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Molecular Identification of *Staphylococcus Aureus* in Imported Frozen and Locally Slaughtered Meat

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Abstract

Key words: *Staphylococcus aureus*, MRSA, Frozen meat, Balady meat

This work was planned to detect and identify *Staphylococcus aureus* in imported frozen meat (Brazilian and Indian) as well as locally slaughtered (Balady) meat using routine bacteriological examination and PCR. A total of 100 samples from Brazalian meat, 75 samples from Indian meat and 50 samples from Balady meat were analyzed for *Staphylococcus aureus*. Our results showed that the prevalence of *Staphylococcus aureus* in Brazilian, Indian and Balady meat was 27%, 26.67 % and 22 %, respectively. Antimicrobial resistances of the *S. aureus* isolates showed sensitivity to penicillin, rifompion, ampicillin and novobiocin, while resistant to oxacillin, sulphatrimethoprim, vancomycin and Cefotoxin. Further, multiple antibiotic resistances were detected in 98% (57/58) of the isolates. Higher multiple antibiotic resistance index was detected from *S. aureus* isolates from the three types of meat and different resistance phenotypes were detected. PCR for the detection of the *mecA* gene of methicillin resistance was positive with the tested oxacillin resistant *S. aureus* isolates. PCR for the detection of the virulence genes; *Sea*, *Seb*, *Sec*, *Sed* and *See* genes, was performed. *Sea* and *Sed* genes were negative, while *Seb*, *Sec*, and *See* genes were detected in 16.66%, 33.33% and 50% of the tested isolates, respectively. These results collectively indicate that Brazilian, Indian as well as balady meat can harbor *S. aureus* with high percentage of resistance to oxacillin and a wide range of multiple antimicrobial resistances.

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1. INTRODUCTION

Meat is considered as an essential part of the human diets in all countries due to its high nutritional quality. It contains high levels of protein, vitamins and minerals (Ogilvie, 2015). Freezing is a method of food preservation that slows the physical, chemical and microbiological activity that causes deterioration in foods (Berry et al., 2008; Adams and Moss, 2008; Ray and; Lounge 2012). Freezing meat subjects it to the risk of contamination from different sources during its journey from the time of slaughter, dressing, processing, handling and freezing until it reaches the consumer, and such contaminations may constitute a public health hazard (USDA, 2015).

Staphylococcus aureus, one of the food contaminants that represent zoonotic risk of transmission to humans, requires understanding its molecular ecology in food, especially that raw milk and raw meat harbor isolates containing multiple toxin genes (Song et al., 2015).

Increasingly common microbiological hazard in food is methicillin-resistant *Staphylococcus aureus* (MRSA). Although prevalence of this pathogen in food is not high, the thread comes from difficulties of treating of infections caused by MRSA (Sciezyńska et al., 2012).

Meat and meat products can be contaminated with different species of bacteria resistant to various antimicrobials. The human health risk of a type of meat or meat product carry by emerging antimicrobial resistance depends on (i) the prevalence of contamination with resistant bacteria, (ii) the human health consequences of an infection with a specific bacterium resistant to a specific antimicrobial and (iii) the consumption volume of a specific product (Presi et al., 2009). It is generally agreed that the internal tissues of healthy slaughter animals are free of bacteria at the time of slaughter. However, under the current practices of meat and poultry processing, it is impossible to guarantee sterility of the final products (Jay, 2005; Odetunde et al., 2011; Schaumburg et al., 2014)

Staphylococcus aureus count (SAC) considered as a one from the most important bacteria can be isolated from frozen imported meat and slaughtered meat

under Egyptian conditions due to its contamination with *S. aureus* during slaughtering and handling of the meat (Biswas et al., 2008).

The presence of methicillin-resistant *Staphylococcus aureus* (MRSA) on meat purchased from retail outlets may allow its spread to households and represents a risk for colonization and possibly infection of consumers. Improved isolation methods have indicated that more than 10% of samples are positive. We aimed to determine rates of MRSA contamination of meat samples, including comparison of fresh and frozen samples, so, characterization of the isolates and determination of their antibiotic susceptibility (Boost et al., 2013; Charles et al., 2006) According to Lee et al., (2008), Ingham et al., 2009; Vázquez-Sánchez et al., 2012).

