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Detection of Enterotoxin genes and some of Enterotoxin influencing factors of *Staphylococcus aureus* isolates in kerbala city

A Thesis

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بسم الله الرحمن الرحيم

{ وَلَسَوْفَ يُعْطِيكَ رَبُّكَ فَتَرْضَى }

صَدَقَ اللّٰهُ الْعَلِيُّ الْعَظِيْم

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Dedication

To the Prophet Mohammed and his progeny (peace be upon them).

To Imam Al-Mahdi Al- Montazer (peace be upon him).

To those...Who I carry his name proudly... And his affection the reason for my success...I ask God to give him long life ,

Dear father

To my angel in life... To the meaning of love and compassion who rejoice the heart for her existence ,

My darling mother

To my soulmate and my life-long companion…To the one with great heart who share my worrier…

My dear husband

To emeritus and my strength, who removed the thorns from my path to pave my way to the science and gave me love, cooperation and support …

My Dear brothers and their families

Who share my concerns and rejoice by my success ,

My Dear sisters

Heba

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Summary

Nasal swab samples were collected from 332 food handlers working in Kerbala. The largest group of food handlers included in this study was the servants in restaurants (n= 95). In addition, there were three additional large groups of food handlers: Chiefs (n=68), Bakery workers (n=66), and Water plant workers (n=59).

The samples were processed according to the standard microbiological procedure. Cultural characteristics, biochemical assays and API staph were used for phenotypic identification of the isolates. Polymerase chain reaction (PCR) targeting *femA* gene fragment was used for the molecular confirmation of the identification. A total of 100 *Staphylococcus aureus* isolates were recovered from the food handlers, representing a prevalence of 30.1%. Asignificantly high prevalence of nasal swab carriage of *S.aureus* were found among the workers of school foods shops (100%), cafes workers, (85.7%), and sweet shop workers (71%).

Multiplex PCR was used to study the prevalence of staphylococcal enterotoxins gene type a, b, c, d and e , the prevalence of enterotoxin genes were 38% among 100 isolates . 16 (16%) were positive for *sea*, 18(18 %) were positive for *seb*, 8(8%) were positive for *sec* and *see*, 6(6%) were positive for *sed.*

In addition, many strains were found to possess multiple enterotoxin genes. 27% positive for one gene, 6% Positive for two genes 4% positive for three genes, 1% positive for five genes. These results indicate that a significant numbers of food handlers carry enterotoxigenic strains and thus imposing a

risk of transmitting those strain to food during food processing or preparing, packaging etc.

Therefore, a specific measure must be put in place in order to prevent food poisoning which would be the result of transmission of *Staphylococcus aureus* to food products.

To study the effect of culture conditions on the toxin production series of experiments were done , the experiments include three factors (salt,pH,glucose) . For this purpose, 2 strains were selected. The first one was a possessing multiple enterotoxin genes, whereas the second one did n't have any of the studied enterotoxin genes.

High Nacl concentration (12%, 14%) were found to induce toxin production but reduce growth rate , whereas near-neutral pH was found to increase both growth rate and toxin production , and glucose concentration (2.5%) were found to induce toxin production but also reduce growth rate.

According to the results, the conditions that are not supportive for toxin productions include low (pH , glucose concentration and Nacl concentration).

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List of Abbreviations

API-STAPH	Analytical Profile Index for Staphylococci identification
aw	water activity
bp	base pair
DNA	DNA Deoxyribonucleic acid
EDTA	Ethylene Diamine Tetra Acetic Acid
EFSA	European Food Safety Authority
KDa	Kilo Dalton
µg/ml	Microgram per milliliter
µL	micro liter
MLST	multilocus sequence typing
O.D.	optical density
PCR	Polymerase chain reaction
PFGE	pulsed field gel electrophoresis
SEs	Staphylococcal Enterotoxins
SEA	Staphylococcal Enterotoxin A
SEB	Staphylococcal Enterotoxin B
SEC	Staphylococcal Enterotoxin C

SED	Staphylococcal Enterotoxin D
SEE	Staphylococcal Enterotoxin E
sea	Staphylococcal Enterotoxin a gene
seb	Staphylococcal Enterotoxin b gene
sec	Staphylococcal Enterotoxin c gene
sed	Staphylococcal Enterotoxin d gene
see	Staphylococcal Enterotoxin e gene
SFP	Staphylococcal Food Poisoning
SFPOs	Staphylococcal Food Poisoning Outbreaks
Spa	staphylococcal protein A
TBE	Tris Borate – EDTA
TE buffer	Tris-EDTA buffer

Chapter one
Introduction

Introduction

New foodborne diseases have emerged due to several causes such as demographic changes , changes in food production and distribution as well as in food choices, microbial adaptation, and inadequate infrastructure. Increase in the opportunities in commerce and travelling resulted in global spread of local regional foodborne diseases (Anderson *et al*., 2001).

Staphylococcal Food Poisoning (SFP) is an intoxication caused by consumption of improperly prepared or stored foods containing adequate amounts of one (or more) preformed enterotoxin (Schelin *et al*., 2011; Niveditha *et al*., 2012).

Staphylococcal intoxication is often associated with the kinds of foods that include poultry , and egg products ,meat and meat products, , milk and dairy products, bakery products,especially cream-filled pastries and cakes, sandwich fillings and salads, because of the capacity of *Staphylococcus aureus* (*S.aureus*) to grow at relatively low water activity (aw=0.86) , so it has been implicated salted food products in food poisoning (Tasci *et al*., 2011).

Staphylococcus aureus is found persistently or temporarily in human nasal microbiota, without causing any symptoms. The presence of these bacteria in food occurs frequently due to inappropriate manipulation of food by carriers of this microorganism (Hatakka *et al*., 2000).

The large numbers of carriers (more than 30-50% of the population), the contamination of food or one of its gradients during handling, storage at unsuitable temperatures , the capacity of the microorganism to develop in a

wide range of pH, free water concentrations, and sodium chloride concentrations- and therefore a wide range of food products- are the main epidemiological features that create the ideal conditions for an outbreak of SFP; work surfaces and equipments used to prepare foods are an important source for indirect contamination (Di Giannatale *et al*., 2011).

Previous studies demonstrated that employees working in the food industry are the main source for spreading foodborne diseases or the epidemic ones (Parashar *et al*., 1998 ; Girish *et al*., 2002). Food handlers have been implicated in a plethora of foodborne diseases. It has been reported that one of the important pathogens often transmitted via food contaminated by infected food handlers is *S.aureus* (Wertheim *et al*., 2005 ; Verkaik *et al*., 2011). Nasal and hand carriage of enterotoxigenic *S. aureus* by food handlers is an important source of staphylococcal food contamination in restaurants and fast food outlets, therefore it is important to detect *S.aureus* carriage among food handlers to prevent possible food contamination by them resulting in food poisoning (Colombari *et al*., 2007; Udo *et al*., 2009).

Many methods have been developed in order to detect the toxins quickly with specificity and sensitivity which include: the following an immunoassay single diffusion tube test, polymerase chain reaction (PCR), an enzyme-linked immunosorbent assay (ELISA), a reversed passive latex agglutination assay (RPLA), and the Ouchterlony double diffusion method (ODD)(Sharma *et al*., 2000 ; Di Pinto *et al*., 2004).

Several factors must be considered when choosing a method for enterotoxin detection, such as sensitivity, specificity, reproducibility, cost, labor, rapidity, convenience, and the number of samples (Harrison, 2015).

Aims of the study:

1. Study the prevalence of *S.aureus* nasal colonization among food handlers in Holy Kerbala City.
2. Simultaneous detection and determination of the classical enterotoxin genes in *S.aureus* isolates.
3. Detection the effect of cultural conditions on the production of enterotoxins by selected *S.aureus* isolates.

Chapter two

Literature Review

2. Literature Review

2.1. Food poisoning

Food borne infections is the consumption of bacteria, their toxins or viruses, which may be existing in already contaminated food, or derived during processing from other foods by cross contamination (from catering staff hands , equipment or surfaces), or, less likely, from carriers. On top of that, poisonous chemicals and/or other harmful substances may also be causes food borne diseases if they are present in food. People can become ill if a pesticide is inadvertently added to a food, or if naturally poisonous substances are used to prepare a meal (Walker & Jones, 2002 ; Winn and Koneman, 2006).

There are three types of food poisoning : Infection , intoxication, and intermediate (Bari & Ukuku, 2015). The oral ingestion of adequate amounts of viable microorganisms cause infection and the onset of symptoms is normally delayed, reflecting the time required for the development of an infection. Enteric viruses, *Salmonella*, *Campylobacter* and *Vibrio* species considered examples of food poisoning that cause infection . On the other hand the ingestion of toxins that have been preformed in the food is called intoxication . Therefore, there is no necessity for live organisms to be present and the onset of the symptoms is rapid. *S.aureus* and *Bacillus cereus* are examples of food intoxication. When live bacteria are ingested and subsequently produce a toxin in the host , it causes intermidate food poisoning , as in the case of *Clostridium perfringens* food poisoning (Teplitski *et al*., 2009).

The food borne diseases in humans caused by more than 250 different kinds of bacteria,viruses, parasites, toxins, metals, and prions (Nyenje & Ndip, 2013). Although more than 50% of foodborne illnesses caused by viruses ; generally hospitalizations and deaths associated with foodborne infections occur due to bacterial agents. The toxin from the "disease-causing" microbe , or the human body's reaction to the microbe itself can cause wide range of infections extends from mild gastroenteritis to life threatening neurologic, hepatic, and renal syndromes (Teplitski *et al*., 2009).

Prevention measures need to prevent or limit contamination all the way from farm to table , although most of the foodborne diseases are preventable but there is no simple one-step prevention measure.The spread of microorganisms can be reduce and foods contamination can be prevented by following a variety of good agricultural manufacturing and kitchen practices. Careful review of the whole food production process can identify the principal hazards, and the control points where contamination, limited, or eliminated. the Hazard Analysis Critical Control Point (HACCP) system is a formal method can be used to evaluate and control the risk in foods (Chipabika, 2015).

The infants, young children, the elderly and the immunocompromised persons are the high risk groups for foodborne diseases (Fleury *et al*., 2008).

2.1.1. Microorganisms causing food poisoning

The causing agents of foodborne illness that have public health importance are listed below

Table(2-1)the microorganisms causing food poisoning (Jahan, 2012).

Bacterial agents	Viral agents	Parasitic agents
Listeria monocytogenes	*Norovirus*	*Cryptosporidium sp.*
Staphylococcus aureus	*Hepatitis A*	*Cyclospora cayetanensis*
Bacillus cereus	*Hepatitis E*	*Giardia lamblia*
Bacillus anthracis	*Adenovirus (Enteric)*	*Entamoeba histolytica*
Clostridium botulinum	*Rotaviruses*	*Balantadium coli*
Clostridium perfringens		
Clostridium difficile		
Salmonella spp		
Shigella spp		
Campylobacter spp		
Escherichia coli 0157:H7		
Yersinia enterocolitica		
Brucella spp		
Vibrio Cholerae		

2.2. *Staphylococcus aureus*

S.aureus is Gram-positive bacterium belonging to the family Staphylococcaceae and is often found as a commensal on the skin, skin glands and mucous membranes particularly in the nose of healthy individuals (Plata *et al*., 2009).

S.aureus is responsible for toxin-mediated diseases, such as the Toxic Shock Syndrome (TSS), Kawasaki's Syndrome and staphylococcal food poisoning . It is an extraordinarily versatile pathogen ,it can cause a large spectrum of infections in humans from mild to severe and fatal , it is also economically important when infecting animals, able to cause superficial lesions and systemic infections, (Leung *et al*., 1993 ; Vasconcelos and Cunha, 2010). In addition it cause endocarditis, septicemia,osteomyelitis, pneumonia ,abscesses, wound infections, impetigo and cutaneous rash.The difference in the clinical manifestations is mostly dependent on the numerous virulence factors produced by each strain (Le Loir *et al*., 2003).

S.aureus in the laboratory may be observed as single cells, in pairs or as grape-like irregular clusters. It is characterized as coagulase and catalase positive, non-motile, non-spore-forming and as facultative anaerobic (Winn and Koneman, 2006). It grows in a medium containing 10% sodium chloride and at a temperature ranging from 18°C to 40°C. Relatively simple biochemical tests can be used to differentiate *S.aureus* and the other staphylococci. It has positive reactions for heat-stable nuclease, alkaline phosphatase and mannitol fermentation (Murray *et al*.,2002).

S.aureus requires B vitamins (thiamine and nicotinic acid), inorganic salts and amino acids as a nitrogen source, especially arginine, cystein, proline and valine for growth. Glutamic acid, leucine and tyrosine are not required for growth, but they are essential for enterotoxin production. Deprivation of any amino acid is much less responsive in SEA production than for SEB or SEC production. Arginine seems to be essential for enterotoxin B production (Medveďová and Valík, 2012).

Research conducted by potter and leistner in (1978) found that *S.aureus* can grow at a water activity as low as 0.854 though enterotoxin is usually not produced below 0.9 (James, 2008).

S.aureus is able to grow in a pH over a range from 4.5 to 9.3 (Bergdoll and Lee Wong ,2006). The organisms is able to utilize several different carbohydrate during respiration however under anaerobic condition (Minor & Marth, 1976).

The cell wall of *S.aureus* is also very thick in comparison with other gram-positive bacteria this increased thickness provides organisms with a very high internal pressure making it nearly impossible for many antimicrobial drugs to enter the cell (Freeman-Cook *et al*., 2006). As with all gram-positive bacteria, Peptidoglycan is the basic component of the cell wall and makes up 50% of the cell wall mass (Waldvogel, 1990). Another cell wall constituent is a group of phosphate-containing polymers called teichoic acids which contribute about 40% of cell wall mass (Knox & Wicken, 1973). Some strains may also exhibit the presence of protein A that can comprise up 7% of

the cell wall and may coat the outside of the cell , both teichoic acid and protein a work to increase the virulence of the microorganisms (Gao & Stewart, 2004).

