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Phylogenetic Tree of Gene Enterotoxin B in *Staphylococcus aureus* Isolated from Dairy Products and Human Stool

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ABSTRACT

Collected 280samples from two different sources, which included samples from dairy products and stool samples for intestinal infection patients suspected of suffering from food poisoning according to the diagnosis of the specialist doctor, distributed on 200 samples of stool samples from patients, and the other source of samples included 80 dairy products. For the period from 1/12/2019 to 1/10/2020.For the isolation of *Staphylococcus aureus* and to detect the *seb* gene and characterize from a genetic and phylogeny, the results showed that the *Staph. aureus* bacteria found in 15 isolates with 20.83% of the dairy product samples, while it was found that it recorded an isolation rate of 9.03% of the total of stool samples from patients.Enterotoxin SEB was detected in *Staph.aureus* bacteria and in both sources (dairy products and stool samples) through the use of (PCR) technique. It was proved that the *seb* gene is present in *Staph.aureus* bacteria, as the presence of the *seb* gene was shown at a rate of 33% and 40% for each isolates of the dairy products and stool samples respectively, then analyzed the genetic tree of the *seb* gene encoding for the production of enterotoxin SEB by means of the Mega X program, and the results of the genetic tree analysis showed a clear genetic variation and mutations with global *Staph.aureus* isolates, as our isolates taken from Stool samples of the patients and dairy products in the NCBI Database) and accession numbers were given in the GenBank.

Key words: *Stahylococcus aureus*, Enterotoxin *seb* gene, Phylogenetic Tree.

INTRODUCTION

The genus *Staphylococci* spp. is one of the most important bacteria spread on the skin, mucous membranes, and upper respiratory tract in humans and in many other mammals. It is also found in the environment, soil and air, as it is found in various products and foodstuffs and shows resistance to different environmentalconditions(Vasconceloes and Cunha, 2010).*Staph. aureus* is of great clinical importance and due to its wide spread in nature and its pathogenicity to humans and other epidemics, it has received great attention from researchers all over the worldalthough it is considered a normal flora, it can become an opportunistic pathogen being a common cause of many disease conditions(Tong *et al*., 2015). It can also be responsible for multiple illnesses including

food poisoning (Larkin *et al*., 2009). Food poisoning with *Staphylococcus* is one of the most common foodborne diseases worldwide and results from ingestion of preformatted staphylococcal enterotoxins (SEs) in food as produced by enterotoxic strains(Kadariya *et al*., 2014).*Staphylococcus aureus* produces enterotoxins (SEs) that can be present in toxic doses in spoiled foods, in addition to bad smell or unusual appearance (Argudin *et al*., 2010). It was found that there are five main types of enterotoxins (classic SEs called SEA, SEB, SEC, SED and SEE). New genes encoding intestinal toxins have been identified and they are classified from SEG to SEU. One or more of these genes are believed to cause 95% of staphylococal food poisoning (Michael and Ahmed, 2013).Therefore, due to the risk of infection with this bacterium to which humans are exposed, and its possession of multiple mechanisms in the event of infection this study aimed at the molecular detection of the *seb* gene, as well as the investigation of the evolutionary relationship and convergence ratios (sequence similarity) between local isolates and global isolates in GenBank by conducting a phylogenetic tree.

MATERIALS AND METHODS

Collection of Samples

This study extended for the period between 1/12/2019 to 1/10/2020. During this study 280 samples were collected from two different sources.It was distributed to 200 stool samples from patients attending consulting clinics and from patients visiting consulting clinics and those in hospital at Al-Diwaniyah Teaching Hospital, Children and maternity hospital and health centers in city of Al-Diwaniyah, who suffer from intestinal infection and food poisoning, depending on the diagnosis of the specialist.As for the other source of samples it included 80 samples of Dairy Products from local vendors and local markets in Different areas in city of Al-Diwaniyah.

Isolation and Identification

Stool samples were cultured by planning method on the cultures media Blood agar, mannitol salt agar and MacConkey agar then incubated for 24 hours at 37 ° C (Forbes *et al*., 200).As for dairy products, the method of dilution was used, depending on what was stated in Stukus (1997),Isolates were diagnosed based on the phenotypic properties of bacterial colonies on solid media, and a group of biochemical tests were used, depending on the methods mentioned(Forbes *et al*., 2007; Macfaddin,2000).Bacterial isolates were also diagnosed using the vitec system to diagnose the species of the genus *Staphylococcus*spp.

