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Isolation and Characterization of Enterotoxigenic Coagulase-Positive and Methicillin-Resistant *Staphylococcus aureus* Contaminating Beef Burger and Hot Dog Sandwiches Retailed in Mansoura City

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ABSTRACT

Objective: The study was designed to assess, isolate and characterize *coagulase-positive S. aureus* and its enterotoxins, in addition to their methicillin resistance contaminating the beef burger and hot dog sandwiches purchased from different fast-food restaurants distributed in Mansoura city**.**

Design: Observational study.

Procedures: A total of 100 meat (50 beef burger and 50 hot dog) sandwiches were bacteriologically and molecularly analyzed to isolate and characterize the contaminating *coagulase-positive S. aureus* and its enterotoxins, in addition to their methicillin resistance.

Results: *S. aureus* was found in 86% of overall tested samples, 90% in beef burger and 82% in hot dog sandwiches, in counts of 5.3×10 - 2.9×10^4, 9.5×10 - 1.9×10^4 and 1×10 - 3.8×10^4 and mean counts of 3.9×10^3, 3.5×10^3 and 4.2×10^3, respectively. Of the 106 confirmed coagulase-positive strains, 14 (13.2%) were enterotoxin producing strains and 47 (44.3%) of them carry the *mecA* gene confirmed their methicillin resistance. Regarding the microbiological quality of samples according to detected counts, 10%, 79% and 11% were acceptable, unsatisfactory, and potentially hazardous ready-to-eat meat sandwiches, respectively.

Conclusion and clinical relevance: The results indicated that tested ready-to-eat meat sandwiches were contaminated with enterotoxigenic and methicillin resistant *S. aureus* and represent a potential hazard to consumers; hence strict hygienic measures at fast-food restaurants are crucial.

Keywords: *Staphylococcus aureus*, MRSA, Enterotoxins, Beef burger, Hot dog sandwiches.

1. INTRODUCTION

Staphylococcus aureus (*S. aureus*), a ubiquitous facultative anaerobic Gram-positive coccus occurs singly, in pairs and irregular clusters, is one of the leading pathogens that causes food poisoning outbreaks, globally **[1]**. The organism is a commensal and opportunistic pathogen causing infections range from minor skin disorders as wound infections, locally invasive diseases as osteomyelitis, cellulitis, sinusitis, and pneumonia, to life-threatening meningitis and septicemia **[2, 3]**.

Staphylococcus aureus is found on the skin and mucous membranes of humans and warm-blooded animals. It is also isolated from some food products such as meat and meat products which considered an important reservoir for this organism and subsequently involved in numerous outbreaks **[4 - 6]**. In processed meats, the contamination with *S. aureus* may be resulted from workers with hand or arm lesions or by coughing. The growth and proliferation of *S. aureus* in foods represent a potential health hazard to the consumers, as many strains produce enterotoxins. Therefore, when large numbers of *S. aureus* organisms encountered in processed meat, it may be due to inadequate sanitation, temperature control or both **[7]**.

Staphylococcus aureus can cause food poisoning by producing staphylococcal enterotoxins (SEs) manifested by vomiting, without or with diarrhea, abdominal cramping, and nausea, with symptoms start from half an hour to eight hours after consumption of contaminated foods **[8]**. SEs are heat resistant and can pass through gastrointestinal tract with no loss of their biological activity because proteolytic enzymes as renin, pepsin and trypsin do not have any effect on SEs **[9, 10]**. The amount of toxins required to produce food poisoning symptoms ranges from twenty Nano grams to one µg corresponding to 10^5 staphylococci CFU/g of the food **[11]**.

Recently, methicillin resistant *S. aureus* (MRSA) is a pathogen of increasing importance because it usually exhibited multiple antimicrobial resistance and considered by the WHO in 2017 as one of the 12 bacterial families that pose the utmost threat to human health **[12]**. The organism can be resistant to numerous antimicrobials and quickly disseminates worldwide. In recent years, MRSA caused about 5400 extra deaths **[13]**. Several foods reported as reservoirs for MRSA strains because

the organisms have been reported from different foods as beef, poultry, milk, and vegetables **[14, 15]**. *Staphylococcus aureus* became MRSA because of the acquisition of the *mecA* or *mecC* gene on the staphylococcal cassette chromosome mec (SCCmec), encodes reduced affinity penicillin-binding protein 2a (PBP2a) allows for cell wall biosynthesis at lethal β-lactam concentrations **[16, 17]**.

The widespread distribution of fast-food restaurants and cafeterias which mostly do not meet the required hygienic measures especially those related to food workers, and due to the increase in hours spent outside homes which enforced large numbers of people to consume meat sandwiches from these places, in addition to the increased health risk and multidrug resistance of MRSA. Therefore, the present study was designed to investigate the prevalence of coagulase-positive, enterotoxigenic, and methicillin resistant *S. aureus* contaminating beef burger and hot dog sandwiches retailed in fast-food restaurants and cafeterias distributed at Mansoura city.