2.2.1. Staphylococcal food poisoning (SFP)

Staphylococcal food poisoning (SFP) is an intoxication that results from the consumption of foods containing adequate amounts of one (or more) preformed enterotoxin (Dinges *et al*., 2000 ; Le Loir *et al*., 2003). The toxins of *S.aureus* are known as enterotoxins because they are able to promote water loss from the small intestinal mucosa resulting in vomiting and diarrhea (Martin *et al*., 2003).

The symptoms of SFP have a rapid commencement (2–8 h),which include nausea, violent vomiting, and abdominal cramping, with or without diarrhea (Tranter, 1990 ; Balaban and Rasooly, 2000 ; Murray, 2005).The disease is usually self-limiting and typically resolves within 24–48 h after onset (Murray, 2005).Complete recovery may sometimes take longer depending on the intensity of symptoms,occussanily, in cases of dehydration, collapse or pallor, hospitalization may be needed (Argudin *et al*., 2010). There are several factors affecting the symptoms including the susceptibility of individual, type of *S.aureus* enterotoxin (SET), amount of consumed food and amount of SETs in the food (Jablonski and Bohach, 1997).Fatalities are rare but deaths have been reported among the elderly, infants and severely weakened persons (EFSA, 2009).

2.2.2.Types of foods involved with staphylococcal food poisoning

Foods requiring much processing by handlers, like salads and sandwiches are commonly contaminated by enterotoxin producing strains of *S. aureus*. Though the organism is heat labile and can be killed during cooking or processing, the toxins are extremely heat stable and can remain after cooking and cause illness (Holmberg and Blake, 1984). Contamination is mainly associated with improper handling of cooked or processed foods followed by storage conditions which allow growth of *S.aureus* and production of the enterotoxins,because *S.aureus* does not compete well with indigenous microbiota in raw foods (Stewart, 2005).

In 2009 the European Food Safety Authority (EFSA) reported that the two main food vehicles in verified outbreaks of food poisoning caused by staphylococcal toxins include the cheese followed by mixed or buffet meals (EFSA, 2011).The large numbers of carriers (more than 30-50% of the population), the contamination of food or one of its gradients during handling, storage at unsuitable temperatures and the capacity of the microorganism to develop in a wide range of pH, free water concentrations, and sodium chloride concentrations and therefore a wide range of food products are the main epidemiological features that create the ideal conditions for an outbreak of SFP. Work surfaces and equipment used to prepare foods are an important source for indirect contamination (Di Giannatale *et al*., 2011).

Five conditions was required to induce Staphylococcal Food Poisoning Outbreaks (SFPOs) these include :

1. A source containing enterotoxin-producing staphylococci: raw materials, healthy or infected carrier.
2. Transfer of staphylococci from source to food: dirty food preparation tools due to poor hygiene practices.
3. Food composition with favorable physicochemical characteristics for *S.aureus* growth and toxinogenesis.
4. Favorable temperature and sufficient time for bacterial growth and toxin production.
5. Ingestion of food containing sufficient amounts of toxin to provoke symptoms.

Most SFPOs arise due to poor hygiene practices during processing, cooking or distributing the food product (Hennekinne *et al*., 2010)

2.2.3. Epidemiology of staphylococcal food poisoning

Staphylococcal food poisoning is a common disease whose real incidence is probably underestimated for a number of reasons, which include misdiagnosis, unreported minor outbreaks, improper sample collection and improper laboratory examination ,the control of this disease is of social and economic importance ,In fact, it represents a considerable burden in terms of loss of working days and productivity, hospital expenses, and economical losses in food industries, catering companies and restaurants (Mead *et al*., 1999 ; Balaban & Rasooly, 2000 ; Le Loir *et al*., 2003 ; Chiang *et al*., 2008,).

Regarding food borne outbreaks, the SFPs caused 5.5% of the total number of reported foodborne outbreaks inside the European Union in 2008 (EFSA, 2009). While in Sweden in the period 2003-2009, 111 cases and 30 SFPOs were reported, representing 1% and 2% of the total reported cases and outbreaks, respectively (Rosengren, 2012).

2.3. Enterotoxin of *staphylococcus aureus*

Staphylococcal enterotoxins (SEs) are synthesized by *S.aureus* throughout the logarithmic phase of growth or during the transition from the exponential to the stationary phase and it may represent a potent gastrointestinal exotoxins (Derzelle *et al*., 2009). It is short single polypeptides chain of approximately 600 to 800 amino acids, with molecular weight of about 26-28 KDa (Muller-Alouf *et al*., 2001). Forming two unequal domains, A and B. The A domain is involved in binding to T-cell receptor sites it contains both ,amino and carboxyl termini, and it is larger than B domine (Dinges *et al*., 2000 ; Argudin *et al*., 2010). B Domain contains a cysteine loop that suggested to stabilize a necessary conformation for the emetic (vomiting) property (Hovde *et al*., 1994). Most of the genes that coding SEs are located on mobile elements such as plasmids,pathogenicity islands or Bacteriophages (Lindsay *et al*., 1998).

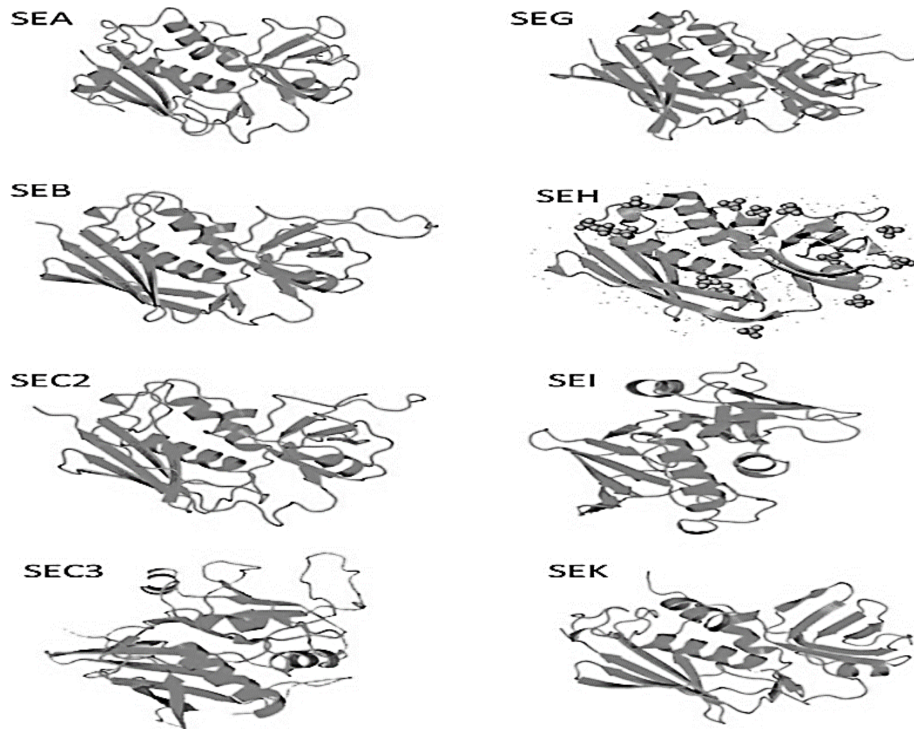

Figure (1-1) 3D structure of various staphylococcal enterotoxins. SEA: Staphylococcal enterotoxin A. SEB: Staphylococcal enterotoxin B SEC2: Staphylococcal enterotoxin C2 .SEC3: Staphylococcal enterotoxin C3 .SEG: Staphylococcal enterotoxin G .SEH: Staphylococcal enterotoxin H (green) with sulfate ions and water.SEI: Staphylococcal enterotoxin I.SEK: Staphylococcal enterotoxin K (Hennekinne *et al*., 2012).

SE proteins may not be completely denatured by mild cooking of contaminated food because it has a remarkable ability to resist heat and acid. 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, the enterotoxins can easily outlast the bacteria that produce them, due to their resistance to inactivation by gastrointestinal proteases including pepsin, trypsin, rennin and papain (Le Loir *et al*., 2003 ; Pinchuk *et al*., 2010).They are active in high nanogram to low microgram quantities (Larkin *et al*., 2009). As little as 20-100 ng SET may be enough to cause SFP (Asao *et al*., 2003).

2.3.1. Type of staphylococcal enterotoxin

There are 24 different types of SEs that have been described, but the toxicity has not been determined for all. SEA, SEB, SEC, SED, and SEE are considered the classical SEs that have been studied and are better understood than the newly described SEs (SEG-SEU) (Schumacher-Perdreau *et al*., 1995 ; Sharma *et al*., 2000).

SEA is the most common toxin in *staphylococcus*-related food poisoning ,while SEB is associated with food poisoning, it has been studied for potential use as an inhaled bioweapon (Ler *et al*., 2006).The second most common staphylococcal toxin is SED which is associated with food poisoning worldwide only very small amounts of this toxin sufficient to induce food poisoning, SEE has also been documented in some cases of food poisoning, while SEF has been implicated in toxic shock syndrome (Pinchuk *et al*., 2010) .SEG, SEH, and SEI were associated with one of the food poisoning outbreaks in Taiwan but they are not studied as well as the others toxins (Chen *et al.*, 2004). SEH has been also involved in massive food poisoning associated with the reconstituted milk consumption in Osaka, Japan in 2000 (Ikeda *et al*., 2005).

2.4 Genetic characteristic of *S.aureus*

The genome of *S. aureus* is a circular chromosome that is 2.8-2.9 Mbp (Mega-base pairs) in size, with a G+C content of about 33%.The chromosome encodes approximately 2700 CDSs (protein coding sequences) as well as structural and regulatory RNAs. It has been proposed that the *S.aureus* genome is composed of the core genome, accessory component and foreign genes (Plata *et al* .,2009).

The core genes are present in more than 95% of isolates, represent 75% of any *S. aureus* genome and determine the backbone of the genome. The organization of the core component is highly conserved and the identity of individual genes between isolates is 98–100%. The majority of core genes are associated with fundamental functional categories of housekeeping functions and central metabolism.The accessory component includes genetic regions present in 1–95% of isolates and accounts for about 25% of any *S. aureus* genome. It typically consists of mobile genetic elements that have or previously had the ability of horizontal transfer between strains. These genetic elements include pathogenicity islands, genomic islands, prophages, chromosomal cassettes and transposons (Lindsay & Holden, 2004).

Plasmids are auto-replicating DNA molecules. Staphylococci typically carry one or more plasmids per cell and these plasmids have varied gene content. Staphylococcal plasmids can be classified into one of the three following groups: (1) small multicopy plasmids that are cryptic or carry a single resistance determinant; (2) larger (15–30 kb) low copy (4–6/cell) plasmids, which usually carry several resistance determinants; and (3) conjugative multiresistance plasmids. Larger plasmids undergo theta replication (a DNA replication mechanism that resembles the Greek letter theta), whereas small plasmids usually replicate by the rolling-circle mechanism (Malachowa & DeLeo, 2010).

2.4.1 Enterotoxin encoding genes

Genes encoding for SE have different genetic supports , most of which are mobile genetic elements . For examples, *sea* gene, composed of 771 base pairs, encodes an enterotoxin A precursor of 257 amino acid residues and

carried by a family of temperate phages, *seb* is an open reading frame encoded the enterotoxin B precursor that consisted of 266 amino acids and chromosomally located in some clinical isolates ,whereas it has been found in a 750 kb plasmid in other *S.aureus* strains. *sec* is encoded by a gene located on a pathogenicity island this gene contains 798-base-pair open reading frame that encodes a protein of 266 amino acid residues, *sed* is located on a plasmid and *see* is carried by a defective phage. The main regulatory system controlling the gene expression of virulence factors in *S.aureus* is the accessory gene regulator that acts in combination with the staphylococcal accessory regulator. But som not all of the SE genes are controlled by the *agr* system. The *seb* ,*sec* and *sed* gnes have been demonstrated to be *agr* dependent , wherease *sea* and *sej* are *agr* independent (Le Loir *et al*., 2003).

SaPIs are mobile pathogenicity islands and are very common in *S. aureus*. They occupy specific sites in the chromosome and carry the genes for many SAgs, including *se*-genes and the toxic shock syndrome (*tst*)gene. The *seb*, *sec* and *sed* genes are regulated by the accessory gene regulator (Agr) system. The Agr system is a quorum sensing system that controls the expression of a number of genes connected to virulence. Quorum sensing is a cell density-dependent system for extracellular signalling between bacteria, and may explain why high bacterial levels are needed before SET is produced (Rosengren, 2012).

2.5. Food handlers and food poisoning

Food handlers are carriers of enteric pathogens (Muhonja &Kimathi, 2014). It plays an important role in food safety and in the occurrence of food poisoning because they may introduce pathogens into food during preparation

(Lillquist *et al*., 2005). The majority of food borne disease outbreaks result from inappropriate food handling practices (Jones & Angulo, 2006).

Food Poisoning affects hundreds thousands of people each year and causes deaths (Muhonja & Kimathi, 2014). A study in the USA suggested that improper food handling practices in food serving establishments contributed to 97% of foodborne illnesses, thus employees should pay attention to their personal hygiene (Howes *et al*., 1996).

The main source of food contamination, via manual contact or through respiratory secretions are food handlers carrying enterotoxin-producing *S. aureus* in their noses or on their hands. In fact, *S.aureus* is a common commensal of the skin and mucosal membranes of humans, with estimates of 60% for intermittent colonization and 20–30% for persistent (Kluytmans & Wertheim, 2005). Food poisoning outbreaks mostly occur when cooked foods are handled by persons who carry the pathogen in their nares or on their skin, (Portocarrero *et al*., 2002).