Bacterial Genomic DNA Extraction

DNA was extracted from the bacteria using a Genomic DNA Extraction kit equipped by the American company Geneaid, and the extraction was carried out according to the manufacturer's

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instructions, and the extracted DNA was examined using a Nano Drop Spectrophotometer for measuring the concentration of nucleic acids, as the DNA was detected for the purpose of Determine the DNA concentration and measure its purity by reading the absorbance at a wavelength ranging between 260 - 280 nm.

Primers

The Primers for screening for the identification of the enterotoxin *seb* gene were designed based on (Jonhson, 1991). These primers were supplied from Macrogen in Koreaand as shown in Table (1).

Table (1)Primers used in this study.

Primer	Sequence		Amplicon
seb gene	**F**	TCGCATCAAACTGACAAACG	**478**
	R	GCAGGTACTCTATAAGTGCC	

Prepare PCR Master Mix

The polymerase chain reaction mixture was prepared using the kit AccuPower® PCR PreMixEquipped by the Korean company Bioneer according to company instructions and as shown in Table (2).

Table (2) components of the polymerase chain reaction.

PCR Master mix		Volume (**ML**)
DNA template		5
Primers	F. primer	1.5
	R. primer	1.5
PCR water		12
Total Volume		20

Gel Electrophoresis

Theelectrophoresis of PCR product of the enterotoxin *seb* gene through gel electrophoresis,By using an agarose gel prepared at a rate of 1.5% under a voltage of 100 volts and a current of 80 amperes for an hour, for the purpose of reading the result of the PCR product.

DNA Sequence Method

A phylogenetic tree analysis was performed to determine the genetic sequence of the enterotoxin *seb* gene in *Staph.aureus* in some samples by molecular genetic analysis using the phylogenetic tree program (Mega X).And calculate the evolutionary distance of strains by the maximum method using (UP GMA), by performing genetic tree analysis between local *Staph.aureus* isolates and global standard isolates, then inserting *Staph.aureus* isolates specific to the seb gene NCPI-GenBank to obtain an accession number in the genebank (GenBank).

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RESULTS AND DISCUSSION

Dairy products and stool samples were cultured on culture media, and bacterial isolates were diagnosed based on the phenotypic characteristics of the bacterial colonies on solid media, The results of this study showed that the number of *Staph.aureus* isolates from dairy products was 15, with an isolation rate of 20.83%. Through these results, we conclude that the presence of these bacteria in a high percentage in these products is an important indicator because it may result in cases of food poisoning as a result of eating these products, When comparing the results of our study with other studies in terms of the percentage of isolation of *Staph.aureus* bacteria from dairy products, we find that it is close to what Santana obtained (2010), as it was found that its isolation rate reached 18.80%, while we find it little compared to what Al-Khafaji and his group obtained (2013) in the samples of dairy products taken from the local markets in Baghdad as their isolation rate reached 48%,As for stool samples the results showed that *Staph. aureus* bacteria formed an isolation rate of 9.03%. The results of this study in terms of isolating *Staph.aureus* bacteria from stool samples agree with the study of Kates and his group (2018), as the rate of isolation reached 9.3%, while This isolation rate does not coincide with what Akingbade and his group (2013) have found, as they found that the isolation rate reached 4.4%. The reason for the discrepancy in these isolation rates may be due to the difference in the size of the samples and may also be attributed to differences in the environmental conditions from which the samples were isolated.

Detection of Gene *seb* Encoding Enterotoxins SEB

The *seb* gene encoding for the production of enterotoxin SEB was investigated in both sources of isolates taken through the use of PCR technique, it was found that the gene amplification product after it was carried on the agarose gel 1.5% and examined under ultraviolet radiation contained five isolates of *Staph. aureus* isolated from dairy products on the *seb* gene at 33% and with a product 478 bp (Fig 1),also found that *Staph.aureus* isolates from stool samples taken from patients were contains of the *seb* gene by 40% with a product size 478 bp (Fig 2).This result was different with Blaiotta and his group (2004) as they did not record the presence of this gene in any of the samples dairy products samples, while the percentage of isolation of this gene from stool samples of patients was higher than that obtained by Shin and his group (2016) as they found that this gene formed an isolation rate of 1.3% from fecal samples associated with food-borne diseases. The production rate of the *seb* gene may be due to various factors, including the type of strain of *Staph.aureus*, which causes infection (Jumaily and Saeed, 2014).