2. MATERIALS AND METHODS

2.1. Collection of samples

A total of 100 meat (50 beef burger and 50 hot dog) sandwiches were purchased from different fast-food restaurants and cafeterias distributed in Mansoura city. Each sample was represented by a sandwich packed individually in a previously marked, clean polyethylene bag, then transferred with a minimum of delay -in icebox- to the laboratory of Meat Hygiene, Food Hygiene and Control Department, Faculty of Veterinary Medicine, Mansoura University, wherein the bacteriological analyses were completed.

2.2. Isolation and identification of S. aureus [18]

Ten grams of the meat part of each sample (beef burger or hot dog sandwich) were excised using a sterile scalpel then homogenized for one min, in a laboratory blender (Moulinex, France), with 90 ml of 0.1% sterile peptone water (Oxoid CM0009) for attaining the original dilution, from which, 1 ml was transferred to sterile test tubes containing 9 ml of the same diluent to prepare serial dilutions (up to 10^{-4}). 0.2 ml of selected dilutions were transferred and evenly spread onto dried surfaces of duplicate plates of Baird-Parker selective agar (Oxoid CM0275) supplemented with egg-yolk tellurite emulsion. The plates allowed to dry then incubated for 48 hours at 37 °C. Characteristic colonies exhibiting typical morphology, grey black to jet-black, shiny, circular, smooth, convex, 2–3 mm in diameter with a narrow white entire margin and may show opaque zones surrounded by zone of clearing extended 2–5 mm in the opaque medium, were considered a presumptive *S. aureus.* The colonies were counted as an initial count of *S. aureus* until confirmation. The top part of five suspected colonies was inoculated into test tubes containing 5 ml of sterile brain heart infusion broth (Oxoid CM0225) then incubated at 37 °C for up to 24 hours for confirmation **[19, 20]**: through Gram staining (Gram positive cocci arranged in characteristic irregular clusters resembling bunches of grapes), catalase activity (positive) and salt tolerance (growth on mannitol salt agar). Coagulase test (tube method) was performed to the confirmed strains as follow: From the previously inoculated brain heart infusion (BHI) broth, 0.1 ml was transferred to tubes contain 0.3 ml of reconstituted rabbit plasma then incubated the for 24 hours at 37 °C. Tubes were observed after 3 h for fibrin clot formation (coagulase positive). Tubes which did not show clot formation had further incubation for 20 hours and then marked as coagulase negative or positive. *S. aureus* produces a clot, gelling either the whole contents of the tube or forming a loose web of fibrin. Finally, the total *S. aureus* count per gram of each examined sample was then calculated and recorded.

2.3. Detection and typing of enterotoxin [21]

The clear culture supernatant fluid, obtained from individual isolates, was tested serologically by Reverse Passive Latex Agglutination technique "RPLA" using kits for the detection of SEs A, B, C and D (SET-RPLA, Denka Sekeu Ltd, Japan). The sensitivity of this test kit in detection of enterotoxins is 0.5 ng/ml of test extract. The test was performed in microtiter plate (v-type) arranged so that each row consists of 8 wells and each test sample needed the use of 5 rows of wells. Using a pipette, 25 µl of diluent were placed in each well then, the sample was picked up simultaneously with 5 diluents (25 µl each) and two-fold diluents of the test sample were carried out along each of the 5 rows except the last well of each row contained 25 µl of diluent only. 25 µl quantities of latex suspensions sensitized separately with anti-enterotoxin A, B, C, and D were added to the wells of each of 1st, 2nd, 3rd, and 4th row of the plate, respectively while 25 µl of control latex were added to each well in the fifth row of the plate using, then mixing the contents. The plate was covered and left undisturbed at room temperature for 24 hrs. Each well in each row was examined for agglutination.

2.4. Molecular characterization of isolated S. aureus

2.4.1. Genomic DNA Extraction

DNA from individual colonies of presumptive *S. aureus*, after overnight culture on BHI agar plates, was obtained using QIA amp mini kit (Qiagen, Germany, GmbH), according to the manufacturers' instructions. The supernatant containing the genomic DNA was transferred to sterile tube and stored at -20 °C until used for PCR.

2.4.2. DNA Amplification of S. aureus virulence genes [22]

A multiplex PCR for identification of the thermonuclease (*nuc*) and methicillin resistance (*mecA*) virulence genes was performed essentially by using specific primers (Pharmacia Biotech) (Table 1).