Because *S.aureus* does not compete well with indigenous microbiota in raw foods, contamination is mainly associated with improper handling of cooked or processed foods, followed by storage under conditions which allow growth of *S.aureus* and production of the enterotoxin(s) (Stewart, 2005).

People in the food production and food service industries should be well trained and motivated to follow good personal hygiene practices, to use correct hand washing procedures and to follow these procedures while working in order to prevent the spread of infection (Martinez-Tome *et al*., 2000). Hand washing sinks can be sources of pathogenic bacteria because they

usually contain stagnant water that supports the growth of microorganisms, which can be transferred to hands during hand hygiene practices (Griffith *et al*., 2003).

2.5.1. Carriage of *staphylococcus aureus* among food handlers

S.aureus is fairly ubiquitous in nature and can be found in water, raw milk, air, decaying vegetation and sewage, though the mucous membrane and skin of mammals including humans, are its primary reservoir (Freeman-Cook *et al*., 2006).

It is colonies the skin and mucosae of human beings and several animal species . Although , multiple body sites can be colonized in human beings but the anterior nares of the nose is the most frequent carriage site for *S.aureus* (Wertheim *et al*., 2005). Of the possible skin colonization sits, rectal carriage seems to be the most significant because it may lead to colonization of the gastrointestinal tract (G.I.). Research has shown that people who carry *S. aureus* in both the nasal and rectal region are more likely to develop an infection than those who only exhibit nasal carriage (Squier *et al*., 2002).

In carries , the main habitat of *S.aureus* is the nasopharynx, a site where strains can persist as transitory or persistent members of the normal microbiota without causing any symptomatology (Hamdan-Partida *et al.*, 2010).

S.aureus nasal carriage may be classified into three different groups; persistent carriers (~20 %) presumed to always carry the bacterium; intermittent carriers (~60 %), who sometimes carry the bacterium; and non-carriers (~20 %), presumed never to carry the bacterium (Kluytmans *et al*.,

1997). These different group can be further broken down by population demographics including gender ethnicity ,age and overall health. A data study carried out at the medical university of South Carolina found that men are more likely than women to harbor *S.aureus.* However , there are no gender-based carriage different in infants (Mainous *et al*., 2006). Children have higher persistent carriage rates than adults (Noble *et al*., 1967 ; Armstrong-Esther, 1976). Rates vary substantially with age, falling from approximately 45% during the first 8 weeks to 21% by 6 months (Francis *et al*., 2005).

Lastly, carriage of *S.aureus* can be affected by the overall health of the population. HIV-positive people or those suffering from chronic disease such as AIDS have increased carriage rates, probably due to decreases immune function though the reason is still unclear because of the extensive use of antibiotic in these demographic groups (Kluytmans *et al*., 1997).

2.5.2. Transmission of *Staphylococcus aureus*

The high percentage of the human population who carries *S.aureus* on their skin and nares makes humans routs is considered the most important transmission routes into the food, also *S.aureus* can be transferred through air, dust and food contact materials (Kluytmans & Wertheim, 2005).

S.aureus can be spread through skin fragments or respiratory droplets produced when people cough or sneeze next to people, other sources of contamination with *S.aureus* may include meat grinders, knives , storage containers, and cutting blocks. Inadequate refrigeration during storage or a prolonged use of warming plates (in case when the food is hold below 60°C or the food is prepared too much in advance) when the food is served

considered high risk factors causing possible *S.aureus* intoxication. Animals are considered another way of transfering *S.aureus*, through especially in raw foods. The skin or feathers are regularly contaminated with *S.aureus* and contamination of dressed carcasses is common and is unavoidable (AlBoort *et al*.,2012).

There are many factors that can be serve as vehicles in the transfer of *S.aureus* to foods which include air, dust, and food contact surfaces that have been frequently incriminated in staphylococcal intoxication (Wieneke *et al*., 1993 ;Tamarapu *et al*., 2001).

Food poisoning bacteria may be brought into the food premises by either food handlers, raw foods, insects, rodents, animals and environmental pollution including soil and dust (Chipabika, 2015).

2.5.3. Control measure food handlers

The word hygiene usually refers to cleanliness and especially to any practice which leads to the removal or reduction of harmful infectious agents (Jumaa, 2005). Hand-washing which is a simple and effective way to cut down on cross-contamination is too often forgotten (Rippel, 2002).

The level of personal hygiene in the visited shops needs rapid intervention from both of the responsible authorities and food handlers to ensure the safety of their food products (Bakr *et al*., 2004).

The US FDA stated that food employees shall clean their hands and exposed portions of their arms for at least 20 seconds while paying attention to removing soil from underneath the fingernails and creating friction on the

surfaces of the hands and arms, finger tips, and areas between the fingers (Fawzi *et al*., 2009).

None of the handlers observed to wore gloves during handling of food products but instead use bare hands, knives or the wrapping plastic sheaths. A considerably significant amount of bacteria have been found on bare hands compared with the amount of bacteria on gloved hands (Cakıroglu &Ucar, 2008).

Everyone, including farmers and growers, manufacturers and processors, food handlers and consumers have responsibility to assure that food is safe and suitable for consumption because effective hygiene control is vital to avoid the adverse effects to human health and economic consequences of foodborne illness, foodborne injury and food spoilage (Chipabika, 2015).

2.6. Typing of *Staphylococcus aureus*

To prevent and control outbreaks of *S.aureus,* and for epidemiological investigations, appropriate typing methods are needed. Numerous methods both phenotypic and genotypic, have been used for the typing of *S.aureus* (Mulligan & Arbeit, 1991). Numerous techniques are available to differentiate *S.aureus*, and isolates. Historically, isolates were distinguished by phenotypic methods, including antibiotic susceptibility testing and bacteriophage typing. Both methods have limitations, as genetically unrelated isolates commonly have the same antibiogram, and many *S.aureus* isolates are non-typeable by phage typing (Shopsin & Kreiswirth, 2001).

The classification of isolates is mostly based on molecular methods, which usually provide better discriminatory power than phenotypic methods

(Faria *et al*., 2008). The most used methods for typing of *S.aureus* are pulsed field gel electrophoresis (PFGE), regarded as gold standard, multilocus sequence typing (MLST) and staphylococcal protein A(spa). The choice of method depends on the problem to be solved and the epidemiological situation in which the method is going to be used, as well as the time and geographical extent of its use (David *et al*., 2013).

There are three related reasons for carrying out *S.aureus* typing. Firstly, typing allows the detection of transmission events and patterns, from within a single healthcare facility, to between continents, and so is central to the practices of infection control and public health microbiology. Secondly, typing allows the inference of virulence and resistance properties of specific isolates and so can directly influence clinical decisions. Finally, typing reveals the population structure and provides insight into the general principles of the species natural history (Aires de Sousa & de Lencastre, 2004).

2.7. Detection of staphylococcal enterotoxin

Many methods have been developed in order to detect the toxins quickly with specificity and sensitivity such as: an immunoassay single diffusion tube test, Polymerase chain reaction (PCR), an enzyme-linked immunosorbent assay (ELISA), a reversed passive latex agglutination assay (RPLA), and the Ouchterlony double diffusion method (ODD) (Sharma *et al*., 2000 , Di Pinto *et al*., 2004).

Several factors must be considered when choosing a method for enterotoxin detection, such as sensitivity, specificity, reproducibility, cost, labor, rapidity, convenience, and the number of samples (Harrison, 2015).

2.7.1. Biological assays for detection of enterotoxins

Biological assays were employed in the detection of staphylococcal enterotoxins. It is involved the administration of samples suspected of containing a toxin to human volunteers or susceptible animals. Biological assays have also involved the administration of the sample suspected to contain the toxin via a catheter into the stomach of the susceptible animal. The animal is observed for at least five hours, and if it develops any emetic response, then the sample is considered to contain the toxin. Initially, in order to establish a link between the enter toxicity of foods and organisms isolated from foods, human volunteers were used. Later on, young cats were used in most of the bioassays, some of the earlier assays were involved with oral administration of the pure toxins to cats and studying the response in the animals (Panneerseelan & Muriana, 2008).

One advantage of bioassays is that the biological activity of the toxin can be detected. In 1953, a monkey challenge test was developed and it tested the effect of administering toxins (culture filtrates) orally to monkeys (Surgalla et al., 1953). However the use of monkeys became limited as these animals were very expensive, difficult to maintain in captivity, and were also not readily available. The bioassays had many disadvantages. The use of animals became difficult as some of the animals did not produce emetic responses when toxins were administered orally. TShe emetic action of enterotoxins on cats and found that emesis did not occur when toxin was administered orally. Cats and dogs showed emetic response only after intravenous inoculation (Kocandrle *et al*., 1966).

The animal assays were also complicated and the results were not confirmatory. In case of intravenous injection of cats and dogs, the emetic response was also caused due to some non specific components. Besides, these disadvantages, the cost of the animals and their maintenance was very high. Hence, these tests became unreliable and the need for more specific and sensitive assays lead to the development of serological assays (Panneerseelan & Muriana, 2008).

2.7.2. Enzyme-linked immunosorbent assay (ELISA)

The ELISA method is commonly used because reagents are commercially available and sensitivity of the test is reliable. Clarisse *et al* .(2013) was used a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) to detect SEA in dairy products and different foods. The authors found that this method was quick, easy, and could be used for routine food product SEA quantification.

The cost of the test is high and detection of SEs is currently limited to the classical types, so therefore it is not an ideal method for hospitals or the food industries to use (Harrison, 2015).

The ELISA detection range varied based on the enterotoxin type being detected, but for all SEs the detection concentration was much lower than the ranges reported for the ODD test (Adesiyun *et al*., 1992).

2.7.3. Reversed passive latex agglutination assay (RPLA)

The RPLA test is the method that continues to be superior (Zouharova & Rysanek,2008).When compared to other methods for detecting

enterotoxins. The SET-RPLA kit is commercially available and is sensitive and specific for the classic types of SEs (Harrison, 2015).

The incubation period is also less than 24 hours and the results can be viewed with the naked eye, unlike the ELISA. In 1988, Fijukawa and Igarashi developed a method for a rapid RPLA test with high-density latex particles that uses only a 3 h incubation time, but further testing has not been presented. Their data suggested that their method was highly specific and sensitive for detection of SEs. There are still some limitations related to cross reactions that lead to false positives (Harrison, 2015).

Compared RPLA and immunoblotting methods for SE detection. Their results showed that SET-RPLA is quick, sensitive, and specific for the enterotoxins tested (SEA-SED) and recommended that it continue to be used to test numerous food samples for routine monitoring (Di Pinto *et al*., 2004).

The RPLA test detected a range from 0.5 to 2.25 ng/mL (Adesiyun *et al*., 1992).

2.7.4. The Ouchterlony double diffusion method (ODD)

The ODD test was developed by Orjan Ouchterlony, and many versions and modifications have been performed (Ouchterlony, 1968).

Double diffusion refers to the fact that both an antigen and an antibody are diffusing through a gel. When a reaction between the two take place, a line of white precipitate forms in the gel giving a positive reaction. This test was developed to determine the concentration of antigen or antibody needed for

detection or to match an antigen with its appropriate antibody. A modification of the ODD test has been developed by (Casman,1960). this method places the antiserum in a centrally located well in a shallow layer of agar in a petri dish and antigens are added to peripheral wells (Harrison, 2015).

The modified ODD test could only detect concentrations of 5μg/mL (Adesiyun *et al*., 1992).

2.7.5. Molecular methods

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude,generating millions copies of a particular DNA sequence (Joshi & Deshpande, 2011).

PCR is commonly used for enterotoxin gene detection, or for confirmation of results from other tests, it is a good way to detect the genes for the enterotoxins, but it is limited to the gene and not the production of the toxin (Morandi *et al.*, 2007). It assays used to identify the pathogen and its enterotoxin genes in food samples can be made in hours rather than days, with high sensitivity and method accuracy, allowing for the detection of very low concentrations of micro-organisms (Najera-Sanchez *et al.*, 2003).

Detection of SE genes by PCR allows the determination of potentially enterotoxigenic *S. aureus* irrespective of whether the strain produces the toxin or not the inability to detect the enterotoxin by immunological methods may occur due either to low level production of enterotoxin or to mutation in the coding region or in a regulatory region. For this reason, PCR may be

considered more sensitive than methods that determine SE-production as immunological methods (Zschöck *et al*., 2000 ; Holeckova *et al*., 2002).

The PCR assay can detect not only live but also damaged and dead microorganisms in food subjected to thermal processing (Najera-Sanchez *et al*., 2003). It is a rapid, sensitive and specific identification method for the genes responsible for toxins produced by *S.aureus* (Mehrotra *et al*., 2000a ; Anvari *et al*., 2008).

Multiplex PCR is a widespread molecular biology technique for amplification of multiple targets in a single PCR experiment. In a multiplexing assay, more than one target sequence can be amplified by using multiple primer pairs in a reaction mixture. As an extension to the practical use of PCR, this technique has the potential to produce considerable savings in time and effort within the laboratory without compromising on the utility of the experiment (Shen *et al*., 2010).

Real-time fluorescence PCR assays is another molecular methods for detecting and toxinotyping nine enterotoxin genes from *S.aureus.* Real-time PCR assays developed on the LightCycler system (LC-PCR) are a powerful tool for rapid detection and toxinotyping of the enterotoxin genes *sea* to *sej* from *S.aureus*. The work offers a very quick, reliable and specific alternative to conventional block cycler PCR assays to identify the enterotoxin profile of toxigenic *S. aureus* (Letertre *et al*., 2003).

Restriction Fragment Length Polymorphism (RFLP) is an alternative method to PFGE. This method is based on polymerase chain reaction amplification of the variable region of the coagulase gene followed by (***Alul***)

and (***CfoI***) restriction enzyme digestion followed by analysis of restriction fragment length polymorphism patterns. This method is fast, easy and not expensive, however cannot type coagulase negative strains (Shatnawi, 2009).

