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Original Research Article

PREVALENCE OF ENTEROTOXIGENIC AND MULTI-DRUG-RESISTANT *Staphylococcus aureus* IN READY TO EAT MEAT SANDWICHES

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Abstract: Due to recent spread of multiple drug resistant pathogens, this study was peformed to investigate the presence of multi-drug resistant enterotoxigenic *Staphylococcus aureus* (*S. aureus*) in some ready to eat meat products (RTE). For this, one hundred and forty samples of ready to eat meat sandwiches were collected from restaurants and street vendors in Zagazig city, Egypt. *Staphylococcus aureus* is one of the most important food poisoning bacteria in RTE. The counts were 3.31 ± 0.49, 2.86 ± 0.36, 3.28 ± 0.24, 3.92 ± 0.41, 2.52 ± 0.11, 3.64 ± 0.39 and 3.12 ± 0.35 $\log_{10}$ CFU/g in examined kofta, luncheon, burger, shawarma, hawawshi, liver and sausage sandwiches, respectively. The examined sandwiches were categorized into good (32.1%), acceptable (32.9%), unsatisfactory (26.4%) and potentially hazard (8.6%). About 72.7 % of examined *S. aureus* strains carry one or more staphylococcal enterotoxin (*se*) genes and *mec*A gene detected in 81.8% of coagulase positive *S. aureus*. The antibiogram showed that 100% of *S. aureus* isolates were resistant to kanamycin, 92% for penicillin and neomycin, 84% for oxacillin and erythromycin and 68% for ampicillin and nalidixic acid. The average of multi-antibiotic resistant (MAR) index of isolated *S. aureus* was 0.59. Moreover, five isolates were resistant to all tested antibiotic.

Key words: *Staphylococcus aureus*; methicillin resistant; enterotoxin; ready to eat

Introduction

Foodborne illness is increased of worldwide. This may in part related to a change in a system of food production such as least processing as well as changing consumer needs for ready-to-eat (RTE) sandwiches (1). Ready to eat meat linked with foodborne disease outbreaks have been associated with various foodborne micro-organisms (2,3). The number of microorganisms in raw ingredients introduced in RTE meat sandwiches is important, however, many factors such as processing, handling, storage and display may impact the microbio-logical load of final RTE meat sandwiches at the point of sale (4). Delicatessen foods such as vegetable salads and sauces have also been incriminated in foodborne illness outbreaks. These foods are usually prepared by hand and this through contact may lead to an increased prevalence of contamination with potential foodborne bacteria, such as *S. aureus* (5). Antimicrobial resistance is a significant public health concern all over the world. The virulent and antibiotic-resistant bacteria cloud be trans-mitted to

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humans by the consumption of RTE meat sandwiches and the presence of these bacteria such as *S. aureus*, indicating poor hygienic measures which may produce a health risk for consumers (6) especially, multi antibiotic resistant (MAR) strains which are more risky and of greater food safety attention (7). Treatment of infectious disease caused by MAR bacteria needs alternatives to conservative antibiotics and the search for recent antibiotics is becoming critical (8). Methicillin-resistant *S. aureus* (MRSA) has newly emerged as a health anxiety and currently causes about 94,000 invasive infections annually in the United States of America, leading to an estimated 18,650 deaths (9). Moreover, MRSA has been found in several species of meat-producing animals, poultry (10) and cattle (11). Detection of MRSA in these animal products, illustrates the importance of exploring meat products as potential sources for transmission of MRSA from the farm animals for the meat product consumer. Therefore, the present study was conducted for determination of Enterotoxigenic *S. aureus* in addition to antibiogram for antibiotic sensitivity in most popular RTE meat sandwiches in Egypt.

Material and methods

One hundred and forty samples of ready to eat sandwiches were collected randomly from different restaurants in Zagazig city, Sharkia Governorate, Egypt, at different sanitation levels. The samples were collected from May 2017 to April 2018. The collected samples represented by 20 each of kofta, luncheon, burger, shawarma, hawawshi, liver and sausage sandwiches. Each collected sample was kept in a separate sterile plastic bag and preserved in an ice box then transferred to the laboratory under an aseptic condition without undue delay.

