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PCR Detection of Enterotoxins and Methecillin Resistances Genes in *Staphylococcus aureus* Isolated from local food in Sulaimania City

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Abstract

Present study was carried out to evaluate bacterial contaminated foods in local markets of Sulaimania city. A total 250 samples were examined for isolation and identification of *Staph. aureus*, that producer to enterotoxins and resistances to methicillin . The samples included dressing of cake, soft chesses (white), ready to eat foods, red meat, and poultry. The results appeared that: the incidence of Staphylococci were identified in 175 (70%) of the total samples from those; 104(42%) were positive for *Staph. aureus* which had the ability to grow on the mannitol salt agar media, the most contaminate foods were dressing of cake 51% and soft chess 45% , While red meat was 44% and poultry 48% as well as ready to eat foods which contaminated with 35% of the samples. According to biochemical testes; *Staph. aureus* isolates had the ability to produce many enzymes like protease lipase, lethicinase , Coagulase , Catalase, also could fermented mannitol anaerobically and Hemolysin production, while all the isolates were oxidase negative. The production of enterotoxin tested by culture methods for *Staph. aureus* isolates showed that 57.14% of the isolates were enterotoxin producer. The producer isolates were confirmed by PCR to detect the existence of (*sea*, *seb* and *sec*) genes, so 55%of the tested isolates possessed these genes. Sensitivity to antibiotics applied to *Staph. aureus* isolates that enterotoxin producer revealed different percentage of sensetivity to different antibiotics, the isolates appeared sensitivity toward pencillin, methicillin, vancomycin. The Methicillin resistance *Staph. aureus* which tested by disk diffusion methods was 81.6%, but by PCR was 73.3%.

Key word: *Staphylococcus aureus* , Enterotoxins, MERSA.

INTRODUCTION

Staphylococcus aureus is an important mammalian pathogenic bacterium that has long been recognized for its propensity to cause serious and invasive diseases. *Staphylococcus aureus*, a gram-positive bacterium, is one of the most common clinical and foodborne pathogens worldwide (Foster 2005; Cagatay *et al.* 2007) It is an opportunistic bacterium frequently part of human micro flora, causing disease when the immune system becomes compromised (Aires de Sousa *et al*., 2004). The food products contaminate by this bacteria often causing food-borne intoxications due to the production of enterotoxins (Loir *et al.*, 2003; Rode *et al.*, 2007). *S. aureus* produces many important virulence factors including SEs, numerous staphylococcal enterotoxins have been described and ingestion of these enterotoxins, causes a rapid onset of nausea and vomiting within 1–6 hours. Less than 200 ng toxin is sufficient to cause symptoms. Generally, *Staph. aureus* counts of 100,000cells/g food are necessary to appear the symptoms; may be severe, they usually resolve within a day and serious complications, hospitalization, and death are rare, In some circumstances ingestion of staphylococci can cause enteritis It was

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reported that more than 70% of *S. aureus* strains produced one or more enterotoxins (Jorgensen *et al.* 2005). Several SEs have been characterized according to serological analysis and classification, and most of their genes have been sequenced (Akineden *et al.* 2001; Omoe *et al.* 2002; Thomas *et al.* 2006). Five of these Staphylococci enterotoxins (SEs); SEA to SEE are recognized as major causes of foodborne illness with SEA, SEB and SEC being the most frequently implicated in foodborne outbreaks (Balaban and Rasooly 2000).It is a well-known foodborne pathogen that produce heat-stable enterotoxins during the growth on a variety of foods, including meat and poultry products, eggs, cream-filled pastries, potatoes, and some salads, Vegetables are less commonly cited as vehicles for *Staph. aureus*. To date, 21 staphylococcal enterotoxins (SEs) or enterotoxin-like proteins (SEls) have been identified and designated SEA to SEV . SEs is short, extracellular proteins that are soluble in water. They are heat resistant (depending on the SE type, SE concentration and food matrix) and highly stable to proteolytic enzymes, such as pepsin, trypsin chymotrypsine, rennin and papain . Enterotoxins stimulate the release of serotonin in the gut. The serotonin acts on neuron receptors in the gut, stimulating the vomiting center in the brain via the vague nerve. Staphylococcal enterotoxins (SEs) are emetic toxins and that cause of food poisoning in humans. SEs has been classified as members of the pyrogenic toxin superantigen family because of their biological activities and structural relatedness. The ability of *S. aureus* to grow and produce SEs under a wide range of conditions is evident from the variety of foods implicated in staphylococcal food poisoning (Balaban and Rasooly,2000; Le Loir *et al*., 2003). The term Methicillin resistant *Staphylococcus aureus* (MRSA) is used for bacteria of *Staph. aureus* that are resistant to methicillin , that have resistant to beta-lactam. MRSA was recognized as the bacteria associated with hospital acquired staphylococcal infections (CFSPH, 2011).