DNA amplification was performed, in a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany), in a 50 µl reaction volume consisted of 2 µl DNA template; 0.6 µM of each forward and reverse primers; 1 U of Taq DNA polymerase; 2 mM MgCl2; dATP, dCTP, dGTP, and dTTP at 200 µM each; and 1x PCR buffer (pH 8.0). The reaction mixture was overlaid with 30 µl of light mineral oil and heated to 94°C for 4 minutes. Parameters for amplification are 30 sec at 94°C (denaturation), 30 sec at 55°C (annealing), and 1 min at 72°C (extension). Subsequently, primers *nuc* 1 (0.2 µM each) were added to the reaction mixture and PCR was continued for 20 cycles, then multiplex PCR were completed with a final extension at 72°C for 5 min.

The amplified DNA fragments were analyzed in 1x TBE buffer stained with ethidium bromide by 1.5% of agarose gel electrophoresis (Applichem, Germany, GmbH) then visualized on UV transilluminator. A 100 bp DNA Ladder (Qiagen, Germany, GmbH) was used as a marker to define fragment sizes.

2.5. Statistical analysis

The achieved data were processed statistically and presented as mean ± standard error, using **SPSS [25]**.

3. RESULTS

Table 1: Primers used for detection of virulence genes of isolated *S. aureus*.

Target Gene	Oligonucleotide sequence (5′ → 3′)	Amplicon size (bp)	Reference
Nuc	F: 5′ GCGATTGATGGTGATACGGTT 3′ R: 5′ AGCCAAGCCTTGACGAACTAAAGC 3′	270	**[23]**
mecA	F: 5′ TAGAAATGACTGAACGTCCG ′3 R: 5′ TTGCGATCAATGTTACCGTAG ′3	533	**[24]**

F: forward primer; R: reverse primer; bp: base pair.

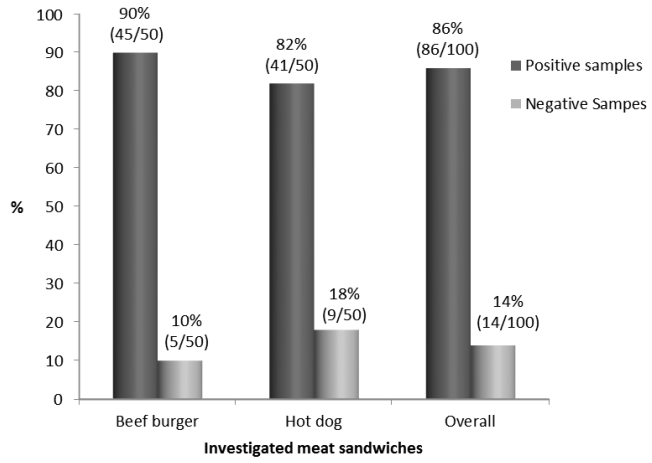

Figure 1: Incidence of *S. aureus* contamination in investigated sandwiches.

Table 2. Detected counts of *S. aureus* in investigated sandwiches.

Meat product	Minimum	Maximum	Mean ± SE*
Beef burger	9.5×10	1.9×10^4	$3.5\times10^3 \pm 5.4\times10^2$
Hot dog	1×10	3.8×10^4	$4.2\times10^3 \pm 8.9\times10^2$
Total	5.3×10	2.9×10^4	$3.9\times10^3 \pm 5.2\times10^2$

*Standard Error

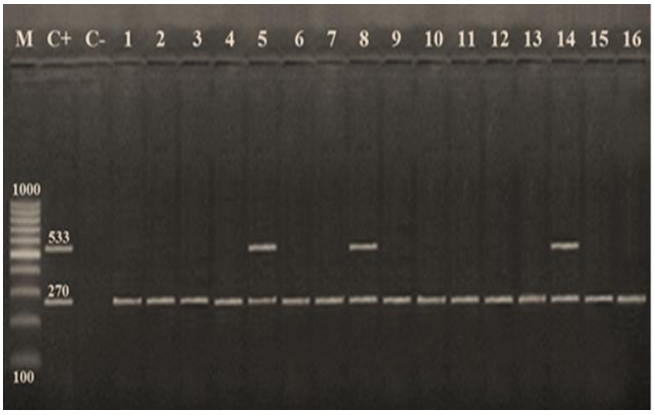

Figure 3. Agarose gel electrophoresis for multiplex PCR products of thermonuclease (*nuc*) (270 bp) and methicillin (*mecA*) (533 bp) virulence genes of isolated *S. aureus.* **Lane M:** A 100 bp DNA ladder marker; **Lane C+:** Control positive *S. aureus* strain for *nuc* and *mecA* genes; **Lane C-:** Control negative; **Lanes from 1 to 16:** Positive *S. aureus* strains for *nuc* gene; **Lanes 5, 8 & 16:** Positive *S. aureus* strains for both *nuc* and *mecA* genes.