Isolation and identification of Staphylococcus aureus

Staphylococcus aureus was isolated as follows: 25 g of each sandwich core (meat, vegetables and sauce) were mixed with 225 ml of peptone water (Oxoid, Basingstoke, Hampshire, CM0009B, UK) and homogenized in a stomacher (Lab-Blender 400, PBI, Milano, Italy). The samples were then inoculated onto Baird-Parker agar (Oxoid CM 275) and incubated at 35°C for 24 and 48 h. The samples characteristic colonies (gray-black, surrounded by a dull halo) were considered to contain coagulase positive *S. aureus* (CPS) (12). Five colonies from each Petri of presumed *S. aureus* isolates were examined by Gram-staining and catalase test. Gram and catalase-positive isolates were further identified according to American Public Health Association (APHA) (13).

Determination of Staphylococcal enterotoxin and mecA genes

After overnight culture on nutrient agar plates, two colonies were suspended in 20 ml of sterile distilled water and the suspension was then heated at 100 ºC for 20 min. Accurately, 50-200 µl of the culture were placed in Eppendorf tube for DNA extraction using QIA amp kit according to Shah et al., (14).

Table 1: Primer sequences of *S. aureus* used for PCR identification system

Oligonucleotide sequence (5′ → 3′)	Product size (bp)	Target gene	References
F- TTGGAAACGGTTAAAACGAA **R- GAACCTTCCCATCAAAAACA**	120	*sea* (F)	(15)
F- TCGCATCAAACTGACAAACG **R- GCGGTACTCTATAAGTGCC**	478	*seb* (F)	
F- GACATAAAAGCTAGGAATTT **R- AAATCGGATTAACATTATCC**	257	*sec* (F)	
F- CTAGTTTGGTAATATCTCCT **R- TAATGCTATATCTTATAGGG**	317	*sed* (F)	
F- GTA GAA ATG ACT GAA CGT CCG ATA A **R- CCA ATT CCA CAT TGT TTC GGT CTA A**	310	*mecA*(F)	(16)

Amplification of enterotoxin genes of S. aureus

Ten µl of DNA sample was diluted in 990 µl of nuclease free water for PCR. The genomic DNA samples were amplified in a reaction mixture (25µl) containing 13.25 sterile dH_2O, 2.5 ml 10 x buffer, 0.63 ml 10mMNTPs, 1ml 25Mm $Mgcl_2$, 1.25µl primer F(20pmol/ml), 1.25µl primer R (20pmol/ml) and fill up to 25µl PCR grade water. Concerning the primers used for demonstration of *S. aureus* enterotoxins (sea, seb, secand sed) (Table 1), the multiplex amplification was performed on a thermal cycler (Master cycler, Eppendorf, Hamburg, Germany). The initial denaturation for 5 min at 95°C followed by 30 cycles of denaturation (94°C for 2 min), annealing (55°C for 1 min.), and extension (72°C for 2 min). A final extension step (72°C for 5 min) was performed after the completion of the cycles (17).

Amplification of mecA genes of S. aureus

The Preparation of uniplex PCR Master mix according to Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit. The 12.5µl Emerald Amp GT PCR master mix (2x premix), 4.5µl PCR grade water, 1µl forward primer (20 pmol), 1µl reverse primer (20 pmol) and 6µl template DNA (Table 1). The primary denaturation was at 94˚C for 5 min, secondary denaturation was at 94˚C for 30 min, annealing was at 50˚C for 30 sec, extension was at 72˚C for 30 sec, the cycle number was 35 and final extension was at72˚C for 7 min. Amplified products were analyzed by 3% of agarose gel electrophoresis (Applichem, Germany, GmbH) in 1x TBE buffer stained with Ethedium bromide and captured as well as visualized on UV transilluminator at 254nm. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes (16).