There has been an enormous increase in the isolation of MRSA strains that has been attributed to the widespread use of methicillin in clinical settings and in food animal production facilities for decades (Panlilio *et al.* 1992; Oliveira *et al.* 2002). In addition, the gene encoding methicillin resistance (*mecA*) has been widely used as a molecular marker of methicillin resistance in detection and typing of *Staphylococcus* spp. (Jonas *et al.* 2002; Francois *et al.* 2003).

Materials and Methods

Sample collection

Two hundred and fifty (250) food samples were collected from local markets from the period November to April. Each sample were transported to the laboratory in insulated boxes and tested within 24hour.

Isolation and Characterization of MRSA

Ten gram of each food samples were incubated overnight at 35°C in 100 ml of a selective enrichment broth consisting of Mueller-Hinton broth (oxoid) supplemented with NaCl to a final concentration of 7% (wt/vol) and 2 µg of oxacillin /ml. After centrifugation for 5 min at 6000 × *g*, the supernatant was discarded and bacterial sediment was re-suspended in the remaining broth, around 100 µl., for primary conventional identification of 5 and 10 µl were sub-cultured on mannitol-agar containing 7.5% NaCl and on Mueller-Hinton agar supplemented with 4% NaCl and 6 µg of oxacillin /ml, respectively. Agar plates were incubated at 35°C for 24 hour. *Staph. aureus* was identified by tested for production of catalase, coagulase, oxidase, Lipase, Lecithinase, Haemolysin and Protease production, according to (Levinson and Jawetz,2000; Harely and Prescott, 2002).

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Production of Enterotoxin

The colonies of purified Staph. *aureus* were stabbing into brain heart infusion slant, after incubation for 24 hrs. at 37C°, one loop full of the growth from the slant was transferred to 5 ml tubes of serial saline solution in order to obtain a $3x10^8$ cell/ml, Four drops of this aqueous culture suspension were spread over the surfaces of plates of brain heart infusion agar which supplemented with phenol red, and then the medium incubated at 37C° for 24 hr. A positive result (enterotoxin production) was indicated by changing in the media color (Cencigoga *et al*., 2003).

PCR for identification of *Staph. aureus*

1- Genomic DNA Extraction

The selected isolate to the Genomic DNA extraction are enterotoxin production and MRSA isolate, the subjection of Genomic DNA extraction was according to the Bioneer kit manufacturing company information and procedures. DNA samples were dissolved in Tris-EDTA buffer (10 mM Tris chloride, 1 mM EDTA[pH 8.0]).

Primers. Oligonucleotides ranging from 18- to 24-mers were selected from the published DNA sequences of the *S. aureus* genes for PCRs, two primers sets were prepared: set A was designed to amplify *sea*, *seb*, *sec*, whereas set B was designed to amplify *mecA*. The primer sequences used in the multiplex PCRs are described in Table 1.

