different *S. aureus* pathogenicity islands (SaPIs). Strains harboring different SaPIs carrying seb were reported to vary in SEB levels produced. To date, five different allelic variants of SEB have been described that vary in biological activity. Also, the location of seb gene on mobile genetic elements can result in horizontal gene transfer between the strains of *S. aureus*. For example, it may locate on the chromosome in some clinical isolates, whereas it has a plasmidic location in other strains of *S. aureus*.[30,31]

Clade I, subgroup one, display relationship between seb and the enterotoxin of *Streptococcus pyogenes.* This may be due to similarity in the biological activity in both toxins Based on the strong pyrogenic effect, which was believed to be the primary characteristic of the toxins.[32] The amino terminus of the purified streptococcal superantigen was more homologous to the amino termini of staphylococcal enterotoxins (SEB, SEC1, and SEC3) than to those of pyrogenic exotoxins A, B, C or other streptococcal toxins.[33] The streptococcal proteins are more similar to some of the SEs than some of the SEs is to each other.[34]

Subgroup two, bind bovine *S. aureus* enterotoxin C with *S. delphini* and *S. epidermidis* exotoxins, all of these species return to coagulase-positive staphylococci group which share major common pathogenic characteristics. In subgroup three, we can realize the association between *S. aureus* enterotoxin (SEG, SEA, SEE), which causes food poisoning outbreaks but in different levels of severity.[35] SEs is monomeric proteins produced in a precursor form possessing typical bacterial signal sequences that are cleaved to release the extracellular mature toxins. Within the enterotoxin family, SEA, SEE, and SED fall into one group based upon amino acid identity (52-83% amino acid identity), while SEB and the SECs fall into another group (62–64% amino acid identity) [34].

## CONCLUSION

The increasing prevalence of *S. aureus* and its emerging antibiotic resistance in milk and cheese is a part of the serious problem for public health. The phylogenetic analysis of the SEB protein sequence was found to share a high degree of similarities and highly conserved regions with those detected USA. Also, it found to share a significant degree of similarities with other types of staphylococcal enterotoxins (SEE, SEC, SEG, and SEA) and the exotoxins of *S. epidermidis, S. delphini* and even *Streptococcus pyogenes*. This study will deliver a new informations about seb gene evolution in *S. aureus* compared with other studied species, which helpful for further functional gene analysis.

## ACKNOWLEDGMENT

The authors are grateful to Al Muthanna University for providing all laboratory and other facilities.

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