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Molecular Divergence of *Staphylococcus Aureus* Isolated from Pork Byproducts Based on Coagulase Gene Polymorphism and The Presence of Enterotoxigenic genes

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Abstract
Background: *Pork by-products are appeared to be a source of consumer coagulase positive S. aureus strains food poisoning.*
Material and Methods: *The genetic lineage of coagulase positive Staphylococcus aureus (coa) recovered from 20 imported and 30 locally manufactured pork byproducts in Cairo was checked using PCR, and restriction fragment length polymorphism analysis (RFLP).*
Results: *A total number of 27 S. aureus were isolated (54%), the coa gene was identified in 17 out of that isolates (62.9%) with molecular sizes ranging from 195 bp to 1196bp. The coa RFLP analysis using BsuRI revealed 4 different band patterns related to the source of isolates. The detection of 7 enterotoxigenic strains out of the 27 S. aureus isolates suggested the public health importance.*
Conclusion: *There were different polymorphism patterns between local and imported isolates with the detection of enterotoxigenic genes represent a public health concern.*
Keywords: *Staphylococcus aureus - pork byproducts - coa gene – enterotoxins- RFLP.*

I. Introduction

Staphylococcus aureus is a bacterium of critical significance, causing a variety of diseases in both animals and humans and its ability to resist different environmental forms [1]. *S. aureus* usually carry many virulence factors that include structural and secretory products which play importantrole in pathogenesis [2]. Coagulase is an extracellular protein that has generally been utilized to differentiate *S. aureus* from the less virulent staphylococci (Coagulase negative Staph- CNS) [3]. Two virulent factors were secreted by *S. aureus*, coagulase (*coa*) and von Willebrand element restricting protein (vWbp), both"*coa*" and "vWbp" together are required for the formation of abscesses and promote the non-proteolytic activation of prothrombin and cleavage of fibrinogen, reactions that are inhibited with specific antibodies against each of these molecules."*coa*" specific antibodies confer protection against abscess formation and *S. aureus* lethal bacteremia.Therefore, coagulase may be used as vaccine antigens to elicit antibodies that protect against *S. aureus* infection [4]. Several molecular procedures have been usedfor identification and comparison of *S. aureus* isolates in epidemiological studies. Among these methods, coagulase gene (*coa* gene) typing which considered a simple and effective strategy for typing *S. aureus* into various subtypes [5].

Another concept of pathogenicity related to *S. aureus* is the ability of some strains produce the enterotoxins (SEs) which are considered a prime source of food intoxication that occurs after consumption of different foods, particularly ready to eat meat, contaminated with *S. aureus* either through unhygienic handling or later improper storage. (SEs) are strong gastrointestinal exotoxins produced by *S. aureus* during the phases of growth, they are resistant to heat treatment, acidity, and proteolytic enzymes, so keeping their effectiveness in the bowel after ingestion [6]. Pork and pork byproducts are supposed to be possible sources of SEs because pig skin is commonly colonized by *S. aureus* [7].

Following up the previous concepts, our study was conducted to cover the following points: Isolation and identification of *S. aureus* from local and imported pork by-products in Egyptian pork markets, molecular detection and polymorphism of coagulase gene (*coa*) from *S. aureus* isolates, besides molecular detection of major enterotoxin expressing genes.

II. Material And Methods

2.1. Sampling

A total of 50 samples (imported pork by-products, n =20 and local pork by-products, n =30 of three different manufacture factories). All samples were purchased between April and June 2016, from different pork markets in Cairo governorate, Egypt. The samples were transferred to the laboratory of microbiology in National Research Center, Giza in a container containing ice cubes under aseptic condition.

2.2. Isolation and identification of *S. aureus*

Twenty gram samples were collected aseptically, 10-fold diluted with sterile quarter-strength Ringers Solution (Oxoid Ltd, London, UK) and homogenized for 90 seconds using the laboratory blender Stomacher 400 (Seward, London, UK). The cultures were incubated in 37°C for 24 h. Then the samples were subcultured on Baird–Parker agar and incubated at 37°C for 24-48 h. The presumptive colonies of *S. aureus* were identified by Gram stain, catalase, coagulase (both the slide and tube) and DNase tests [8,9]. The isolates were subjected to further testing using API STAPH IDENT 32 Staph (Biomerieux, Marcy l'Etoile, France).