PCR conditions, two sets of primer mixes were prepared according to the master mixes of components from the GeneAmp kit (Perkin-Elmer, Norwalk, Conn.), with slight modifications to the given instructions. primer setA contained 200 mM deoxynucleoside triphosphates; 5 ml of 103 reaction buffer (100 mM Tris-HCl(pH 8.3), 500 mM KCl); 1.5 mM MgCl2; 20 pmol (each) of *sea*, *seb*, *sec*, *see*, and *femA* primers; 40 pmol of *sed* primer; 2.5 U of *Taq* DNA polymerase (AmpliTaq DNA polymerase; Perkin-Elmer), and 10 to 1,000 ng of template DNA. The volume of this mix was adjusted to 50 ml with sterile water. Multiplex primer set B included the same constituents as in set A except for the MgCl2 concentration (2.0 mM) and the primers, 20 pmol each for *mecA*. Evaporation of the reaction mixture was prevented by addition of 100 ml of sterile mineral oil.

DNA amplification was carried out in (Perkin-Elmer) thermocycler with the following thermal cycling profile: an initial denaturation at 94°C for 5 min was followed by 35 cycles of amplification (denaturation at 94°C for 2 min, annealing at 57°C for 2 min, and extension at 72°C for 1 min), ending with a final extension at 72°C for 10 min.

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Table 1 the designed primer sequences

Taret gene & primer name	Nucleotide sequence	Amplicon size(bp)	Reference
Sea SEAf SEAr	 5´ATTAACCGAAGGTTCTGTAGA3´ 5´TTGCGTAAAAAGTCTGAATT3´	552	Tang *et al*.2006
Seb SEBf SEBr	 5´TGTATGTATGGAGGTGTAAC3´ 5´ATAGTGACGAGTTAGGTA3´	270	Sharma *et al*.2000
Sec SECf SECr	 5´ACCAGACCCTATGCCAGATG3´ 5´TCCCATTATCAAAGTGGTTTCC3´	371	Cremonesi *et al.2005*
mecA MECAf MECAr	 5´GTAGAAATGACTGAACGTCCGATAA3´ 5´CCAATTCCACATTGTTTCGGTCTAA3´	310	Perez-Roth *et al*2001

RESULTS AND DISCUSSION

In this study, 250 food samples were taken from sulaimania local markets and examined for *staphylococcus aureus*, the samples included; (dressing of different cake (60), dairy product(60),and ready to eat food (60), meat (35) and poultry (35),as appeared in table 2. Out of these food samples, 175(70%) of the total samples were contaminated with bacteria Staphylococci which had the ability to grow on the mannitol salt agar media which is considered as a selective and differential media for identifying genus *staphylococcus (*Benson 2001), form the total samples as shown in table 2; 104(42%) samples were appeared doubted *S. aureus* that grew on mannitol salt agar and had the ability to ferment mannitol forming golden colonies , biochemical tests were conducted, the characters of isolated bacteria were determined as appeared in table 3 .

Table 2: Sources and number of *Staphylococcus aureus* isolates.

Food types	Number of samples	Number of samples Contaminated with *Staph. aureus*	Number of samples contaminated with *Staphylococci*
Red meat	35	12	40
Poultry	35	14	35
Cake with dressing	60	35	47
Dairy products	60	27	33
Ready-to eat food	60	16	20
Total	250	104(42%)	175(70%)

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For further identification; the catalase test was performed and all isolates gave positive results which differentiated Staphylococcus from genus Streptococcus which gave negative results. While the isolates gave negative result to the oxidase test preformed to differentiate Staphylococcus from genus Micrococcus that usually gives positive result (Brooks *et al*.,2007).