2.2. Bacterial culture and identification:

The bacterial isolates were characterized by studying their morphological, cultural, and biochemical characteristics according to Collee et al., (1996) and Quimn et al., (2002) as well as their motility according to Cruick shank et al., (1975). Films were prepared from fresh cultures, stained with Gram stain and examined microscopically for the morphological characteristics of the isolate. The colonial morphology on Paired Barker agar, MacConkey agar and Sorbitol Maconkey agar were studied. Catalase test, oxidase test, urease test, coagulase test, DNase test, haemolysis, pigment production, aerobic fermentation of mannitol, maltose and acetone production were carried as further identification according to Quinn et al., (2002).

2.3. Antimicrobial susceptibility testing of *S. aureus*

The Antimicrobial susceptibility testing by using disc diffusion method was carried out according to the Clinical laboratory standards Institute (CLSI, 2012). The following antibiotics were used: Oxacillin (OX; 1 µg), Oxytetracyclin (OT; 30 µg), Cefotoxin (FOX; 30 µg), Sulphatrimethoprim (SXT; 25 µg), Penicillin G (P; 10 µg), Ampicillin (AMP; 10 µg), Vancomycin (VA; 30 µg), Rifampicin (RD; 5 µg), Novobiocin (NV; 30 µg) and Cefazolin (KZ; 30 µg) (Rocha *et al.*, 2014).

2.4. Polymerase Chain Reaction (PCR) for detection of *S. aureus* virulence and antibiotic resistance genes

This work aimed to the detection and identification of *Staphylococcus aureus* and its antimicrobial susceptibility in frozen meat of either imported (Brazelian and Indian meat) as well as Balady meat using ordinary bacteriological methods as well as PCR.

2. MATERIALS AND METHODS

2.1. Sample collection:

A total of 100 Brazalian meat samples, 75 Indian meat samples and 50 Balady meat samples. The Brazalian and Indian samples were collected from the container of the ship, while the Balady meat samples were collected from super markets and butcher-shops at Alexandria Province. The samples were collected aseptically and were kept frozen until arrived at the laboratory, Egypt.

From pure cultures, DNA was extracted by phenol-chloroform method according to Shambrook *et al.*, (1989). PCR (Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit) was prepared by adding 12.5 µl of Emerald Amp GT PCR master mix (2x premix), 4.5 µl PCR grade water, 1 µl forward primer (20 pmol), 1 µl reverse primer (20 pmol) and 6 µl template DNA to a total volume of 25 µl. Primers used for the detection of the different genes and cycling conditions are listed in Table (1).

2.5. Statistical analysis:

The statistical analysis was made using Chi^2-test according to (SAS, 2004).

3. RESULTS AND DISCUSSION

3.1. Sensory evaluation of different types of meat samples:

Our results on Sensory evaluation of different types of examined meat samples cleared that all examined samples showed good color and odor by a percentage of 100 %. While its consistency showed differences among the examined meat samples. The Indian, Brazilian and Balady meat samples showed consistency of 93.34 %, 90 %, and 88 %, respectively (Table. 2). These results could be attributed to the refrigeration and freezing that resulted in decreasing the growth of bacteria so preserving the color, odor and consistency of the meat. These results are similar to that of Berry et al., (2008) where he observed that freezing slows the physical, chemical and microbiological activity that causes deterioration in foods. In contrast, the balady meat could sometimes be exposed to bad handling and bad storage of the meat that will affect the consistency level of the balady meat.

Table (1): Oligonucleotide primers sequences

Gene	Primer	Primer sequence (5'-3')	Length of amplified product	Reference
Sea	GSEAF-1	GGTTATCAATGTGCGGGTGG	102 bp	**Mehrotra *et al*., 2000**
	GSEAR-2	CGGCACTTTTTTCTCTTCGG		
Seb	GSEBF-1	GTATGGTGGTGTAACTGAGC	164 bp	
	GSEBR-2	CCAAATAGTGACGAGTTAGG		
Sec	GSECF-1	AGATGAAGTAGTTGATGTGTATGG	451 bp	
	GSECR-2	CACACTTTTAGAATCAACCG		
Sed	GSEDF-1	CCAATAATAGGAGAAAATAAAAG	278 bp	
	GSEDR-2	ATTGGTATTTTTTTTCGTTC		
See	GSEEF-1	AGGTTTTTTCACAGGTCATCC	209 bp	
	GSEER-2	CTTTTTTTTCTTCGGTCAATC		
mecA	mecA-1	GTA GAA ATG ACT GAA CGT CCG ATA A	310 bp	**McClure *et al*., 2006**
	mecA-2	CCA ATT CCA CAT TGT TTC GGT CTA A		
16SrRNA	16SrRNA.*F*	CCTATAAGACTGGGATAACTTCGGG	791 bp	**Mason *et al*., 2001**
	16SrRNA.R	CTTTGAGTTTCAACCTTGCGGTCG		
blaZ	BlaZ.F	ACTTCAACACCTGCTGCTTTC	173 bp	**Duran *et al*., 2012**
	BlaZ.R	TGACCACTTTTATCAGCAACC		

Table (2): Statistical analytical results of sensory evaluation of frozen (Brazilian and Indian) and Balady meat (n= 225).