2.8. Factor affecting the production of staphylococcal enterotoxin

Enterotoxin production required growth of *S.aureus*, although of enterotoxin production does not always accompany growth, and in a few cases toxin production has been observed in non-replicating cell cultures (Wallin-Carlquist *et al*., 2010). Enterotoxin production is affected by key parameters which either prevent it or stimulate it, many studies have been performed to identify these parameters in laboratory media and in diverse food products. It seems that enterotoxin production regulated by a multifaceted network of environmental and genetic factors (Schelin *et al*., 2011).

2.8.1. Temperature

The minimum and maximum temperatures for enterotoxin production was (10 -45)°C respectively (Bergdoll & Lee Wong ,2006) and the optimum between (34–40)°C (Schelin *et al*., 2011).

Foods were subjected to temperature abuse (>10°C) must be considered potential candidates for staphylococcal enterotoxin production, with the ensuing possibility for a food poisoning outbreak. The literature suggests that the amount of enterotoxin synthesized by *S.aureus* decreased dramatically when the microorganism is grown at 20 to 25°C, even though final cell densities are similar over a wide range of temperatures. However, even at the lower temperature ranges, an extended temperature abuse period could allow sufficient toxin synthesis to pose a food poisoning risk (Smith *et al*., 1983).

2.8.2. pH

pH values between (4 -10) considered sutible for growth of most staphylococcal stains and the optimum pH (6 – 7) (Schelin *et al*., 2011). The pH range tolerated is reduced when other cultural parameters become non-optimal. For example, the lowest pH values that supported growth and SE production in anaerobic cultures were 4.6 and 5.3,while the lowest pH that permitted growth and SE production by aerobically cultured *S. aureus* strains was 4.0 (Smith *et al*., 1983). growth of *S.aureus* declined with lower pH values (Lanciotti *et al*., 2001).

SEC was produced in the pH range between (4.00 -9.83) when *S.aureus* was inoculated at a high concentration (108 CFU ml-1) without added salt . The optimum pH for production was approximately 5 , while the presence of 12% Nacl resulted in growth but not enterotoxin production. Given the high concentration used it is not clear how these results might translate to more realistic inoculum concentrations. In pH and oxygen tension controlled culture, the optimum SEA production has been reported at pH (6.5-7.0) (Paulin *et al*., 2012).

2.8.3. Water activity (aw)

S.aureus are able to grow over a much wider water activity range than other food-associated pathogens ,thus water activity has great importance. The bacteria can grow at a minimum aw of 0.83–0.86 equivalent to about 20% NaCl (Hennekinne *et al*., 2012). Low aw conditions were shown to restrict the production of SEB more than SEA (Qi and Miller, 2000).

Growth was occurred over a wide range of aw values than that allowing enterotoxin production. The aw conditions for SE production are somewhat

different than those for growth, depending on the type of toxin. SEA and SED production occurs under nearly all aw conditions allowing growth of *S. aureus* as long as all other conditions are optimal. Production of SEB is very sensitive to reductions in aw and hardly any is produced at aw 0.93 despite extensive growth. The effect of aw on SEC production follows the same pattern as SEB production (Ewald & Notermans, 1988 ; Qi & Miller, 2000).

In laboratory media and in food, the conditions for growth and SE production may differ to some extent. Studies on the osmoadaptive strategies of *S.aureus* have revealed that the cells respond by accumulating certain low molecular weight compounds termed compatible solutes when grown in a low aw medium. Glycine betaine, carnitine and proline have been shown to be principal compatible solutes accumulated within osmotically stressed *S.aureus* cells, and their accumulation results from sodium-dependent transport systems (Gutierrez *et al*., 1995 ; Qi & Miller, 2000).There is strong evidence that compatible solutes stimulate not only growth but also toxin synthesis. For example, SEB production was significantly stimulated at low aw when proline was available in the broth (Qi & Miller, 2000).

2.8.4. Oxygen

S.aureus is facultative anaerobic bacteria that can grow in the absence of oxygen even if its growth has been slowed down. The generation time of *S.aureus* during the exponential phase of growth at 37 °C in brain heart infusion medium (a rich laboratory medium) is around 35 min under aerobic conditions but takes 80 min under strict anaerobic conditions (Belay &Rasooly, 2002).

SE production is also higher under aerobic than anaerobic conditions (Belay and Rasooly, 2002). For example, SEH production is maximal for a pH of 7 and an aeration rate of 300 $ml \cdot min^{-1}$ and a strong decrease is observed in anaerobic conditions (Stewart & Hocking, 2003).

2.8.5. Salt

Salt inhibit the growth of *S.aureus* despite the fact that *S.aureus* is a halotolerant bacteria compared with other pathogens. It can tolerate Nacl concentrations between (2.5%- 20%), but its growth is dramatically impaired at high salt concentrations (Orden *et al*., 1992).

Chapter three

Materials and methods

3. Materials and Methods

3.1. Materials

3.1.1. Apparatuses and Equipments

Table (3-1) shows the apparatus and instruments that were used in the experiments of the study:

Table (3-1): Apparatuses and Equipments

No.	Equipment	Company	country of origin
1	Autoclave	Hirayama	Japan
2	Benson Burner	Jenway	Germany
3	Centrifuge, Cooling centrifuge	Hettich	Germany
4	Digital camera	Sony	Japan
5	Distilled	GFL	Germany
6	Electrophoresis horizontal system Electrophoresis vertical system	Cleaver Scientific Ltd	UK
7	Eppendorf tubes	Merk	Germany
8	Hot plate	Labinco	Holland
9	Incubator	Fisher scientific	Germany
10	Inoculating loop	Loop Shandon	England
11	Micropipettes	Slamed	Germany
12	Micropipettes	Oxford	Germany
13	Millipore filter unite (0.3)mμ	Gallenkamp	England
14	Thermo cycler, Power supply	Cleaver Scientific Ltd	UK
15	pH-meter	WTW inolab	Germany
16	Refrigerator	Vestel	Turkey

17	Sensitive balance	Kern	Germany
18	Shacking incubator	Lab Tech	Korea
19	Spectrophotometer	APEL	Japan
20	UV trans illuminator	Cleaver Scientific Ltd	UK
21	Vortex shaker test tube shaker	Gemmy	USA
22	Water bath	GFL	Germany

3.1.2. Chemical and Biological materials

The following Chemical and Biological materials were used in this study:

Table (3-2): Chemical and Biological materials

No	Chemicals and biological materials	Company	country of origin
1	Absolut ethanol	Fluka chemika	Switzerland
2	Acetic acid	Fluka	Switzerland
3	Acrylamide, Bis-acrylamide, B-Mercap to ethanol	Bio basic INC	Canada
4	Agarose, HCL	BDH	England
5	Ammonium per sulfate	BIO BASIC INC	Canada
6	Api staph test Kit	BioMerieux	France

7	Boric acid	Fisher scientific international	U.K
8	Bromophenol blue	Fluka	Switzerland
9	DNA Ladder, PCR premix	BIONEER	Korea
10	Ethidium Bromide	Oxiod	England
11	Ethylenediaminetetraacetic acid disodium salt	HIMEDIA	INDIA
12	EthyleneDiamineTetra Acetic acid	BDH	England
13	Formalin	EDUTEK	India
14	Glacial acetic acid	FINE CHEM LIMITED	India
15	Glucose	BDH	U.K
16	Glycerol	FISHER	U.K
17	Glycine	Thomas baker	India
18	Methanol	HIMEDIA	INDIA
19	Nacl	thomas Baker	India
20	Primers	BIONEER	Korea
21	Protein marker	Promega	USA
22	SDS	Romil	Cambridge

23	Sliver nitrate	CDH	INDA
24	Sodium acetate anhydrous		
25	Sodium carbonate anhydrous		
26	Sodium hydroxide	Thomas-Baker	USA
27	Sodium thiosulphate pent-hydrate	CDH	INDA
28	TEMED(Tetramethylethylenediamine)	HIMEDIA	INDIA
29	Tris base	SIGMA-ALDRICH	U.S.A
30	Tris-HCl	HIMEDIA	INDIA

3.1.3. Culture Media:

The following culture media were used throughout this study:

Table (3.3): culture media used in this study

No.	Media	Company and country of origin	Advantage
1	Brain Heart Infusion agar	HIMIDIA/ India	Bacterial isolation
2	Brain Heart Infusion Broth		Bacterial inoculate
3	Mannitol Salt Agar		Bacterial isolation
4	Nutrient Broth		Bacterial inoculate

3.1.4. Rapid multi test system

Table (3.4): Rapid multi test system

Test	**Manufacture(Origin)**
API-STAPH System	BioMerieux (France)

3.1.5. PCR Materials

3.1.5.1. Master Mix

Table (3.5): Master Mix

***AccuPower®* PCR Premix**	**Source**
AccuPower® PCR Premix is the powerful technology for convenient and easy to perform DNA amplification.it contains DNApolymerase, *dntps, a tracking dye and reaction buffer in a premixed format, freeze-dried into pellet. The patented chemical stabilizer of this product enables to maintain the activity of pre mixture for over a month even when stored at room temperature (25c), over 2 years in freezer.	BIONEER

* deoxynucleotide triphosphates

3.1.5.2 Amplicon sequence and products

Table (3.6) Amplicon sequence and products

Gene		Primer sequences(5́-3́)	Size of amplified product(bp)	Reference
Sea	F*	GGTTATCAATGTGCGGGTGG	102	(Mehrotra *et al*.,2000a)
	R**	CGGCACTTTTTTCTCTTCGG		
Seb	F*	GTATGGTGGTGTAACTGAGC	164	
	R**	CCAAATAGTGACGAGTTAGC		
Sec	F*	AGATGAAGTAGTTGATGTGTATGG	451	
	R**	CACACTTTTAGAATCAACCG		
Sed	F*	CCAATAATAGGAGAAAATAAAAG	278	
	R**	ATTGGTATTTTTTTTCGTTC		
See	F*	AGGTTTTTTCACAGGTCATCC	209	
	R**	CTTTTTTTTCTTCGGTCAATC		
FemA	F*	AAAAAAGCACATAACAAGCG	132	
	R**	GATAAAGAAGAAACCAGCAG		

*Forward ** Reverse

3.1.5.3. Molecular Weight Marker

Table (3.8): Molecular Weight Marker

DNA marker	Description	Source
25/100 bp	25/100bp mixed DNA ladder is specially designed for determining the size of double strand DNA from 25 to 2000 base pairs. The DNA ladder consists of 17 double strand DNA fragments ranging in size from 25 to200pb in 25bp increments and additional fragment of 300,400,500,600,700,800,900,1000,2000bp.the150,500,1000,2000bp bands are approximately two to three times brighter for easy identification	BIONEER

Table (3-9) Broad range protein molecular weight markers

Protein marker	Description	source
10/225 KDa	The broad range protein molecular weight markers consist of nine precisely sized recombinant proteins of molecular weight 225, 150, 100, 75, 50,35,25,15 and 10 KDa .each protein is present at a concentration of 0.1µg/µl, except for 50 KDa protein, which is present at 0.3µg/µl and serves as a reference indicator, having triple the intensity of the other protein. All other proteins appears with equal intensity on the gel.	promega

3.2. Methods

3.2.1. Culture Media:

All media were prepared according to the instructions of the manufacturing company. Sterilization of culture media and solutions were achieved by Autoclaving at121℃/1 pound for 15min after adjusted pH with 0.1N NaOH or 0.1N Hcl to 7.2 other solutions that destroy by heat were sterilized by Millipore filtration through 0.22 and 0.45µm filters: the following media were used in this study:

3.2.1.1. Brain heart infusion agar

It was used for reactivated of the bacteria.

3.2.1.2. Brain heart infusion broth

It was used for Salinity, Glucose and pH Experiments.

3.2.1.3. Mannitol salt agar medium

It was considered as selective and differential medium for the genus *Staphylococcus* (Benson, 2001).

3.2.2. Sample collection

Nasal swabs from food handlers were examined for *S.aureus* isolation,the nasal swabs were collected in collaboration with the Public Health Laboratory that belongs to the Health Directorate of Holy Kerbala Province. The nasal swabs were processed according to the standard microbiological procedures for the isolation of *S.aureus* , nasal swab

specimens were obtained by using sterile dry cotton-wool swabs , both anterior nares (left and right)were swabbed by rubbing the swab four times around the inside of each nostril while applying an even pressure and rotating the swab without interruption.

3.2.3. Culturing of swabs for *staphylococcuss aureus* isolation

After swabs were collected from people working as food handlers ,each swabs were cultured on selective media Mannitol salt agar then incubated at 37 C° for 24 hrs. The color changed from pink to bright yellow, when the bacteria was mannitol fermented which shows a positive result, while the unchanging color of the medium was negative result (MacFaddin, 2000).The identification depending on its morphological and cultural characteristics (mannitol fermentation, colony shape, size, color, borders, and texture) and then it was examined under the microscope after making smear from pure colony on clean slid and stain with Gram's stain for observation arrangement and reaction bacteria with stain (Collee *et al.,* 1996; MacFaddin, 2000).

3.2.4. Primary identification steps

3.2.4.1. The Preparation of Gram's Stain Stains, Reagents, Solutions and Emulsions used in the identification of bacterial isolates

This stain was used to differentiate Gram-negative from Gram positive bacteria and to study microscopic properties that was carried out according to (Benson, 2001).

3.2.4.2. Biochemical tests

3.2.4.2.1. Catalase Test

Hydrogen peroxide (H_2O_2) 3% was prepared for detecting catalase production.One or two drops of catalase test reagent (3% H_2O_2) were placed on a slide; a growth from the center of a fresh pure colony from nutrient agar plate was mixed with the reagent on the slide. The formation of bubbles indicates a positive result (Benson, 2001).