Table 3. Biochemical characteristic and some virulence factors of *Staph. aureus* isolates

Biochemical tests	%positive samples
Catalase	100(104)
Oxidase	0(0)
Coagulase	100(104)
Anaerobically fermented mannitol	100(104)
Lipase	72(69)
Lecithinase	22(21)
Hemolysin production	77(74)
Protease	62(60)

Staph. aureus isolates examined to coagulase tube test, all the isolates (104) gave positive reaction, Anaerobic utilization of mannitol was carried out to distinguish *Staph. aureus* from other staphylococci , the result showed that the all the isolates;104 (100%) had the ability to fermented mannitol sugar anearobically, According to lipase production, there were 72 (69%) of isolated bacteria were shown positive results. This corresponds with Hammer *et al*., (2005). Staphylococci possess lipolytic enzymes which render them resistant to the bactericidal lipids of human skin (Kusumaningrum *et al*., 2003). Furthermore, bacterial lipases have been encouraged bacteria to survive pasteurization temperatures, thus they are heat stable enzymes, which is the reason for them being a main source of spoilage in heated fat containing food products (Braun *et al*.,2001).The lecithinase least synthesized enzyme was 22 (21%) and this also corresponds with research of Hammer *et al*., (2005), The reason for lower lecithinase production may be due to the fact that the *Staph. aureus* isolates were from foods.

Staph. aureus produced protease 62 (59%) as shown in table 3, *Staph. aureus* can produces extracellular proteases, including metallo-serine and cysteine proteases (Dubin, 2002), reports suggest that proteases also play a role in the transition of *Staph. aureus* cells from an adhesive to an invasive phenotype by degrading bacterial cell surface proteins, such as fibronectin binding protein and protein A, and possibly contributing to the dissemination of infection (Karlsson and Arvidson, 2002).

According to hymolysin production, 77 (74%) of the isolates on sheep blood agar was positive indicated by a wide zone of complete haemolysis around the bacteria colonies indicating β-haemolysis, or a wide zone of incomplete haemolysis around the bacteria colonies indicating α-haemolysis.

Detection of enterotoxigenic isolates of *Staph. aureus* by PCR

According to PCR method for detection of *sea, seb, sec,* genes found that 33(32%) of isolates produced enterotoxins table 4, 20 isolates produce enterotoxin type C, shown in figure 1. 8

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isolates produced enterotoxin type A as shown in figure 2 , 5 isolates produce enterotoxin type B as shown in figure 3. Among these isolates 3 isolates produced two kinds of enterotoxin (type A and C),and only one isolate produce enterotoxin type A with B.

Soriano *et al* ., (2002) studied the incidence of enterotoxigenic *Staphylococci* and their toxins in foods, Out Of 504 food samples, 19 (3.8%) yielded strains of enterotoxigenic Staphylococci, and these strains produced enterotoxins C (SEC), D (SED), B (SEB) and A (SEA),moreover, SEA, SEB and SEC were isolated from three hamburger samples.