Condition	Meat type	Organoleptic properties	Frozen meat			
			Normal		Abnormal	
			No	%	No	%
Frozen	Brazilian meat	Colour	100	100	0	0
		Odour	100	100	0	0
		Consistency	90	90	10	10
	Indian meat	Colour	75	100	0	0
		Odour	75	100	0	0
		Consistency	70	93.34	5	6.66
Non-frozen	Balady meat	Colour	50	100	0	0
		Odour	50	100	0	0
		Consistency	44	88	6	12

$Chi^2 = 15.34^{**}$ **= Significant at ($P < 0.05$)

Consumers purchase meat products after making judgments about the quality and price of the meat. They often use color and consistency to judge the ultimate tenderness and the expected taste of the meat cuts. However, such quality attributes are very difficult to quantify from visual assessment. Because most meat products, and fresh meats in particular, are purchased based upon a visual inspection of the product, abnormal coloration has an adverse effect on the salability of the product**.** Thus in case of the tested meat samples from the three different sources, there was no abnormal colorations or physical changes that could affect the visual assessment of the consumers.

3.2. Incidences of *Staphylococcus aureus* among examined meat samples

Higher percentage of *S. aureus* isolation was observed in Brazalian meat (27%), followed by Indian meat (26.67%) and balady meat (22%) (Table. 3). These results could be attributed to the handling and long period of long journey that the meat take till it reaches the consumer subjecting the meat to higher contamination than the balady meat, which takes a short period till it reaches the consumer. Our results agree with those of Castillo et al., (1998) where they reported that water wash and trim treatments caused spreading of the contamination to other areas of the carcass surface. Also, Jay, (2005) reported that the internal tissues of healthy slaughter animals are free of bacteria at the time of slaughter. However, under the current practices of meat and processing, it is impossible to guarantee sterility of the final products.

Table (3): Incidences of *Staphylococcus aureus* among examined samples (n = 225):

Condition	samples	Number	*Staphylococcus aureus* +ve
			Number (%)
Frozen	Brazilian meat	100	27 (27)
	Indian meat	75	20 (26.67)
Non-frozen	Balady meat	50	11 (22)
Total		225	58 (25.78)

$Chi^2 = 7.57^*$ *= Significant at ($P < 0.05$)

Our results agreed with those of Sedeh et al., (2007) who examined bacteriologically 10 raw frozen boneless beef samples and found that the main bacterial isolates were *Enterobacteraceae*, coliform and *Staphylococcus aureus*.

Our results where 100 % of our *Staphylococcus aureus* isolates were coagulase positive are also similar to those obtained by De ber et al., (1999) as he reported the percentage of *S. aureus* among coagulase positive isolates of 97% and 2% were identified as *S. intermedius* and 1% as *S. hyicus*.

3.3. Antimicrobial sensitivity of *Staphylococcus aureus* isolated from meat

Staphylococcus aureus isolates were highly susceptible to penicillin, rifompion, ampicillin and novobiocin. While of intermediate susceptibility to Cefazolin and Oxytetracyclin. In contrast, the isolates showed high resistance to oxacillin, sulphatrimethoprim, vancomycin and Cefotoxin (Table. 4). Our results agreed with those of Sciezyńska et al., (2012) who showed that *S. aureus* was of high sensitivity to penicillin, rifompion and ampicillin, while in contrast to our results, of lower sensitivity to sulphatrimethoprim, oxacillin, vancomycin and Cefotoxin. Multiple antibiotic resistance (MAR) could be detected with 98 % (57/58) of the S. *aureus* isolates giving a wide variety of resistance phenotypes among the isolates (Table. 5). In addition, our results agree with those of Inge et al., (2007) who explained that *Staphylococcus aureus* isolates are resistant to methicillin in meat (MRSA) has emerged as a risk factor for patients in general population and particularly in immunocompromised patients. In fact, it can produce serious infections that may then be seen as septicemia. However, transmission of MRSA from food to people can represent a serious problem only for immunocompromised people. Vancomycin is the elective antimicrobial commonly used in case of MRSA infection, but *S. aureus* strains with reduced sensibility to vancomycin also emerged. As shown in Table (4), the resistance of *Staphylococcus aureus* isolates to vancomycin was high and this could pose a threat to treatment of MRSA. Further, the MAR index was high within the three types of meat with an average reaching 0.7 (Table. 6).