3.2.4.2.2. Coagulase test

Several colonies of bacteria were transferred with a loop to a tube containing 0.5 ml of rabbit plasma. The tube was covered to prevent evaporation and incubated at 37°C over night. The test was read by tilting the tube and observing the clot formation in the plasma. Negative test results in the plasma remained free-flowing with no evidence of a clot (Collee *et al.,* 1996).

3.2.4.3. Confirmatory identification

3.2.4.3.1. API Systems (BioMerieux)

This system consists of strips containing dehydrated substrates in individual 20 micro tubes. These tests were reconstituted by adding to each micro tube an aliquot of API-STAPH medium that had been inoculated with the bacterial suspension The strip was then incubated for 8-24 h at 35-37 ˚C, after incubation, metabolism produced color change that are either spontaneous or revealed by the reagents addition. Identification of the staphylococcal species was made by using indicators, and differential charts

supplied by the manufacturer. The reactions were read according to the reading table and the identification is obtained by referring to the analytical profile

3.2.5. Detection of enterotoxin gene by multiplex polymerase chain reaction

3.2.5.1. Preparation of Buffers and Solutions

The following solutions and reagents were used in the present study, those require sterilization were autoclaved at 121°C for 15 minutes. Millipore filter (0.3µm) was used for sterilization of heat sensitive solutions such as glucose solution. The pH was adjusted using 1M NaOH and 1M Hcl.

3.2.5.2. Buffers used in DNA extraction

3.2.5.2.1. Tris-EDTA buffer (TE buffer) pH 8.0

It was prepared as 10X buffer by mixing 10 ml of 1M Tris- Hcl buffer with 50 ml of D.W and then adding 0.37 gm of EDTA. The pH was adjusted to 8, then the volume was completed to 100 ml with D.W. The final concentration was 10X, then autoclaved at 121°C for 15 minutes, and stored at 4°C until used. Working solution of 1X TE buffer (pH 8.0) was prepared by diluting the stock solution 10X (Sambrook & Russell, 2001).

3.2.5.3. Buffers used for Agarose Gel Electrophoresis

3.2.5.3.1. Working solution of Tris-Borate-EDTA (TBE buffer)

Tris-borate-EDTA buffer was prepared by dissolving 5.4 gm tris base ,2.75 gm boric acid and 1 ml of 0.5 M EDTA and the volume brought to 1L (Sambrook & Russell, 2001).

3.2.5.4. Ethidium Bromide

It was (5mg/ml) prepared by dissolving 0.05 gm of ethidium bromide in 10 ml of D.W and stored in dark reagent bottle (Sambrook and Russell, 2001).

3.2.5.5. Multiplex Polymerase Chain Reaction Assay for detection of staphylococcal enterotoxin gene

Multiplex PCR is a widespread molecular biology technique for amplification of multiple targets in a single PCR experiment. In a multiplexing assay, more than one target sequence can be amplified by using multiple primer pairs in a reaction mixture.

3.2.5.5.1. Preparation of Primers

The primers listed in table (3-6) were used in this study; these primers were provided in a lyophilized form, dissolved in sterile distilled water to give a final concentration of 100 pmol/ µL as recommended by provider and stored in deep freezer until used in PCR amplification. At first, stock solution (100 pmol/µl)for each primers was prepared by dissolving the lyophilized primer into sterile distilled water and the working solution prepared by added 10 µL from each primer and add 90µL sterile distilled (Mehrotra *et al.*,2000a).

3.2.5.5.2. DNA Extraction

DNA was extracted by boiling method, (3-5) colonies of overnight bacterial culture on brain heart infusion agar (BHI) were transferred into a microfuge tube containing 300 µl sterile distilled water. Tubes then is incubated in boiling water bath for 5-10 minutes. Tubes then centrifuged for 10 minutes at 12000 rpm. Supernatant was transferred into new sterile tubes and kept freeze until used in PCR amplification (Zhang *et al*., 2004).

3.2.6. Polymerase Chain Reaction (PCR) Technique

3.2.6.1. Gene's selection

Five genes were selected to be amplified multiplex PCR technique *sea*, *seb*, *sec, sed and see* (Mehrotra *et al*.,2000a).

3.2.6.2. PCR Premix

It was provided by the supplier (BIONEER), the PCR premix consists of: 1U Top DNApolymerase, 250 µM each: dntps (dATP, dCTP, dGTP, dTTP) 10 mM Tris-HCl (pH 9.0)30 mM KCl, 1.5 mM MgCl2, stabilizer and tracking dye

3.2.6.3. PCR assay

All of 100 samples of the bacterial isolates were analyzed by PCR.To each premix tube , the following reagents were added ; 2 µL of each of Forwared and Reverse primers 5 µL of DNA samples , and the volum adjusted to 20 µL by adding sterilized double distilled water.

To detect *fem A* gene fragment the PCR cycling conditions were as follows: denaturation at 95˚C for 5 min ,followed by 30 cycles of 96 ˚C for

1min ,52 ˚C for 30 sec ,72 ˚C for 10 min. The PCR products were resolved on electrophoresis.

3.2.6.4. PCR Amplification

The extracted DNA, primers and PCR premix (AccuPower, Bioneer), were thawed at room temperature , vortex and centrifuged briefly to bring the contents to the bottom of the tubes. PCR Mixture was set up in a total volume of 20µL included 5µL of PCR premix, 0.5µL of each primer and 3µL of template DNA have been used. The rest volume was completed with sterile de-ionized distilled water. Negative control contained all material except template DNA. PCR reaction tubes were centrifuged briefly to mix and bring the contents to the bottom of the tubes, and placed into thermo cycler PCR instrument where DNA was amplified as indicating in the tables.

Table (3-10) Program used for multiplex PCR to amplify enterotoxins genes *sea*, *seb*, *sec, sed and see*

Stage	**Temperature (time) (min)**		**Refrences**
Initial denaturation	94˚C (5)		(Mehrotra *et al*.,2000a)
Denaturation	94℃ (2)	35 cycle	
Annealing	57 ˚C (2)		
Extension	72 ˚C (1)		
Final extension	72 ˚C (7)		

3.2.7. Preparation of Agarose Gel

Agarose gel was prepared in 1.5 % concentration for quality of the extracted DNA, by dissolving 1.5 gm of agarose powder in 100 ml of 0.5X TBE buffer, and melted, then the agarose gel was cooled to 50-60 ˚C , 5 µL of ethidium bromide dye was added with mixing, agarose was poured out into the gel jar to prevent bubble formation, then cooled to 20 ˚C.When agarose gel was poured, several wells were carefully made with a comb at one side of the gel about 5-10 mm away from the end of gel; after final solidification. The comb was carefully removed; the jar was put in the electrophoresis tank.3 microliters of the 100 bp DNA ladder were placed in the first left well of the agarose electrophoresis gel. 7 µL of each DNA sample was add , then the electrophoresis tank closed with its special lid, and electric current was matched (70 volt for 1 h) (Al-Khafaji.,2013).

3.2.8. Analysis of production of enterotoxin

In this study, the effect of different culture condition on the production of enterotoxin by *S.aureus* was studied . T test was used for statistical analysis for each experiments .The following culture condition were included

3.2.8.1. Effect of pH on growth and enterotoxin production of *staphylococcus aureus*

In this set of experiments , the effect of pH value was studied. For this purpose , bacteria was grown on brain heart infusuion agar (BHI) and incubated at 37 ˚C for 24 hr. On the second day 4-5 colonies were transferred into brain heart infusuion broth and incubated for anthor 24hr . On the third day , 1 ml of broth was transferred to 50 ml brain heart infusion broth with

different pH value. Atotal of 4 pH value were studies ,4.5,5.5,6.5,8.5. The bottles containing the 50 ml (BHI) broth then were incubated for 48 hr at 37˚C using shaker incubated. After incubation , the optical density for each bottle was measured by measuring the absorbance at 600 nm using spectrophotometer. The culture then centrifuged and the pellets were collected and stored in freezer until used later on .

3.2.8.2. Effect of salt on growth and enterotoxin production of *staphylococcus aureus*

In this set of experiments , the effect of Nacl concentration was studied. For this purpose , bacteria was grown on brain heart infusuion agar (BHI) and incubated at 37 ˚C for 24 hr. On the second day 4-5 colonies were transferred into brain heart infusuion broth and incubated for anthor 24hr . On the third day , 1 ml of broth was transferred to 50 ml brain heart infusion broth with different Nacl concentration. Atotal of 4 Nacl concentration were studies 8%, 10%, 12%, 14% . The bottles containing the 50 ml (BHI) broth then were incubated for 48 hr at 37˚C using shaker incubated. After incubation , the optical density for each bottle was measured by measuring the absorbance at 600 nm using spectrophotometer. The culture then centrifuged and the pellets were collected and stored in freezer until used later on .

3.2.8.3. Effect of glucose on growth and enterotoxin production of *staphylococcus aureus*

In this set of experiments , the effect of glucose concentration was studied. For this purpose , bacteria was grown on brain heart infusuion agar (BHI) and incubated at 37 ˚C for 24 hr. On the second day 4-5 colonies were

transferred into brain heart infusuion broth and incubated for anthor 24hr . 20% glucose solution was prepared by (dissolved 20 gm glucose in 100 ml distill water) filtration through 0.3 cellulose membrane atotal of 4 glucose concentration were studies ,0% ,0.5% ,1.5% ,2.5% . Withdraw amount of (BHI) broth was equal to the amount was added . On the third day the bottles containing 50 ml (BHI) broth then were incubated for 48 hr at 37˚C using shaker incubated. After incubation , the optical density for each bottle was measured by measuring the absorbance at 600 nm using spectrophotometer. The culture then centrifuged and the pellets were collected and stored in freezer until used later on .

3.2.9.1. Method of protein extractions

3.2.9.1.1. Extraction of whole cell protein (wcp)

The bacteria was activated on (BHI)agar and incubated overnight at 37˚C then some colony was taken and suspended in 3ml BHI broth. The sample had been incubated again for 48h at 37 ˚C were further centrifuged for 3min at 12100 rpm.the collected cells were washed three times with sterile distilled water and stirred after adding 25µl SDS sample buffer. The protein were denatured in boiling water for 5min.the supernatant was then centrifuged again for 3 min at 12100 rpm.collected in an eppendorf tube and kept at -50c until the electrophoresis was carried out (Laemmli, 1970).

3.2.9.2. Buffer used in vertical electrophoresis

A- Preparation of 1x electrophoresis buffer and polyacrylamide gel

Electrophoresis buffer was prepared by mixing 7.5 gm tris base, 36.25 glycine and 2.5gm SDS and the volume was brought to 2.5 L distilled water.

The first step was needed to prepare buffer solution for polyacrylamide, preparation of 40% acrylamide .Acrylamide was prepared by dissolving 37.5 gm acrylamide and 1g of bisacrylamide in 100 ml distilled water (Akbar *et al*., 2012).

B-Preparation of 1M Tris pH 8.8 ,1M Tris pH 6.8 , 20% SDS buffer

Tris buffer was prepared by dissolving 30.25g tris hydrochloride in 200 ml distilled water. Then the volume was brought to 250 ml, the pH of this solution was modified by addition of sodium hydroxide and measured pH by pH meter until reached to pH 8.8.

Tris buffer was prepared by dissolving 30.25 tris base in 200 ml distilled water .Then the volume was brought to 250 ml, pH of this solution was modified by addition of Hcl and measured pH by pH meter until reach to pH 6.8.

SDS buffer was prepared by dissolving 20 gm of SDS in 50 ml distilled water then the volume was brought to 100 ml distilled water, Saved in refrigerator (Akbar *et al*., 2012).

C-Preparation of resolving gel 10% and stacking gel

Table (3-11) material was needed for resolving gel

1M tris pH 8.8	3.750 ml
20% SDS	0.025ml
40% acrylamide	2.50 ml
dH2O	3.755ml
10% ammonium per sulfate	100µl
TEMED	10µl

The materials in the table (3-11) were mixed in gas vacuum tube so that to prevent oxygen that interference with polymerization of gel except ammonium per sulfate and TEMED were added before pour gel. In this time set the vertical electrophoresis .

Table (3-12) material was needed for stacking gel

1M Tris pH 6.8	0.63 ml
20% SDS	0.025 ml
40% acrylamide	0.83ml
H_2O	3.450ml
10% ammonium per sulfate	50µl
TEMED	5µl

The materials in the table (3-12) were mixed in vacuum tube and poured on resolving gel (Akbar *et al*., 2012).

3.2.9.3.The polyacrylamide gel was poured

A-Gel sandwich was assembled

1- Plates, spacer, comb were cleaned and rinsed well

2-glass plate was assembled in sandwich

3- Slide plate was into plate clamp and vertically stand.It must ensure that the glass plate sandwich was (1-2)mm below the edge of the plate clamp by gently sliding the clamp up before sealing the assembly. This prevents the unpolymerised resolving gel solution from leaking out of the sandwich

4-This is checked when distilled water was poured between two slide plate to ensure don't leaking out of sandwich (Akbar *et al*., 2012).

B-Separating gel was poured

1- For routine protein analysis, 12% resolving gel was poured. large quantity of proteins required lower gel percentages (<10%) while smaller proteins and peptides required higher acrylamide percentages (>15%)

2-100 µl of 10% aps and 6 µl TEMED was added ,

3-The materials was mixed well by inverting tube carefully , so that to avoid oxygen interfere with polymerization of gels

4- The solution was poured into the gel sandwich by used a Pasteur pipette untilled desired level (5.5-6.5 cm)

5- 70% ethanol or 0.01% SDS was added over the gel gently

6-The gel was allowed to polymerized between (30-60) minutes at room temperature or until interface appears. If the gels was not to be run on same day. They can be removed from the clamps and covered with resolving gel buffer and stored at 4c for (2-3) days (Akbar *et al*., 2012).