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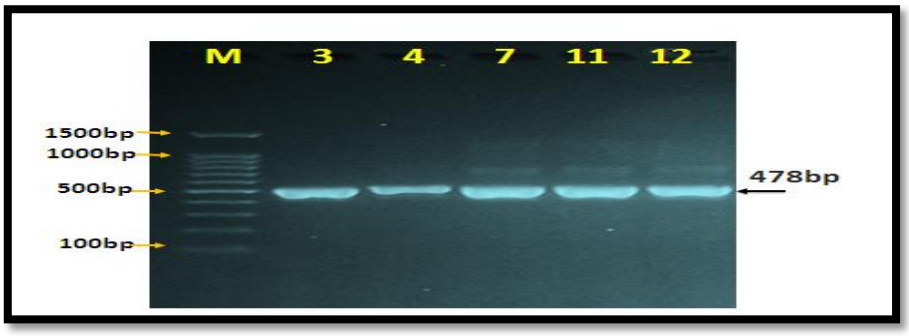

Figure (1) electrophoresis of agarose gel (1.5%), voltages (100) and voltage difference (80) ampere for one hour, which shows the results of the PCR examination for the detection of enterotoxin *seb* in *Staphylococcus aureus* isolated from dairy products. M (Marker ladder 1500-100bp) represents isolates (3,4, 7,11,12) test positive bacterial isolates with a product 478bp.

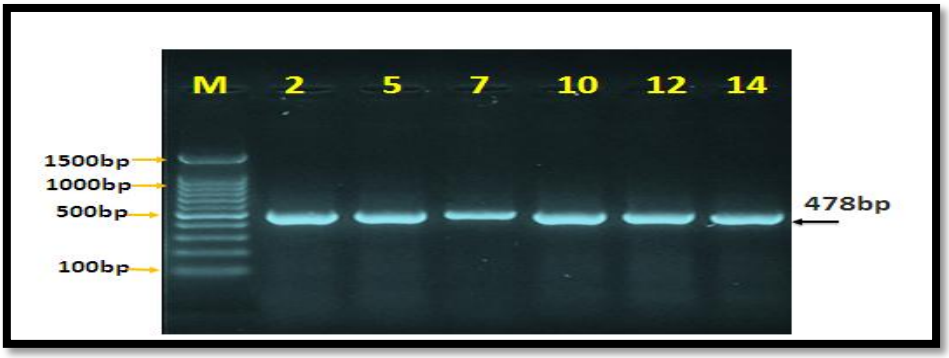

Figure (2) electrophoresis of agarose gel (1.5%), voltages (100) and voltage difference (80) ampere for one hour, which shows the results of the PCR examination for the detection of enterotoxin *seb* in *Staphylococcus aureus* isolated from stool samples. M (Marker ladder 1500-100bp) represents isolates (2, 5,7,10,12,14) test positive bacterial isolates with a product 478bp.

Phylogenetic Tree Analysis of the *seb* gene encoding for the production of enterotoxin SEB

A phylogenetic tree was analyzed for the *seb* gene that encodes for the production of enterotoxin (SEB) in *Staph.aureus*, which was isolated from stool samples from patients and samples of Dairy Products.The data were analyzed sequence based on the tools of the National Center for Biotechnology Information (NCBI), using NCBI Blast by comparing them with the specific source sequences (RefSeq).(Fig 3) shows the results of analyzing the genetic tree for the gene in local *Staph. aureus* isolates and global standard isolates mediated by the Mega X program, as two *seb* gene samples were taken, one from the stool samples of the patients and the second from the samples of dairy products (milk), and after comparing the results with global isolates, the gene in our local isolates showed clear genetic variation (Variation and Mutations), as our isolates taken from stool samples of patients and dairy products were recorded in the center's database The National Bioinformatics Technology (NCBI Database) and accession numbers were given in GenBank (MW084650) and (MW084651) for each of the stool samples and dairy products respectively.