Table (4): Antimicrobial sensitivity of *Staphylococcus aureus* isolated from meat.

Drug	Resistance
Sulphatrimethoprim (Sxt)	65.5 %
Penicillin G (P)	20.6 %
Cefotoxin (FOX)	53.4 %
Oxacillin (OX)	81 %
Vancomycin (VA)	77.5 %
Novobiocin (NV)	36.2 %
Cefazolin (RZ)	37.9 %
Oxytetracyclin (OT)	56.8 %
Ampicillin (Amp)	27.5 %
Rifompion (RD)	15.5 %

Table (5): Resistance phenotypes of *Staphylococcus aureus* isolated from meat.

Sample Number	Resistance Phenotype	MAR index
	OX, VA, NV, KZ, OT, RD	0.6
	SXT ,FOX ,OX ,VA ,OT ,AMP	0.6
	SXT ,FOX ,OX ,VA ,NV ,OT.	0.6
	SXT ,OX ,VA ,KZ ,OT.	0.5
	SXT ,VA ,KZ.	0.3
	FOX ,OX ,VA ,OT ,RD.	0.5
	SXT ,FOX ,OX ,VA ,OT ,RD.	0.6
	P ,FOX ,NV ,OT ,RD.	0.5
	SXT ,OX ,VA ,KZ.	0.4
Brazilian meat	FOX ,OX ,VA ,NV ,OT.	0.5
	SXT ,FOX ,OX ,VA ,OT ,AMP,RD.	0.7
	SXT ,FOX ,OX ,VA ,OT.	0.5
	SXT ,OX ,VA ,OT ,AMP.	0.5
	SXT ,OX ,VA ,NV ,OT.	0.5
	SXT ,FOX ,OX ,VA ,KZ AMP.	0.6
	SXT ,OX ,VA ,NV ,KZ ,OT ,RD.	0.7
	SXT ,FOX ,OX ,VA ,NV ,AMP.	0.6
	FOX ,OX ,VA ,OT ,AMP ,RD.	0.6
	SXT ,FOX ,VA.	0.3
	SXT ,FOX ,OX ,VA ,KZ.	0.5
	SXT ,VA ,OT.	0.3
	SXT ,FOX ,OX ,VA ,OT ,AMP.	0.6
	OX ,VA.	0.2
	SXT ,OX ,VA.	0.3
	P ,FOX ,NV ,OT.	0.4
	SXT ,OX ,VA ,KZ ,OT.	0.5
	OX, NV ,KZ.	0.3
Indian meat	SXT ,FOX ,OX ,NV.	0.4
	P ,FOX ,OX ,VA ,NV.	0.5
	SXT ,P ,NV ,AMP.	0.4
	OX ,NV ,KZ ,OT.	0.4
	SXT ,VA ,KZ.	0.3
	P ,FOX ,OX ,VA ,OT ,AMP.	0.6
	SXT ,P ,OX.	0.3
	SXT ,P ,OX ,NV ,KZ.	0.5
	KZ.	0.1
	P ,OX ,VA ,NV ,KZ ,OT ,RD.	0.7
	P ,OT ,AMP.	0.3
	SXT ,FOX ,OX ,VA ,KZ.	0.5
	SXT ,P ,FOX ,OX ,VA ,NV.	0.6
	SXT ,OX ,VA ,NV.	0.4
Balady meat	VA ,NV.	0.2
	SXT ,OX ,VA ,NV ,AMP.	0.5
	OX ,VA ,OT.	0.3
	SXT ,OX ,VA ,KZ ,OT.	0.5
	P ,FOX ,OX ,KZ ,AMP.	0.5
	FOX ,OX ,KZ.	0.3

Table (6). Multiple antibiotic resistance index of *Staphylococcus aureus* isolated from meat

MAR index	Number of isolates
0.1	1
0.2	2
0.3	9
0.4	6
0.5	13
0.6	9
0.7	3

3.4. Detection of different genes of *Staphylococcus aureus* isolated from meat by PCR.

The PCR results cleared that all tested isolates were positive for the 16SrRNA gene (Table. 7, fig. 1a), which indicated similarities and great relationships between the isolates from meat. Same finding was concluded by Forsman et al., (1997), Mendoza et al., (1998), and Kuzma et al., (2003) who reported that PCR based on 16S-23S RNA intergenic spacer region sequences had been successfully applied for the identification of *S. aureus*. Thus, PCR assay using species-specific *S. aureus* primer could be used as specific tool in diagnosis of *S. aureus* and used effectively for monitoring *S. aureus*

contamination in meat because of its high specificity and sensitivity *(Goto et al., 2007)*.