C- The stacking gel was poured (3.75%)

1-The aqueous layer was poured off from separating gel and rinse with distelled water

2-The components was combined for stacking gel

3-The stacking solution was poured on top of separating gel

4-The comb was insert into stacking gel and take care to avoid forming bubbles on the ends of the teeth

5-The gel was allowed to polymerize 30-60 minutes or until ready (Akbar *et al*., 2012).

E- The gel was clamped onto electrophoresis tank

1-The comb was removed carefully from the gel

2-The gel/glass plate sandwich was placed into electrophoresis core. The short glass plate should face the center, or inside of the core.

3-The core was placed assembly into the running tank

4- The electrophoresis buffer was added to the core. The Buffer should be added to the top of the assembly.1-2 inches of electrophoresis buffer was added to the running tank.

5-Wells were rinsed out wells with buffer in preparation for sample loading (Akbar *et al*., 2012).

3.2.9.3.Preparation of Sample

Table (3-13) material was needed for sample buffer preparation

0.06M tris	0.0948 gm.
Glycerol	250µl
SDS	0.05gm
β-mercaptoethanol	0.125µl
Bromophenol blue	0.001 gm.

1-Protein samples was dissolved rapidly in room temperature water path

2- Sample buffer was added to protein samples

3-The sample was heated in the 95°C dry bath for 5-10 minute

4-The sample was transported to cooling centrifuge for 5 minute

5-The sample can be stored at 20 °C for 6 month for future runs (Akbar *et al*., 2012).

3.2.9.4. Separation of protein samples by PAGE and staining the gel to visualize protein bands

A- Separation of protein samples by PAGE

1-The sample was loaded into wells using micropipette or Hamilton™ syringe normally 25 µl of sample can be loaded into each well

2-Every well was loaded with the same volume of sample

3- The empty wells were filled will 1x sample dye

4-Electrodes were attached so that protein well move towards the anode

5-The gel was run at 100-200 volte until dye front reach to the bottom of the gel. The running time was vary, depended on the percentage cross linking and buffer composition.

6-The pour supply was turned off when the tracking dye reaches the bottom of the gel, and the power cables was disconnected (Akbar *et al*., 2012).

B- Staining the gel to visualize protein bands

1-Electrodes were disconnected, the electrophoresis buffer was removed from the tank and keep the buffer then gel sandwich was removed from tank

2-Side spacer was removed and the plates were gently separated so that the gel left on one plate

3-Gel was washed with distilled water

4-The gel was transported to tip box containing fixing solution (Akbar *et al*., 2012).

3.2.9.5.Preparation of Sliver staining for polyacrylamide gel

A-Preparation of fixing solution and sensitizing solution

Fixing solution was prepared by mixing 40 ml absolute ethanol and 10 ml acetic acid ,the volume was brought to 50 ml distilled water the gel remind in this step for 1 hour.

Sensitizing solution was prepared by mixing 6.8 gm sodium acetate, 0.2 gm sodium thiosulphate pentahydrate and dissolved in 30ml absolut ethanol. The volume was brought to 100ml distilled water. 0.5ml formaldehyde was added per 100 ml of sensitizing solution before use, the gel was remind in this step for 1 hour .After this step the gel was washed with distilled water for 3 time each one 10 min (Chevallet *et al*., 2006).

B-Preparation of sliver solution and developing solution

Sliver solution was prepared by dissolving 0.25gm sliver nitrate in 100 ml distilled water then 40 µl of formaldehyde was added to 100 ml of sliver solution before it used the gel was remind in this solution for 1 hour after this step the gel was washed with distilled water for 1min.

The developing solution was prepared by dissolving 2.5gm of sodium carbonate in 100ml distilled water then 20µl formaldehyde was added to

100ml of developing solution before used .the gel was remind in this solution for 10-30 min (Chevallet *et al*., 2006).

C-Preparation of stop solution and preserving solution

The stop solution was prepared by dissolving 1.5 gm of Na_2EDTA in 100ml distilled water.

preserving solution was prepared by mixing 30% absolute ethanol with 4 ml glycerol then volume was brought to 100ml distilled water (Chevallet *et al*., 2006).

Chapter four

Results and discussion

4.Results and discussion

4.1. Isolation and identification of *Staphylococcus aureus* from food handlers

Nasal swabs were obtained from 332 food handlers from different regions of Holy Kerbala City. The food handlers were working in different kinds of jobs like : workers serving in restaurants, Chiefs, workers in food wholesale agencies, bakeries/ pastries, school food shops, cafés, sweets shops, supermarkets, beverages/ice cream shops, seeds shops, food storages,and butchers as shown in table (4.1). The largest group of food handlers included in this study was the servants in restaurants (no= 95). In addition, there were three additional large groups of food handlers: Chiefs (n=68), Bakery workers (n=66), and Water Plant workers (n=59). A total of 100 isolates of *S.aureus* were recovered from food handlers ,and thus the prevalence of *S.aureus* carriage among food handlers in Kerbala was 30.1% .

Isolation and primary identification was performed according to the standard microbiological procedures including culture characteristics on Mannitol salt agar, arrangements of bacterial cells in gram's stain, biochemical reaction (catalase and coagulase) test and API-Staph. In addition, PCR amplification of *femA* gene fragments was used to confirm the identification of strains.

The *femA* gene is a useful marker for confirmation of *S.aureus* because it is universally present only in *S.aureus* and is implicated in cell wall metabolism and pentaglycine-side chain formation (Hubscher *et al*., 2007 ; Al-Talib *et al*., 2009). The primers pair used in this study is amplifying a 132bp fragment within the *femA* gene shown in figure (4.1). In this study, this

primers pair was successfully amplified the desired band (132 bp) from the *S.aureus* isolates recovered food handlers. Rapid methods for the identification of bacterial pathogens such as PCR become an inevitable goal for medical microbiology laboratories , despite the fact that most of the conventional culture methods are still useful and valid. Rapid methods take 3 hours to confirm a positive culture ,while conventional culture and biochemical tests would take at least 5 days. Rapid diagnostics are really important to patients for appropriate treatment and reduce the hospitalization and medical cost. In addition, the overall preparation process is not tedious and low cost since direct boiling method are being used for DNA extraction from cell culture.

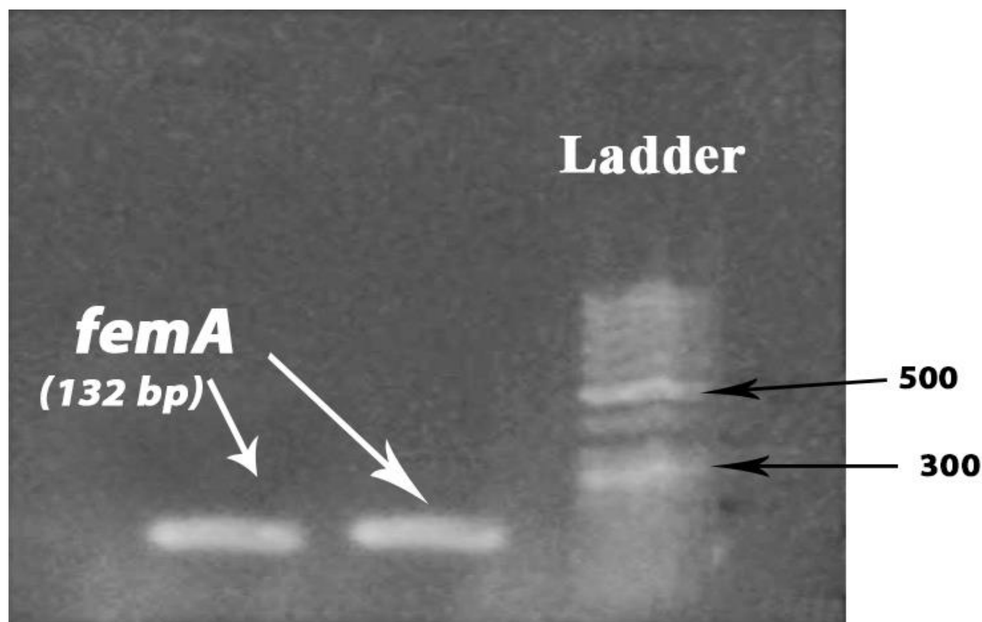

Figure (4.1) PCR amplification of *femA* gene of *S. aureus* (132bp), visualized by 1.5% agarose electrophoresis and staining with ethidium bromide for 1 h at 70 vol.

4.2. Nasal Carriage of *Staphlococcus.aureus* among the Food handlers

It was found that 24 of the Servant in restaurant (25.2%) were carrying *S.aureus* in their nasal cavity and this was comparable to the carriage percentage among the Chiefs (23.5%). However, a high carriage percentages were found among workers in Sweets shops, cafés and Bakery/Pastry shops (71.4%, 85.7% and 34.8%), respectively. In contrast, only 1 out of 11 workers in seeds shop was found to carry *S.aureus* in his nasal cavity. Unfortunately, the numbers within the rest groups were too small and thus is not enough to draw any inferential statistics about the prevalence of *S.aureus* nasal carriage among them (Table 4.1)

Table (4.1) Nasal Carriage of *Staphlococcus.aureus* among different groups of the Food Handlers

Source of samples	No of tested samples	No of positive samples (%)
Servants in Restaurants	95	24(25.2%)
Chiefs	68	16(23.5%)
Food wholesale workers	1	1(100%)
Bakery workers	66	23(34.8%)
School food shop workers	8	8(100%)
Cafe workers	7	6(85.7%)
Sweets shops workers	7	5(71.4%)
Supermarkets workers	2	2(100%)
Water plant workers	59	9(15.2%)
Pure water factories workers	3	1(33.3%)

Butchers	1	1(100%)
Beverages and Ice-creams shops workers	3	2(66.6%)
Seeds shops workers	11	1(9%)
Food storage workers	1	1(100%)
Total	332	100 (30.1%)

Several authors have studied the presence of *S.aureus* in the nasal cavities of food handlers in this study. It was found that 30.1% food handlers were positive for *S.aureus* which in agreement with Vanderbergh *et al*. (1999) who reported that the isolation of *S.aureus* from nasal cavities, could vary from 20 to 55% in a healthy adult population (VandenBergh *et al*., 1999). Higher *S.aureus* prevalence among food handlers, of 44.6%, 53.2% and 23.1% was noted in Botswana, Kuwait, and South-eastern Anatolia, respectively (Uzunovi *et al*., 2013). These high results may be due to the transmission mode of *S.aureus* through hands, which may become contaminated by contact with colonized or infected individuals or through contact with colonized or infected body sites of other persons. Other factors contributing to transmission include close skin to- skin contact, crowded conditions, and poor hygiene (Dablool & Al-Ghamdi, 2011).

4.3. Multiplex PCR for detection of selected staphylococcal Enterotoxin genes

To ensure that all of the target gene sequences were satisfactorily amplified, the reaction conditions for the multiplex PCR assay were optimized.

To reduce the possibility of occurrence of unwanted bands originating from nonspecific amplification , the primers used in each set had almost equal annealing temperatures. Figure (4.2),(4.3) shows the presence of the amplified products of five bands (*sea, seb, sec, sed,* and *see*) was obtained when of DNAs extracted from different isolates. As shown in figures (4.2) ,(4.3) many strains have reported to possess multiple genes of enterotoxins.

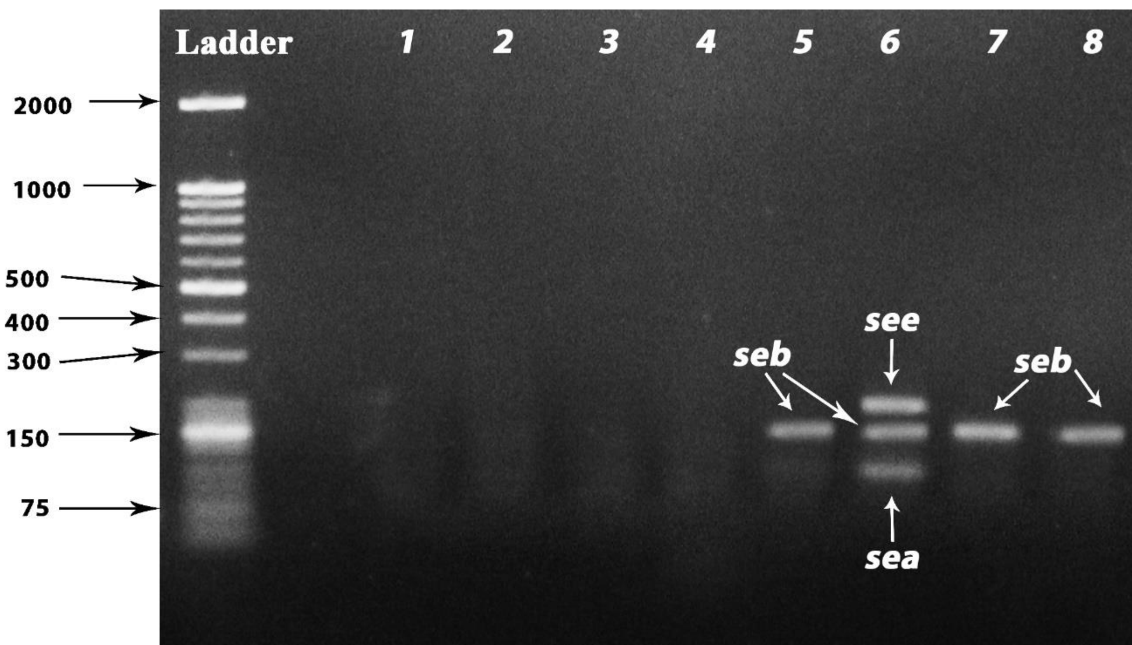

Figure 4.2 PCR amplification for detection of *Staphylococcus aureus* enterotoxins genes. Lane L: 50 bp DNA ladder, Lane 5 shows *seb* (164bp) gene , Lane 6 shows *sea* (102bp) ,*seb* (164bp),*see* (209bp) genes Lane 7,8 shows *seb* (164bp) gene (visualized by 1.5 % agarose electrophoresis and ethidium bromide staining for 1 h at 70 vol); (2-8) isolates from food handlers.