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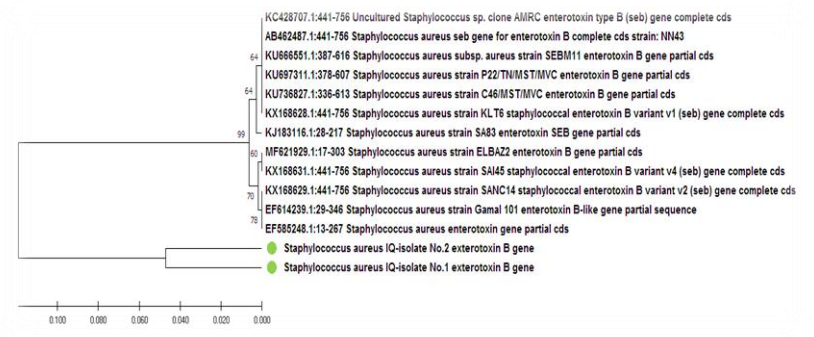

Figure (3): Phylogenetic tree analysis using MEGA X partial sequencing software for the seb gene, in local Staph.aureus isolates with global isolates shown in the genetic tree analysis.

The results presented in Table (3) showed the convergence ratios of our local isolates represented by the two isolates IQ-No.1 and IQ-No.2 from stool samples of patients and samples of dairy products respectively with global isolates, the two isolates gave different proportions that match The reason why our local isolates do not completely match global isolates to biological diversity in many pathogens, which results in the occurrence of large variations. Through this, we conclude that the genetic tree represents an important representative scheme for the relationships between the different types of organisms (Choudhuri, 2014.) These results, which clarified the rates of convergence between *Staph.aureus* isolates and global isolates, came to confirm the correct and accurate diagnosis of our local isolates after they were compared with global isolates, and thus they may be useful from a diagnostic aspect. It is also possible to obtain additional information about the structural and functional relationships of intestinal toxins in particular. There are no studies on the nitrogen base sequences of the *seb* gene at the local level and so this data obtained in our local isolates can be utilized in the NCBI database.

Table (3) Convergence ratios between local and global *Staph.aureus* isolates.

Bacterial Isolates	Sequence ID	Identities	
		(IQ- No.1) MW084650	IQ- No.2)(MW084651
Staph. aureus	KX168631	%80.06	%84.64
Staph. aureus	EF614239	%79.88	%84.42
Staph. aureus	AB462487	%79.75	%83.70
Staphylococcus spp.	KC428707	%79.75	%83.70
Staph. aureus	KX168628	%79.75	%83.70
Staph. aureus	KX168629	%79.75	%84.33
Staph. aureus	KU736827	%81.21	%85.00
Staph. aureus	MF621929	%80.21	%84.43
Staph. aureus	EF585248	81.25%	%85.21
Staph. aureus	KU666551	%82.05	%85.71
Staph. aureus	KU697311	%82.05	%85.71
Staph. aureus	KJ183116	%81.44	%83.25

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The results shown of the alignment analysis in (Fig 4) for the first local isolate of stool samples (IQ-No.1) (MW084650) and (Fig 4) for the second local isolate (IQ-No.2) (MW084651) for dairy products, which showed variations in comparison with global isolates. Through these results, we conclude that there is a variation in the sequence of nucleotides in many sites and this may result in a change in the amino acid sequence. It was found that the enterotoxin SEB shows a high level of variability. In the sequence between different strains, this is attributed to the presence of pathological islets (SaPIs) carrying SEB and correlating them with differences in the levels of production of the *seb* gene (Sato'o et al., 2013, Qasim and Al-Mayali, 2019).