Antibiogram for antibiotic sensitivity of isolated S. aureus

Antimicrobial susceptibility was tested by the single diffusion method according to Deresse et al. (18). Sensitivity discs with variable concentrations were used to determine the susceptibility of the isolated strains (Oxoid Limited, Basingstoke, Hampshire, UK). Agar plate method was applied by using of nutrient agar as a substrate for growth of the tested bacterium for its antibiotic sensitivity. The bacterial culture was uniformly spread on the surface of nutrient agar. Then the antibiotic discs were placed over the surface of inoculated plate. Moreover, the plate was then incubated at suitable temperature (25°C) for 2-7 days and checked for the growth of the bacterium around the antibiotic discs. The maximal inhibition zone for the growth of microbe is said to that antibiotic had a maximum effect on the microbe growth. Therefore, the antimicrobial susceptibility testing was applied according to the guidelines stipulated by National Committee for Clinical Laboratory Standards (NCCLS) (19). Multiple Antibiotic Resistances (MAR) index for each strain was determined according to the formula stipulated by Singh et al., (20) as follow:

MAR index = Number of antibiotics with resistantce profile / the number of used antibiotics.

Isolates classified as intermediate were considered sensitive for MAR index.

Results and discussion

Staphylococcal food poisoning usually occurs in foods that require human handling during preparation and left at room temperature for long periods before consumption. The data illustrated in Table (2) declared that *S. aureus* was detected in 90, 50, 75, 80, 65, 55and 60% with mean counts 3.31±0.49, 2.86±0.36, 3.28±0.24, 3.92±0.41, 2.52±0.11, 3.64±0.39 and 3.12±0.35log_{10} CFU/g in examined kofta, luncheon, burger, shawarma, hawawshi, liver and sausage sandwiches, respectively. The high prevalence of *S. aureus* attributed to the poor personal hygiene during processing of sandwiches, which resulted in higher contamination (21). There were significant differences between the examined sandwiches ($p<0.05$). The lowest mean value detected in hawawshi, which attributed to preparation of hawawshi sandwiches without the addition of tahini sauce or green salads that not previously heat-treated

during stages of preparation. Moreover, the variation could be related to the difference of the cooking system (frying, roasting, grilling, etc.), the microbiological quality of raw ingredient, the shape and size of the meat piece(s), the utensils used for cooking (oven, crock pot, stew pot, grill, etc.), the presence of seasoning ingredients (sauces, spices, vegetables, etc.) (22). *S. aureus* previously isolated from 17.9 % of RTE and count ranged from 2.30 to 3.60 $\log_{10}$ CFU/g in Taiwan (23). Enterotoxigenic *S. aureus* detected in 60% of coriander sauce, 58% of coconut slices and in 86% samples of RTE salads collected from New Delhi and Patiala City, India (24). There is no available regulation for *S. aureus* count in Egyptian RTE food, so the examined sandwiches were categorized according to the Compendium of Microbiological Criteria for Food (25) into good (32.1%), acceptable (32.9%), unsatis-factory (26.4%) and potentially hazard (8.6%). The unsatisfactory plus potentially hazard samples were 49 (45%) carry a significant risk if applied under certain conditions as excessive handling, bad hygienic conditions, time and temperature abuse that help *S. aureus* to proliferate and produce different staphylo-coccal enterotoxin (SEs) that may cause food poisoning when ingested (26). *S. aureus* produces a number (SEs) that responsible for inflammation and hyperemia of the gastro-intestinal mucosa. Moreover, symptoms as nausea, vomiting, and abdominal cramps, and diarrhea rarely occur. These usually start within one to eight hours after ingestion of food contaminated with *S. aureus* and symptoms last one or two days. The illness seldom is fatal (27).