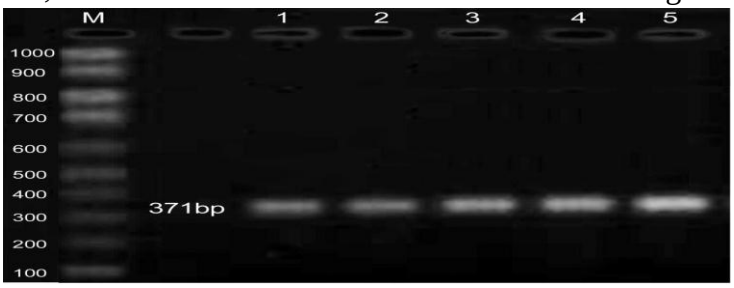

Figure 1: PCR Amplification and Electrophoretic analysis of *sec* genes

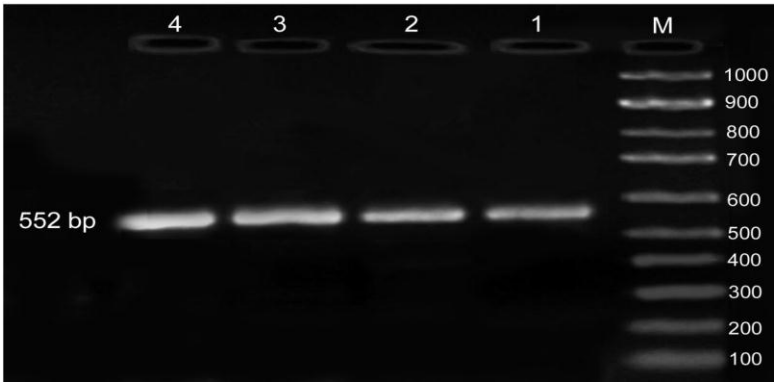

Figure 2: PCR Amplification and Gel Electrophoretic Analysis of sea genes

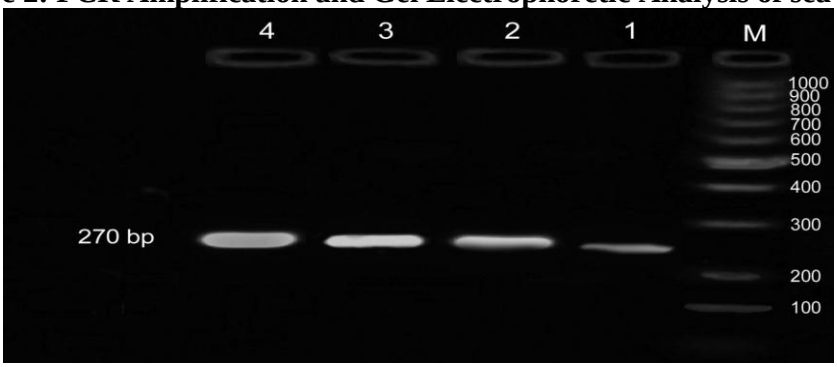

Figure 3 PCR Amplification and Gel Electrophoretic Analysis of seb genes

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Table 4 PCR Detection of *Staph. aureus* Enterotoxogenic isolates

Source of isolates	No. of samples contaminated with *Staph. aureus*	No. of isolates (+Es)	Percent of (+Es)
Cake with Cream	35	10	28.5
Dairy products	27	12	44.44
Ready-to eat food	16	5	31.2
Poultry	14	3	21.4
Red meat	12	3	25
total	104	33	31.73

Type C enterotoxin was the commonest (42.7%) while type D was the least common (2.5%). The type A enterotoxins was recorded in 12.8%of enterotoxins- producing isolates. Staphylococcal enterotoxins (SEs) are encoded by the respective genes (*sea* - *seu*), a strain of *Staph. aureus* can carry two or more genes *seg* and *sei* being the most common pair, the presence of a single SE gene has been reported by Ercolini *et al*.,(2004). Scherrer *et al.*, (2004), found that *Staph. aureus* isolated from sheep's and cow's milk were often producers of SEC. In the present study enterotoxin C was the most frequently detected especially those that being isolated from dairy products. Sanmoon, (2007) found that SE-producing *Staph. aureus* isolates which obtained from milk, (59.6%) of the isolates produced only one type of enterotoxin, and the remaining isolates produced two or more types of toxins. Simple PCR procedures developed for the detection of SE *(sea, seb, sec, sed, see, seg, seh,* and *sei* together) genes directly in spiked food samples (McLauchlin *et al*, 2000).

Methicillin resistance *Staph. aureus* (MRSA)

Detection of MRSA in this study was conducted to the enterotoxgenic *Staph. aureus* isolates, the results showed that 45.1% and 40.3% of the enterotoxigenic *Staph. aureus* were methicillin resistance by disk diffusion methods and by molecular methods (PCR) respectively as shown in table 5 and figure 4 .