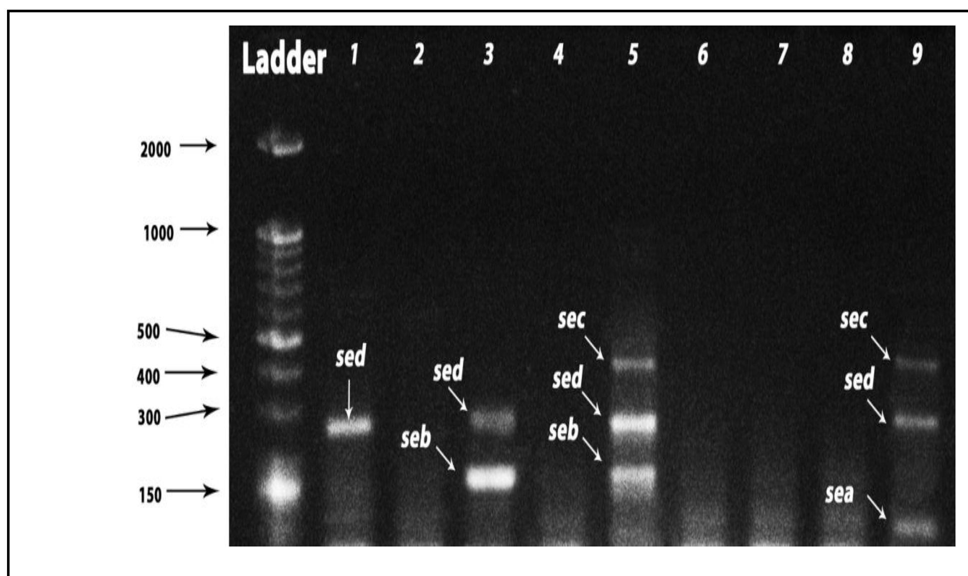

Figure 4.3 PCR amplification for detection of *Staphylococcus aureus* enterotoxins genes. Lane L: 50 bp DNA ladder, Lane 1 shows *sed* (278bp) gene , Lane 3 shows *seb* (164bp),*sed* (278bp) genes Lane 5 shows *seb* (164bp),*sed* (278bp),*sec* (451bp) genes, Lane 9 shows *sea* (102bp),*sed* (278bp),*sec* (451bp) genes (visualized by 1.5 % agarose electrophoresis and ethidium bromide staining for 1 h at 70 vol); (2-9) isolates from food handlers.

4.3.1. Distribution of the enterotoxigenic isolates according to the source of isolates

Table (4.2) summarizes the distribution of the enterotoxigenic isolates according to the sources from which the isolates were recovered. The frequency of the enterotoxigenic genes were ranging from 0 to 62.5%. The highest frequency of enterotoxigenic strains were found in the isolates recovered from workers in school shops (5 out of 8, 62.5%). A half of the strains recovered from Chiefs were enterotoxigenic. Isolates from workers in bakeries and servant in restaurants were also found to show high frequencies of enterotoxigenic genes (43.4% and 41.6% respectively). In contrast, lower frequency of enterotoxin genes were detected among isolates recovered from

Cafés workers (16.6%), Water Plans (11.1%) and Sweets Shops workers (20%).

Table (4.2) Distribution and percentage of enterotoxin genes in *Staphylococcus aureus* isolates

Source	No of positive samples (%)	No of positive enterotoxin genes	No of multiple enterotoxin genes
Servant	24(25.2%)	10(41.6%)	1(10%)
Chiefs	16(23.5%)	8(50%)	4(50%)
Food wholesale workers	1(100%)	0(0%)	0(0%)
Bakery worker	23(34.8%)	10(43.4%)	4(40%)
School food shop	8(100%)	5(62.5%)	2(40%)
Café	6(85.7%)	1(16.6%)	0
Sweets	5(71.4%)	1(20%)	0
Supermarkets	2(100%)	1(50%)	0
Water plants worker	9(15.2%)	1(11.1%)	0
Pure water factory	1(33.3%)	0(0%)	0
Butchers	1(100%)	0(0%)	0
Beverages and Ice-creams	2(66.6%)	0(0%)	0
Seeds	1(9%)	0(0%)	0
Food storage	1(100%)	1(100%)	0
Total	100(30.1)	38%	11

Collectively, the results of this study may indicate that the distribution of the enterotoxigenic strains is not even among the different workers. It is not very clear why that certain group of workers carry isolates with high frequency of enterotoxigenic genes. However, it could be suggested that food handlers who have higher contact with food (such as Chiefs and restaurants workers) have a higher risk to carry enterotoxigenic strains. This, in turn,

might possibly indicate that food play a role in transmitting the enterotoxigenic strains to the workers. According to those results, a strict monitoring should be applied for those group of workers to detect any nasal carriage of the enterotoxigenic strains, in addition to applying measures to decolonize them by appropriate treatment.

This study, detection of enterotoxin genes by PCR showed that 38 (38 %) out of 100 *S.aureus* isolates were positive for one or more of these genes. Prevalence of enterotoxigenic *S.aureus* in food handlers is variable between industries and countries. Prevalence estimates from several small studies range from 2% of food handlers in Italy (Talarico *et al*., 1997). 12% of flight-catering staff in Finland (Hatakka *et al*., 2000), 19% of restaurant workers in Chile (Figueroa *et al*., 2002), to 62% of fish processing factory workers in India (Simon & Sanjeev, 2007).

4.3.2. Frequency of the enterotoxin genes

This study were detected genes encoding the classic (*sea ,seb , sec ,sed,* and *see*) in *S.aureus* strains isolated from food handlers in Kerbala city . In study *seb* was the predominant gene followed by *sea*.

Table(4-3) shows the frequency of enterotoxin genes among the studied isolates. Among the 100 strains isolated from nasal swabs of food handlers, 16 (16%) were positive for *sea*, 18 (18%) were positive for *seb*, 8 (8%) were positive for sec, 6 (6%) were positive for sed and 8 (8%) were positive for *see*.

Table (4-3) Frequency of the enterotoxin genes

Multiple enterotoxin genes	Percentage %	Enterotoxin genes	Percentage %
(*sea,seb,sec*),(*seb,sed*)	1	*Sea*	16
(*sec,sed*) ,(*sed,see*)	1	*Seb*	18
(*sea,sec,sed*),(*seb,see*)	1	*Sec*	8
(*sea ,seb*)	2	*Sed*	6
(*sea,seb,see*)	2	*See*	8
(*sea,seb,sec,sed,see*)	1	(*sea, seb, sec, sed* and *see*)	38

The predominant enterotoxins genes in this study were *seb* followed by *sea* in all isolates. In Kuwait, *S.aureus* strains isolated food handlers were shown have enterotoxin genes *seb* followed by *sea, sec and sed* (Al Bustan *et al*., 1996, Soto *et al*., 1996). The occurrence of multiple genes is carried by the same isolate indicating the pathogenic potential of *S.aureus*.

In this study, *sea* was detected in 16 (16%) strains. However, in other studies, the percentages of positivity to this gene were variable and differ according to the settings and source of the strains geographic region . *Sea* was studied in Switzerland between November and December 2010 and was detected in 13 (26 %) of strains isolated from 50 nasal swabs, in 10 (20%) (out of 50 strains) of strains isolated from clinical cases of infection and 6 (30%) of strains isolated from 20 food poisoning cases (Wattinger *et al*., 2012). In other studies ,*sea* was studied in Poland and was detected in 7 (8.75%) of strain isolated from 80 nasal carriers (Bania *et al*., 2006). In other studies , *sea* was studied in Netherlands, was detected in 20.97 (19.6%) of strain isolated

from 107 nasal swabs of a control (healthy) population (Mehrotra *et al*., 2000b). In other studies *sea* was studied in a tertiary-care hospital (Samsung Medical Center) in Seoul, Korea in period from December 2005 to February 2006 and was detected in 45.03 (47.4%) of strain isolated from 95 nasal swabs of children, and in 14.98 (21.4%) of 70 strain isolated from blood (Peck *et al*., 2009).

Also, *seb* was detected in 18(18%) strains. In Switzerland it was detected in 4 (8%) of strains isolated from 50 nasal swabs, in 11 (22%) (out of 50 strains) of strains isolated from clinical cases of infection and 1 (5%) of strains isolated from 20 sample of food poisoning cases (Wattinger *et al*., 2012). In Poland, it was detected in 4 (5%) of strain isolated from 80 nasal carriers (Bania *et al*., 2006). In Korea it was detected in 4 (5.8%) of strain isolated from 70 from blood (Peck *et al*., 2009). In Netherlands, it was detected in 5 (5.6%) of strain isolated from 107 nasal swabs of a control (healthy) population (Mehrotra *et al*., 2000b).

In this study, *sec* was detected in 8 (8%) strains. *sec* was studied in Switzerland 8 (16 %) of strains isolated from 50 nasal swabs, in13 (26%) (out of 50 strains) of strains isolated from clinical cases of infection and 4 (20%) of strains isolated from 20 food poisoning cases (Wattinger *et al.*, 2012). In Poland, it was detected in 14 (17.5%) of strain isolated from 80 nasal carriers (Bania *et al*., 2006). In other studies , *sec* was studied in a tertiary-care hospital (Samsung Medical Center) in Seoul, Korea in period from December 2005 to February 2006 and it was detected in 1.9 (2.1%) of strain isolated from 95 nasal swabs of children, in 24 (34.3%) of 70 strain isolated from blood (Peck *et al*., 2009). In Netherlands, it was detected in 8 (7.5%) of strain isolated

from 107 nasal swabs of a control (healthy) population (Mehrotra *et al*., 2000b).

Also , *sed* was detected in 6 (6%) strains. In Switzerland 1 (2%) of strains isolated from 50 nasal swabs, in 3 (6%) (out of 50 strains) of strains isolated from clinical cases of infection and 3 (15%) of strains isolated from 20 food poisoning cases (Wattinger *et al*., 2012). In Poland 4 (5%) of strain isolated from 80 nasal carriers (Bania *et al*., 2006). In Korea it was detected in 2 (2.9%) of strain isolated from 70 from blood (Peck *et al*., 2009). In Netherlands, it was detected in 2 (1.9%) of strain isolated from 107 nasal swabs of a control (healthy) population (Mehrotra *et al*., 2000b).

The *see* gene was detected in 8(8%) strains in this study. This result is important because the previous studies showed the presence of this gene only in strains from food. In Switzerland, *see* was found to be absent in the strains isolated from nasal swabs (n=50), clinical cases of infection (n=50) and from food poisoning cases (Wattinger *et al*., 2012). In other studies, *see* was studied in a tertiary-care hospital (Samsung Medical Center) in Seoul, Korea in period from December 2005 to February 2006 and it was absent in the strain isolated from nasal swabs of children (n=95), and strain isolated from blood (n=70) (Peck *et al*., 2009). In other studies , *see* was studied in Netherlands, but not detected in any strains isolated from 107 nasal swabs of a control (healthy) population (Mehrotra *et al*., 2000b). On the other hand, in Brazil , *see* was detected in 2.7 (5.1%) of strain isolated from 54 raw milk samples (Rall *et al*., 2008).

The variation in reported rates results, at least partly, from differences in study populations, sampling and culture techniques, and criteria for the definition of persistent or intermittent carriers (VandenBergh *et al*.,1999).

Nasal Carrier of *S.aureus* have been identified as risk factors for community-acquired and nosocomial infections. Screened 230 donors of diverse ethnic and socioeconomic backgrounds and identified 62 (27%) whose nasal secretions were colonized by *S. aureus* (cole *et al*., 2001).

This study offered novel PCR primers specific for the detection of *sea, seb, sec, sed* and *see* genes of *S. aureus*. These primers could be used for an epidemiological study of the hazardous *S.aureus* in food-poisoning outbreaks. The identification of staphylococcal toxin genes in strains of *S.aureus* by PCR offers a very specific, sensitive, relatively rapid, and inexpensive alternative to traditional immunological assays which depend on adequate gene expression for reliability and sensitivity (cole *et al*., 2001).

4.4. Factor influencing the enterotoxin production, protein extraction and separation by SDS.PAGE was used

To detect the band representative to enterotoxin (~27 KDa) several studies have documented that the enterotoxins have a molecular weight of (~27 KD) (Imani Fooladi *et al*., 2010). And SDS-PAGE was used to detect the presence of the enterotoxin gene production (EI-Jakee *et al*., 2013). In the current study, it was studied the effects of several growth condition on the growth rates of *S.aureus* isolates , and thereafter, studied the effects of those conditions, on the enterotoxin production , for this purpose, a (SANS 13) isolate that showed to possess multiple enterotoxin genes were selected, in

addition an (SANS 8) isolate that shown not to possess any enterotoxin genes were chose for comparison.

4.4.1. Effects of Salt concentration on Enterotoxins production by *Staphylococcus aureus*

Table (4-4) shows the effects of salt (Nacl) concentration on growth of *S.aureus* and its production of the enterotoxins. Four salt concentrations were used in this study (8%, 10%, 12%, and 14%) that represents the normal range of salt concentration where *S.aureus* can grow in. The growth yield was evaluated by optical density at 600 nm while enterotoxin production was judged by the detection of around 27 KDa bands on SDS-PAGE electrophoresis.

As shown in table (4-4) , the 8% salt concentration gave the high growth rate followed by 10% Nacl and the growth rate was decreased in association with increasing the salt concentration. Enterotoxins production showed a positive correlation with salts concentration at 14%, where toxins were detected when bacteria grown in high salt concentration (12%, 14%).