The results declared in Table (3) showed DNA expression of *S. aureus* enterotoxins genes by using multiplex PCR of *sea*, *seb*, *sec* and *sed* enterotoxin genes for characterization of *S. aureus*. It was found 8/11 isolates were enterotoxin producing strains, 4 strains of *S. aureus* carry *sea* gene, 5 strains carry *sec* gene, 2 strains carry *seb* gene, one strain carry *sea* plus *sec* gene and one strain carry *sea* plus *seb* plus *sec*. It is obvious from the results that *S. aureus* strains carry more than enterotoxin genes, and presence of these strains in ready to eat meat imposes potential hazards and may induce food poisoning if the conditions are suitable for staphylococcal enterotoxin production.

These results agree to some extent with those obtained in different studied that used multiplex PCR for detection of *S. aureus* enterotoxin genes (28-30). The amount of SEs required for typical symptoms of food poisoning is very low, ranging from 20 ng to 1µg, which equal 10^5 Staphylococci CFU/g of food (31).

Table 2: Prevalence and count of *S. aureus* in comparison to standard of ready to eat meat (n= 140)

Sandwiches	prevalence	Mean± SD range	Categories			
			good	acceptable	unsatisfactory	Potentially hazard
Kofta	90%	3.31 ± 0.49^{ab} 2.64-4.41	2	4	10	4
Luncheon	50%	2.86 ± 0.36^{bc} 2.12-3.15	10	8	2	0
Burger	75%	3.28 ± 0.24^{ab} 2.32-4.17	5	5	7	3
Shawarma	80%	3.92 ± 0.41^{a} 3.2-4.58	4	5	8	3
Hawawshi	65%	2.52 ± 0.11^{c} 2-3.2	7	8	5	0
Liver	55%	3.64 ± 0.39^{a} 2.99-4.2	9	7	3	1
Sausage	60%	3.12 ± 0.35^{b} 2.80-4.12	8	9	2	1
Total			45 (32.1%)	46(32.9%)	37(26.4%)	12(8.6%)

Means of the same column carrying small superscript letters were significantly different ($P < 0.05$).

Table 3: Enterotoxin genes in examined *S. aureus* isolates from ready to eat meat sandwiches

Staphylococcus aureus	*sea* (120 bp)	*seb* (478 bp)	*sec* (257 bp)	*sed* (317 bp)
1	-	-	-	-
2	-	-	+	-
3	+	+	+	-
4	+	-	-	-
5	+	-	+	-
6	-	-	-	-
7	+	-	-	-
8	-	-	+	-
9	-	-	-	-
10	-	-	+	-
11	-	+	-	-

Table 4: Antibiotic susceptibility of *S. aureus* isolated from ready to eat meat sandwiches

Antibiotic class	Antibiotic	Sensitive	Intermediate	Resistant
Penicillin	Penicillin (P)	-	2 (8%)	23 (92%)
	Ampicillin (AM)	5 (20%)	3 (12%)	17 (68%)
	Oxacillin (OX)	3 (12%)	1 (4%)	21(84%)
Cephalosporin	Cephalotin (CN)	18 (72%)	2 (8%)	5 (20%)
Fluroquinolones	Ciprofloxacin (CP)	11 (44%)	2 (8%)	12 (48%)
	Enrofloxacin (EN)	13 (52%)	4 (16%)	8 (32%)
Quinolones	Nalidixic acid (NA)	2 (8%)	6 (24%)	17 (68%)
Aminoglycosides	Gentamicin (G)	10 (40%)	2 (8%)	13 (52%)
	Neomycin (N)	-	2 (8%)	23 (92%)
	Kanamycin (K)	-	-	25 (100%)
Tetracycline	Oxytetracycline (T)	6 (24%)	3 (12%)	16 (64 %)
Macrolides	Erythromycin (E)	1 (4%)	3 (12%)	21 (84%)
Sulfonamides	Sulphamethoxazol (SXT)	7 (28%)	2 (8%)	16 (64%)
Phenicols	Chloramphenicol (C)	3 (12%)	4 (16%)	18 (72 %)

Table 5: Antibiotic resistance pattern and MAR index of *S. aureus* isolated from ready to eat meat sandwiches