Table 5: Identification of Sensitive isolates of *Staph. aureus* to Methicillin by PCR and Disk methods

Source of isolates	No. of samples contaminated with *Staph. aureus*	No. of isolates (+SEs.)	No. of isolates resistance to Methicillin	
			By disk	By PCR
Cake with Cream	35	10	**19**	**13**
Dairy products	27	12	**15**	**15**
Ready-to eat food	16	5	**6**	**6**
Poultry	14	3	**4**	**3**
Red meat	12	3	**3**	**5**
Total	104	33	47(45.1%)	42(40.3%)

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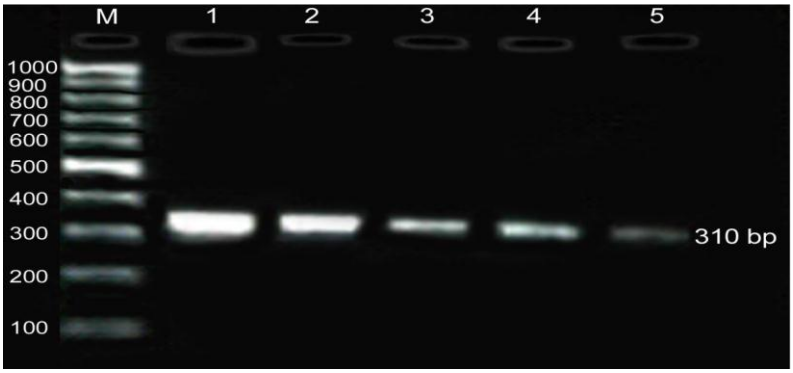
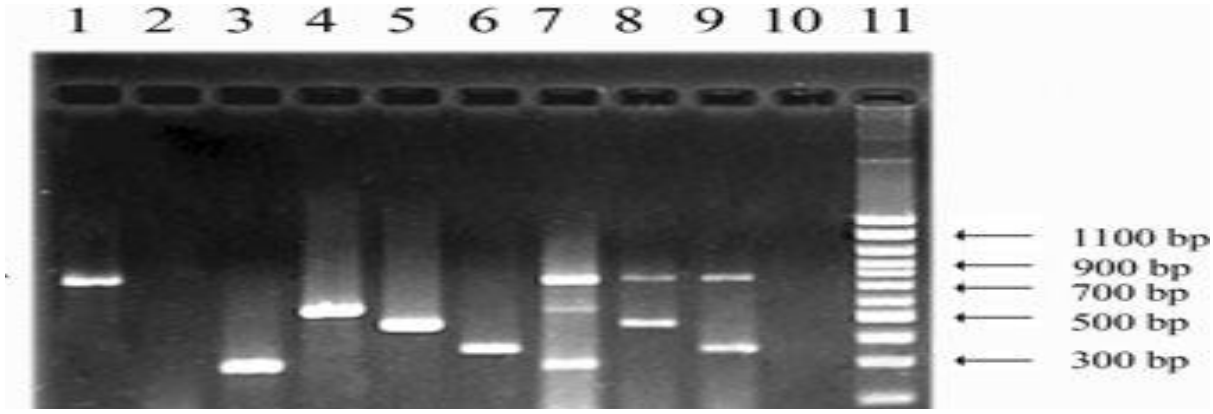


Figure: 4 PCR-Amplification and Electrophoretic analysis of *mecA genes*

To study the combination between *mecA* gene and *sea*, *seb* and *sec* genes, it was found that 8 isolates have *mecA* with *sec genes* , it mean that these isolates have ability to produce enterotoxin type C and resistance to methicillin. However, only 2 isolates have *mecA* with *sea* genes and the
ability to produce enterotoxin type A and resistance to methicillin at the same time as shown in figure 5.

Foods may be contaminated by human strains of MRSA which present in meat processors and other food handlers. Meat may also be contaminated by MRSA carried in animals as demonstrated by a study following pigs from lair age through slaughter to commercial pork products (Molla *et al*., 2010). Some studies detected primarily MRSA strains in foods; indicating that humans were the probable source (Weese *et al*., 2010). Since *Staph. aureus* is a known cause of mastitis in ruminants, several studies analyzed milk from cows with mastitis and detected MRSA, these studies have demonstrated that meat can also become contaminated during slaughter and processing of animals carrying MRSA (Seo *et al.*,2010;Beneke *et al.*,2011; de Jonge *et al.*,2010).



Figuer 5: PCR Amplification and Agarose gel electrophoresis of *sea,seb,sec* and *mecA* genes.

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