All tested isolates were positive for the *mecA* gene that is very important for the detection of methicillin resistance *Staphylococcus aureus* (MRSA) (Table. 7, fig. 1d). Further, all isolates were positive for the *blaz* gene (Table. 7, fig. 1b) that indicates the presence of a heteroro-resistant population of *Staphylococcus* aureus. All tested isolates were negative for the *Sea* and *Sed* genes, while, positive by 16.66%, 33.33% and 50% for the *Seb*, *Sec*, and *See* genes. Ali et al., (2011) analyzed 1070 food samples obtained from retail markets and dairy farms in the Marmara Region of Turkey for the presence of *S. aureus*. Out of 147 isolates, 92 (62.6%) were enterotoxigenic. PCR was used to investigate the presence of staphylococcal enterotoxin genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq* and *seu*), exfoliative toxin genes (*eta* and *etb*) and the toxic − shock syndrome toxin gene (*tst*). The PCR results showed that 53.3% of the isolates contained staphylococcal enterotoxin-like (SEl) toxin genes (*seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*,sea.see *sep*, *seq* and *seu*). Furthermore, *seo*, *sei*, *sem*, *seg*, *seu* and *sec* were found in 37.0, 32.7, 30.4, 29.3, 29.3 and 27.2%, respectively, of the isolates. Our result agree with their PCR results where we detected staphylococcal enterotoxin-like (*see* and *sec)* genes in our *Staphylococcus aureus* isolates indicating the presence of Enterotoxin producing types of *S. aureus* that can cause food-borne disease (Table. 7, fig. 1c).

Our results concluded that, the freezing improve the organoleptic properties of the meat. Further, meat of different sources can harbor the health threating type of *S. aureus*; the methicillin resistance *Staphylococcus aureus* (MRSA). Moreover, applying strict hygienic practices for transporting, storage and handling of meat is essential for supplying meat of high quality and safe for human consumption.

Table (7): Detection of different genes of *Staphylococcus aureus* isolated from meat by PCR.

Samples		Genes							
		16SrRNA	Antimicrobial		Virulence				
			mecA	*blaZ*	*Sea*	*Seb*	*Sec*	*Sed*	*See*
Brazilian	1	+	+	+	-	-	-	-	-
	2	+	+	+	-	-	+	-	+
Indian	3	+	+	+	-	-	-	-	+
	4	+	+	+	-	-	-	-	+
Balady	5	+	+	+	-	+	-	-	-
	6	+	+	+	-	-	+	-	-

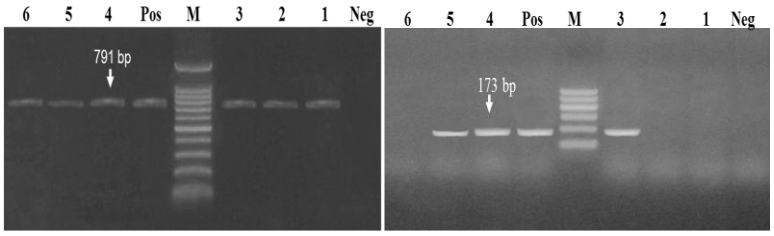

Fig. 1a: 16SrRNA

Fig. 1b: *blaz*

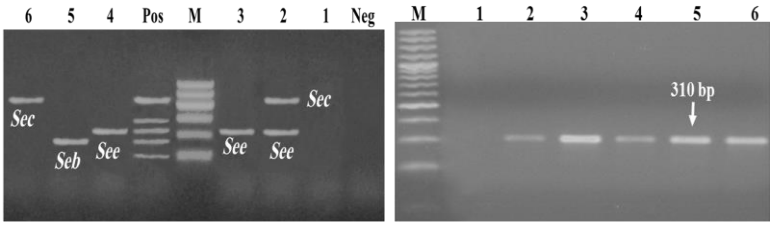

Fig. 1c: Enterotoxins

Fig. 1d: *mecA*

Figure 1. Agarose gel electrophoresis of amplified DNA showing the specificity of the single reactions for the detection of the different genes. **(a)** 16SrRNA gene **(b)** *blaz* gene **(c)** Enterotoxin genes **(d)** *mecA* gene. Pos; positive control, Neg; negative control, M; DNA ladder. 1, 2, 3, 4, 5; 6; *S. aureus* isolated from meat.

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