Resistance pattern	Resistance profile	Number of isolates	Number of antibiotics	MAR
I.	K ,P, N, OX, E, C, AM, NA, T, SXT, G, CP, EN, CN	5	14	1
II.	K ,P, N, OX, E, C, AM, NA, T, SXT, G, CP, EN	3	13	0.92
III.	K ,P, N, OX, E, C, AM, NA, T, SXT, G, CP	4	12	0.85
IV.	K ,P, N, OX, E, C, AM, NA, T, SXT, G	1	11	0.78
V.	K ,P, N, OX, E, C, AM, NA, T, SXT	3	10	0.71
VI.	K ,P, N, OX, E, C, AM, NA	1	8	0.57
VII.	K ,P, N, OX, E, C	1	6	0.42
VIII.	K ,P, N, OX, E	3	5	0.35
IX.	K ,P, N	2	3	0.21
X.	K	2	1	0.071
Average				0.59

Methicillin-resistant *S. aureus* (MRSA) has recently appeared as a health anxiety and causes approximately 94,000 invasive infections annually in the USA, leading to an estimated 18,650 deaths (9). In this study *S. aureus* isolates were tested for the presence of the *mec*A gene, the *mec*A gene detected in 81.8% of coagulase positive *S. aureus* samples.

The results of this study showed a higher prevalence than that recorded by Kitai et al. (32) they detected *mec*A gene in 0.5% from 444 raw chicken meat products sampled from

supermarkets in Japan. Also a study conducted in Korea, including 930 retail meat samples, showed the presence of MRSA in 0.2% detected at chicken meat samples, but not detected in any pork or beef sample (33). Van Loo et al., (34) found two (2.5%) MRSA strains in 79 samples of raw pork and beef.

Regarding to the detection of staphylococcal enterotoxin genes and *mec*A genes, Sergelidis et al. (35) found that MRSA strains isolated from meat and poultry had the ability to produce enterotoxins and produce SEs genes.

The MRSA is transmitted to cooked products due to temperature abuse during storage may result in the multiplication of MRSA. The higher MRSA contamination of various examined RTE sandwiches possible related with the use of antibiotics in animal husbandry (36).

The results in Table (4) shows that 100% of the isolates were resistant to kanamycin, 92% for penicillin and neomycin, 84% for oxacillin and erythromycin and 68% for ampicillin and nalidixic acid, 64% for oxytetracycline and sulphamethoxazol. On contrary sensitivity observed for cephalotin and enrofloxacin by 72% and 52%, respectively. Results were partially in agreement with other studies (37-38).

The multiple antibiotic resistance MAR index of isolated *S. aureus* ranged from 0.071 to 1 with an average 0.59. Moreover, 5 (20%) strains were multi-resistant to all tested antibiotic and 23 (92%) of *S. aureus* isolates are considered as multi antibiotic resistant (Table 5). The variation in the MAR index could be attributed to differences in the sources of samples; geographic distribution, which has differential selective pressures for the antibiotic resistance levels; and test methodologies (39).

The an average of MAR results has shown that MAR higher than 0.2 could be due to contamination from high-risk sources (40), such as humans and farm animals frequently exposed to antibiotics in animals raised for food for different purposes such as prophylaxis, and growth promotion, or therapeutics and these resistant bacteria can be transmitted to human through foods (41), resulting in potential risk to consumers. The high MAR in the current study indicated that the isolates originated from high-risk source samples, therefore monitoring of antimicrobial resistance is essential to identify the effectiveness of new generations of antibiotics. *S. aureus* strains are known to be frequently resistant to antibiotic therapy due to their capacity to produce an exopolysaccharide barrier (42) and carry a wide variety of multi–drug resistant genes on plasmids, which can be exchanged and spread among different species of Staphylococci (43).

Conclusion

In conclusion, RTE meat sandwiches contaminated with high numbers of multi drug resistant and enterotoxin producing *S. aureus*. These findings reflect poor personnel hygiene during processing, which may lead to health problems to consumers.

Conflict of interest

None of the authors have any conflict of interest to declare.

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