Score	Expect	Identities	Gaps	Strand
226 bits(122)	8e-63	257/321(80%)	13/321(4%)	Plus/Plus

```
Query  1    GCATAATGGATACCAATTACGTATTAACAT-TTGAAGTATTACTGTCAGCAAACTTGACG  59
Sbjct  1    ..........A.........-.-.-...-..A.A.............T..GGT.T....A.  56

Query  60   ATGGTAGAAACTTATTATCTATTGAAGT-GAAAC-CATTAG-TAAAAGGGACTGCCCAAG  116
Sbjct  57   ......A...T.........T....C..AC....TA..A..AA.....T......T....  116

Query  117  AAATCTCCTACCTAACTCGTCACTATTTGCCGAAAGCTATAAAACTCTATGAATTTAACA  176
Sbjct  117  ..T.AGAT.....................GT....AA..A...................  176

Query  177  ACTCGCCTTATGAAACGGGGCG-ATTAAATTTATAGAAAGAGAGAATAGCTTTTCACCTG  235
Sbjct  177  ...................ATAT.................T.............GGTA..  236

Query  236  ACATGACGCCTG-ACCAGGAGATTTC-TTGACCAGTCTAAATTTTCTAA-GTAGTTCGAT  292
Sbjct  237  ......T.....C..........AAAT.......A.......A..-...T.AT..A.A..  295

Query  293  GGTCATAAGATGATTGATTCT  313
Sbjct  296  .A.A....AT..G........  316
```

Figure (4) a multi-alignment analysis of the nitrogen base sequences of the *seb* gene in the *Staph.aureus* isolate (IQ-No.1). The multi-alignment analysis showed a match to the global isolates with the sign (*) as well as with substitution mutations in the *seb* gene.

Score	Expect	Identities	Gaps	Strand
311 bits(168)	3e-88	270/319(85%)	7/319(2%)	Plus/Plus

```
Query  5    GCATAATGGATACCATTTAGTATTAATATAGAAG-ATTACTGTCAGCG-ACTTCACGATT  62
Sbjct  1    ..........A....A....-..A..........T........T..G.T.T..G.A...G  59

Query  63   TTAAAAACTTATTATCTATTGA-ATTGAAACTAATAAGAAAAAAGGAACTGCCCAAGAAA  121
Sbjct  60   G......T.........T....CG.AC..................TG.....T......T  119

Query  122  TCGACTACCTAACTCGTCACTATTTGCCGAAAGCTAAAAAACTCTATGAATTTAACAACT  181
Sbjct  120  .A..T.....................GT....AA..........................  179

Query  182  CGCCTTATGAAACGGGATATATTAAATTTATAGAAAGAGAGAATAGCTTTTCACCATGAC  241
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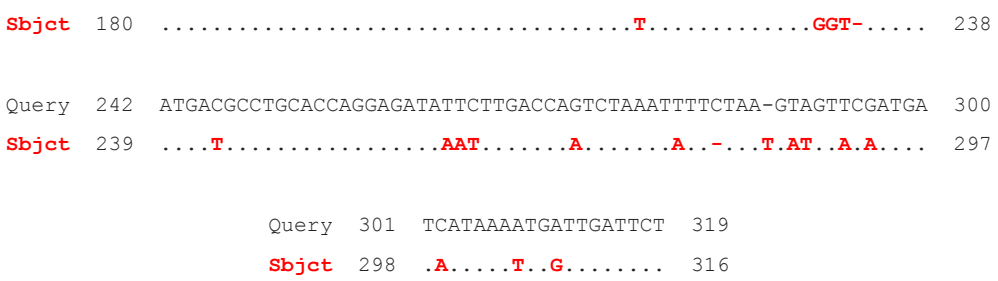

Figure (5) a multi-alignment analysis of the nitrogen base sequences of the *seb* gene in the local *Staph.aureus* isolate (IQ-No.2). The multi-alignment analysis showed a match with the global isolates (*) as well as substitution mutations in the *seb* gene.

CONCLUSION

It was found that *Staph.aureus* bacteria is one of the main pathogens found in dairy products and also its found in hospitals in Diwaniyah city as a pathogen, in addition to being considered a normal flora and thus its presence may result in some health problems,Alsofound from the analysis of the genetic tree of a gene*seb* in local *Staph. aureus* isolateswhen compared with global isolates, showed clear genetic variation and mutations. Therefore, exposure to genetic mutations may cause the emergence of dangerous types in the community of Al-Diwaniyah, which may lead to frequent infections or even difficult treatment.

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