4. DISCUSSION

A total of 100 meat (50 each of beef burger and hot dog) sandwiches were investigated to isolate the coagulase-positive *S. aureus*. Presumptive colonies of *S. aureus* were confirmed by coagulase test and by detecting the thermonuclease (*nuc*) gene in all confirmed isolates, and the counts were finally calculated. Coagulase-positive *S. aureus* organisms were detected in 86 (86%), 45 (90%), and 41 (82%) of overall, beef burger, and hot dog sandwiches, respectively (Figure, 1 & 3). The coagulase-positive counts (minimum, maximum and mean) were 5.3×10, 2.9×10^4 and 3.9×10^3; 9.5×10, 1.9×10^4 and 3.5×10^3; and 1×10, 3.8×10^4 and 4.2×10^3 in overall, beef burger and hot dog sandwiches, respectively (Table, 2). Nearly Similar results were reported by **[26, 27]**. **CDC [28]** stated the food is contaminated with staphylococci by contact with food handlers who carry it and foods that need no cooking as hamburger sandwiches are susceptible to contaminate with *S. aureus* and subsequent toxin production.

Table 3: Incidence of *mecA* and enterotoxin production among isolated *S. aureus* (n=106).

Meat Sandwiches	No. of isolated strains	No. & % of *mecA*-positive Strains		No. & % of enterotoxin-producing strains		Enterotoxin production											
						A		B		C		D		A & C		A & D	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Beef burger	78	36	46.2	10	12.8	4	5.1	2	2.7	-	-	2		-	-	2	2.7
Hot dog	28	11	39.3	4	14.3	-	-	-	-	2	7.1	-	-	2	7.1	-	-
Overall	**106**	**47**	**44.3**	**14**	**13.2**	**4**	**3.8**	**2**	**1.9**	**2**	**1.9**	**2**	**1.9**	**2**	**1.9**	**2**	**1.9**

Regarding the microbiological quality of tested samples as ready-to-eat meat, 10% (10/100), 12% (6/50), and 8% (4/50); 79% (79/100), 78% (39/50), and 80% (40/50); and 11% (11/100), 10% (5/50), and 12% (6/50) of overall, beef burger, and hot dog sandwiches investigated were acceptable (low risk), unsatisfactory (moderate risk) and potentially hazardous (high risk), respectively **[29]**. These results mean that most (79%) of tested sandwiches contaminated with higher counts of *S. aureus* organisms which represent a moderate risk to consumers and 11% of them are potentially injurious to health and/or unfit for human consumption (Figure, 2).

The results showed a higher (86%) contamination level of tested sandwiches and higher detecting counts (mean of 2.9×10^4), besides 11% of samples exceeding the limits recommended by **Health Protection Agency [29]** for ready-to-eat meats indicating poor hygienic measures during preparing, cooking and handling of these sandwiches in restaurants and subsequent potential risk because sandwiches contaminated with SEs may not smell bad or look spoiled and these organisms can multiply in the food and produce enterotoxins that can make people ill. However, *S. aureus* bacteria are killed by cooking; their toxins are not destroyed and will still be able to cause illness **[9]**. It is established that meat products should be free from *S. aureus* enterotoxins according to **Egyptian Organization for Standardization [30]**.

Out of 106 isolates of confirmed coagulase-positive *S. aureus* from all meat sandwiches tested, the gene encoding the methicillin resistance (*mecA*) was detected in 47 (44.3%) while only 14 (13.2) were enterotoxin producing (Figure, 3 & Table, 3). The four enterotoxins detected were SE A (3.8%), and SE B, SE C, SE D, SE A & C, and SE A & D (1.9%, each). The *mecA* gene could be detected in 25% of tested *S. aureus* isolates **[27]**. On the other hand, SE A and SE D could be detected in 3.6% (each) of *S. aureus* contaminating examined street vendor meat samples **[31]**. Similar results were also detected **[26]**. 15-80% of isolated *S. aureus* are enterotoxigenic and ready-to-eat meat products are contaminated during mixing and handling the ingredients **[32]**. Generally, MRSA and its enterotoxins when found indicate lack of hygiene during food production **[33]**, and the overuse of antibiotics in animals and humans lead to increasing the presence of these strains **[34]**.

Conclusion

It can be concluded that investigated beef burger and hot dog sandwiches retailed in Mansoura city showed higher degree of contamination with coagulase-positive, enterotoxin producing and methicillin resistant *S. aureus* organisms. Hence, consumption of such sandwiches may constitute potential health risk. Therefore, strict control measures should be followed to ensure the safety of these meat sandwiches to consumers.

Conflict of interest statement

No conflict of interest.

Research Ethics Committee permission

The research was conducted according to standards of Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University.

Authors´ contribution

Mohammed E. A. collected the samples carried out the practical part. Ramadan H. is a co-supervisor. Abd-Elghany S. M. designed the experiment, supervised the practical work, and revised the manuscript. Mahros M. A. supervised the practical part, drafted, and revised the manuscript.

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