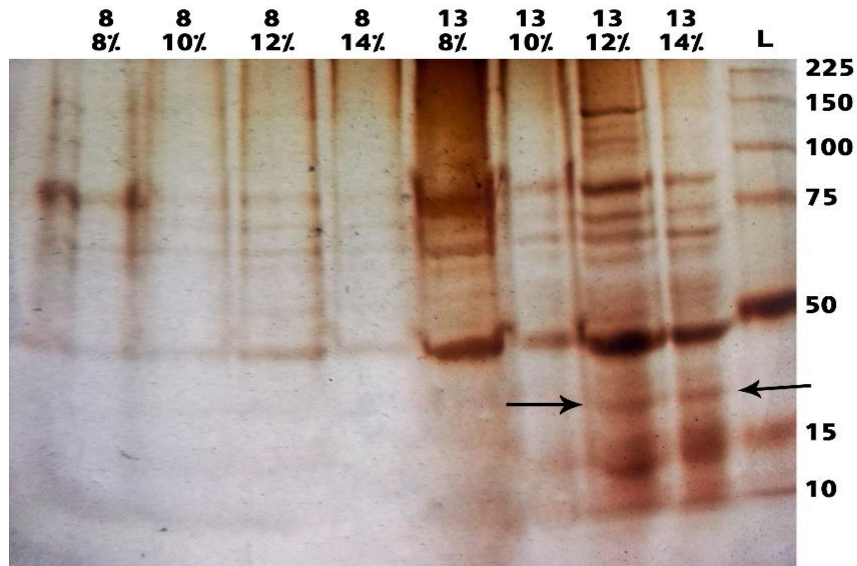

Figure 4.4 Electrophoresis patterns of whole cells extracted proteins from two *S.aureus* isolates (SANS13 and SANS8) grown in media containing different Nacl concentrations (8%, 10%, 12 % and 14%). The band representing the enterotoxin (~27 KDa) was shown in patterns of SANS13 grown in 12% and 14% Nacl).

These results indicate two things; first, the toxin production could be increased in high salt concentrations, and second, toxin productions may be not related the growth rate of bacteria. In other words , it is not necessary that the better growth condition to be associated with toxin productions. This results may be explained on the basis that the expression of stress proteins was induced due to the exposure of microorganisms to sub-lethal concentrations of salt , the induce occur in a profile similar to that of stress protein expression induced by heat shock. the Mar (multiple antibiotic resistances) operon can be induce by many environmental stresses, this operon known to regulate the expression of a large number of genes, including the efflux pump (the arcAB efflux pump) (Alekshun and Levy, 1999 , McMahon *et al*., 2007).

These results are very important and may have implications as it is known that adding salt is common methods to preserve foods. In addition to that some kinds of foods are prepared with high salt concentration.

Salt has often been incorporated as an antimicrobial agent in meat, meat products, or brine solutions. Historically, salt can be used both as an additive and preservative in foods, and abundant information on it can be found in the literature (Hajmeer *et al*., 2006).

Additionally , according to results, these kinds of foods have more risk to be contaminated with enterotoxigenic *S.aureus*. These result are consistent with previous studies (Vijaranakul *et al*., 1997 , Scybert *et al*., 2003 , Arney DR & CJC., 2005, Ganjian *et al*., 2012). Who found that *S.aureus* could grow gradually at salt conditions range from 5. 0% to 35. 0% (wt/vol).

And thus, it could be concluded that using high concentrations of salt in food preservation can lead to the increase the production of enterotoxins by *S. aureus.*

Table (4-4) effect of salt concentration on growth and enterotoxin production

Strain	Nacl	Optical dencity (OD) 600nm	Enterotoxin production
SANS13	8%	1.390	-
	10%	1.318	-
	12%*	1.220	+
	14%*	0.794	+
SANS8	8%	1.440	-
	10%	1.410	-
	12%	0.930	-
	14%	0.943	-

***(**12,14)% Nacl concenteration was shown highly significant differences at ($P \leq 0.01$)

4.4.2. Effects of pH on Enterotoxins production by *Staphylococcus aureus*

Table (4-5) shows the result of studying the effect of pH on growth rate and enterotoxin production of *S.aureus* isolate. This experiment showed that the better growth rates were seen at range of pH between pH 6.5 to 8.5 and no growth could be detected at pH 4.5 .

In addition, enterotoxin production was detected at pH 5.5 and 6.5 and not detected at pH 8.5, these results indicates that low pH (acidic environment) and high (alkaline environment) are not supportive for toxin production, and enterotoxin are only produced at pH near the neutral range.

These results were consistent with previous studies which found that optimal conditions for SEB production were achieved with pH control at 7.0 (Metzger *et al*., 1973, Carpenter and Silverman, 1974). In addition, another study reported that pH of 6.8 give higher yields of enterotoxins B and C than either pH 6.0 or 5.3 (Reiser and Weiss, 1969).

These result are very important because it may be used to suggest a way for selecting environment not supportive to toxin production. These environment may be used for preserving food (acidic or alkaline) rather than adding sugar or salts.

SE production decreases in acidic pH and it is optimal in neutral pH. Usually, SE production is inhibited in pH below 5. Substances used to acidify the medium may have more or less effects at a given pH. For example, acetic acid has a greater inhibitory effect than lactic acid on SE production (Le Loir *et al*., 2003).

Table (4-5) effect of pH on growth and enterotoxin production

Strain	pH	Optical dencity (OD) 600nm	Enterotoxin production
SANS13	4.5	0	-
	5.5*	1.340	+
	6.5*	1.594	+
	8.5	1.530	-
SANS8	4.5	0	-
	5.5	1.550	-
	6.5	1.650	-
	8.5	1.803	-

*5.5 and 6.5 pH was shown highly significant differences at ($P \leq 0.01$)

4.4.3. Effects of glucose concentration on Enterotoxins production *by Staphylococcus aureus*

Table (4-6) shows the effects of different glucose concentration (0%, 0.5%, 1.5% and 2.5%) on growth rate and enterotoxin production of *S.aureus.* The result of this experiment showed that better growth concentration range from 0.5% to 1.5%). However, enterotoxin production was detected at glucose concentration of 2.5% and not detected in lower glucose concentration.

This result indicates that glucose concentration is important for enterotoxin production. And glucose may induced the production of the toxin by the bacteria through a certain mechanisms.

Regarding the increased growth rate with addition of glucose to the medium, the results of this study are consistent with previous study that showed that the presence of glucose and glycerol in the medium also resulted in a rapid increase in the specific growth rate (Jarvis *et al*., 1975).

One study has shown that glucose had little effect on growth and toxin production at levels from 0 to 0.3% but suppressed bacterial growth and, more extensively, toxin production at a level of 3% (Schlievert and Blomster, 1983).

Adding sugar to food is common and is done for different purposes such as food preservation or for production of sweat foods etc , therefore, the results of this study may suggest that food with added sugar entails higher risk of containing enterotoxin.

SE production, especially for SEB and SEC Shown to be inhibited by glucose (Bergdoll, 1989). This inhibitory effect has been attributed to a drop in pH, as a consequence of glucose metabolism. Glucose and low pH indeed have an inhibitory effect on *agr* expression thus, these observations could be correlated with *agr*-dependent synthesis of these SEs (Regassa *et al*., 1992; Novick, 2000).

Table (4-6) effect of glucose concentration on growth and enterotoxin production

Strain	Glucose concentration	Optical dencity (OD) 600nm	Enterotoxin production
SANS13	0%	1.745	-
	0.5%	1.966	-
	1.5%	2.000	-
	2.5%*	1.720	+
SANS8	0%	1.617	-
	0.5%	1.849	-
	1.5%	1.789	-
	*2.5%	1.766	-

*2.5% glucose concentration was shown highly significant differences at ($P \leq 0.01$)

Conclusions
&
Recommendation

Conclusions

1- The prevalence of nasal carriage of *Staphylococcus aureus* is high among food handlers in Holy Kerbala city, and prevalence of enterotoxin genes among the *Staphylococcus aureus* isolates is also high. Therefore, strict measures are necessary to protect food poisoning outbreaks.

2- The production of enterotoxins by *S.aureus* increases in preserved food due to high concentrations with studied rangs of salt in food preservation .

3- Low pH (acidic environment) and high (alkaline environment) are not supportive for toxin production, and enterotoxin are only produced at pH near the neutral range.

4- The enterotoxin produce at 2.5% glucose concentration

Recommendations

1. Conduct detailed study to detect staphylococcal enterotoxin in food that processed by food hundlers were positive for staphylococcal enterotoxins.
2. More attention is needed on surveillance program on food handlers and the use of molecular methods to detect nasal carriage of enterotoxigenic *S.aureus* strains.
3. Commitment to healthy conditions and good hygiene is necessary when preparing meals in restaurants and Cafes.Regular food testing is required in restaurants and punish the restaurants that does not apply good healthy conditions if Preparation of food.Regular testing of workers in restaurant kitchens is required to make sure that no one has pathogenic bacteria and provide health education to them, and forcing them to wear headscarves and masks, gloves and clothes for work
4. Using multiplex PCR assay in detecting the presence of newly described staphylococcal enterotoxin genes is advised, because there is no available immunoassay to detect them because of the difficulty to purify them.
5. Because high concentrations of salt in food preservation contributes to the spread of S.aureus that cause food poisoning, using moderate concentration of salt is recommended .

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الخلاصة

تم جمع 332 مسحة انفية من المتعاملين مع الاغذية العاملين في كربلاء المقدسة,كانت اكبر مجموعة من المتعاملين مع الاغذية في هذة الدراسة هم النادلون في المطاعم (n=95)عينة بالاضافة الى ثلاث مجموعات اخرى وهم الطباخين (n=68), وعمال المخابز (n=66) , وعمال معامل المياه (n=59).

تم التعامل مع العينات بحسب طرق العمل القياسية للاحياء المجهرية , تم تشخيص العزلات مظهريا بالاعتماد على الخصائص المزرعية للعزلات والفحوصات الكيموحيوية بالاضافة الى اشرطة API staph وتم ايضا تشخيص العزلات جينيا باستخدام تفاعل البلمرة المتسلسل (PCR) حيث تم استهداف الجين *fem A* ,تم الحصول على 100عزلة من جرثومة *S.aureus* من المتعاملين مع الاغذية وشكلت نسبة (30.1%) ,حيث كانت منتشرة بشكل ملحوظ بين عمال حوانيت المدارس (100%), وعمال المقاهي (85.7%) , وعمال معامل الحلويات (71%).

تم أستخدام تفاعل البلمرة المتسلسل المتعدد لغرض دراسة أمتلاك جينات السموم المعوية لجرثومة *S.aureus* نوع a,b,c,d,and e .ان عدد العزلات الجرثومية التي اعطت نتيجة موجبة لجينات السموم المعوية كان عددها 38(38%)عزلة من مجموع 100 عزلة ,حيث اعطت 16(16%) عزلة نتيجة موجبة للجين *sea*, و18 عينة (18%) اعطت نتيجة موجبة للجين *seb* , بينما كانت عدد العزلات الموجبة للجينين *sec*و*see* هي 8 عينة (8%) , اما بالنسبة للجين *sed* فقد كان عدد العزلات الموجبة 6 عزلات (6%).

بالاضافة الى ان العديد من السلالات تمتلك جينات لسموم معوية متعددة حيث كان 38% من العزلات موجبة لواحد او اكثر من الجينات ,27% من العزلات موجبة لجين واحد فقط و6% من العزلات كانت موجبة لجينين بينما 4% فقط من العزلات كانت موجبة لثلاث جينات و1% من العزلات موجبة لخمسة جينات.

أظهرت نتائج هذة الدراسة الى ان عدد كبير من المتعاملين مع الاغذية يحملون سلالات منتجة للسموم المعوية وهذا يشكل خطر على الصحة العامة اذ من الممكن ان تنتقل تلك السلالات الى الاطعمة خلال عملية طهي الطعام وتحضيرة او خلال عملية تعليب الطعام...الخ ,لذلك يجب وضع اليات محددة لغرض منع تسمم الطعام الذي ينتج من انتقال جرثومة *S.aureus* الى منتجات الطعام .

الخلاصة

لغرض دراسة تأثير الظروف الزرعية المختلفة على انتاج الذيفان فأن سلسلة من التجارب قد اجريت, هذه التجارب تشمل ثلاث عوامل وهي (الملح ,الرقم الهيدروجيني والكلوكوز) ولهذا الغرض تم اختيار سلالتين , السلالة الاولى تمتلك جينات لسموم معوية متعددة , بينما السلالة الثانية لاتمتلك اي جينات للسموم المعوية.

وجد ان التركيز العالي من ملح كلوريد الصوديوم (12%,14%) يحفز من انتاج الذيفان لكن يخفض معدل النمو , وبالنسبة لتأثير الرقم الهيدروجيني فقد لوحظ ان هناك زيادة في كل من معدل النمو وانتاج الذيفان قرب الرقم الهيدروجيني المتعادل,وتركيز الكلوكوز (2.5%) يحفز من انتاج الذيفان ولكن يخفض معدل النمو.

وطبقا لهذة النتائج فأن الظروف التي لاتدعم انتاج الذيفان هي الرقم الهيدروجيني المنخفض وتركيز ملح كلوريد الصوديوم المنخفض وتركيز الكلوكوز المنخفض

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء / كلية العلوم

قسم علوم الحياة

الكشف عن جينات السموم المعوية وبعض العوامل المؤثرة على السموم المعوية لعزلات المكورات العنقودية الذهبية في مدينة كربلاء

رسالة مقدمة الى

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وهي جزء من متطلبات نيل درجة الماجستير في علوم الحياة

من قبل

هبة مهدي محمود موسى

بكالوريوس علوم حياة / جامعة كربلاء (2012)

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