2.3. DNA extraction and PCR assay

DNA extracted from *S. aureus* strains by using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

2.4. PCR detection of (*coa)* gene

The *coa* gene was amplified by using two sequences of the primers, Forward primer: 5′ CGA GAC CAA GAT TCA ACA AG 3′ and reverse primer: 5′ AAA GAA AAC CAC TCA CAT CA 3′ [10]. Primers were utilized in 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. Amplification was conducted in thermal cycler, which was adjusted as follows, an initial denaturation at 94˚C for 5 min. The cycling proceeded for 30 cycles of denaturation at 94˚C for30 sec, annealing at 55˚C for45 sec, and extension at 72˚C for45 sec with a step of the final extension at 72˚C for10 min.

2.5. PCR Product analysis

The PCR reaction mixtures were analyzed by electrophoresis on a 1.5% (w/v) agarose gel in the presence of 100- bp DNA ladder (Fermentas Life Science, EU) [11].

2.6. Coagulase gene typing by RFLP method

S. aureus strains positive(*coa*) gene was subjected to restriction fragment length polymorphism. PCR amplified (*coa*) gene product were digested with BsuRI enzyme,Preparation of restriction master mix according to Thermo FastDigest® BsuRI Cat. No. FD0154 were shown in table (1).

Table 1. Master Mix preparation for BsuRIenzyme

Component	**Volume/reaction**
10X FastDigest Green buffer	2 µl
BsuRI enzyme	1 µl
PCR product	10 µl
Water, nuclease –free	17 µl

The restricted fragments were separated in 1.5% agarose gel.

2.7. Multiplex PCR for enterotoxins

Primers were utilized in a 50- µl reaction containing 25 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 5 µl of water, and 10 µl of DNA template.

Table 2. Multiplex PCR for enterotoxins

Target gene	Primers sequence	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Sea	GGTTATCAATGTGC GGGTGG	102	94˚C 5 min.	94˚C 30 sec.	50˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	[12]
	CGGCACTTTTTTCTC TTCGG							
Seb	GTATGGTGGTGTAA CTGAGC	164						
	CCAAATAGTGACGA GTTAGG							
Sec	AGATGAAGTAGTTG ATGTGTATGG	451						
	CACACTTTTAGAAT CAACCG							
Sed	CCAATAATAGGAGA AAATAAAAG	278						
	ATTGGTATTTTTTTT							

	CGTTC							
See	AGGTTTTTTCACAG GTCATCC	209						
	CTTTTTTTTCTTCGG TCAATC							

III. Results

A number of identified nine *S. aureus* isolates were recovered from 20 imported pork by-products samples with an incidence of 45%,while 18 *S. aureus* were isolated from local pork by-products (18/30) with an incidence (60%)respectively, therefore the overall incidence of *S. aureus* isolates was 27/50 (54%) .Our study detected *coa* gene in 17 / 27 *S. aureus* strains (imported pork by-products, no=6 and local, no=11) with percentage (62.9%) as presented in photo (1).

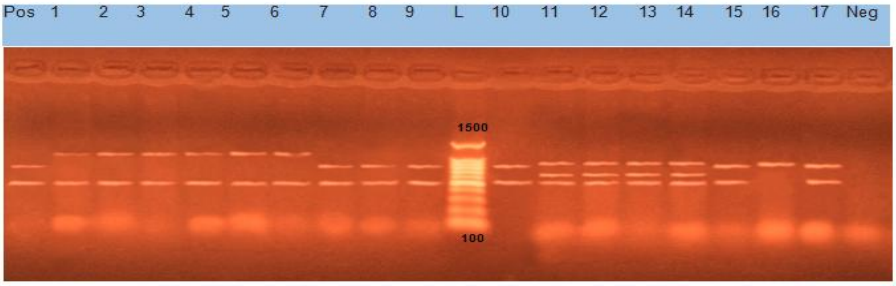

Photo 1: Amplified PCR product of *coa* gene L: 100bp ladder. Lane Neg: negative control. Lane Pos: positive control. Lanes 1-6: represented imported samples isolates, Lanes (7-10), (11-14), (15-17): represented local samples isolates obtained from factories no 1,2, and 3 respectively. The *coa* gene PCR products of 17*S. aureus* produced different size fragments which ranged from 491 bp to 1183 bp approximately as explained in the table (3).

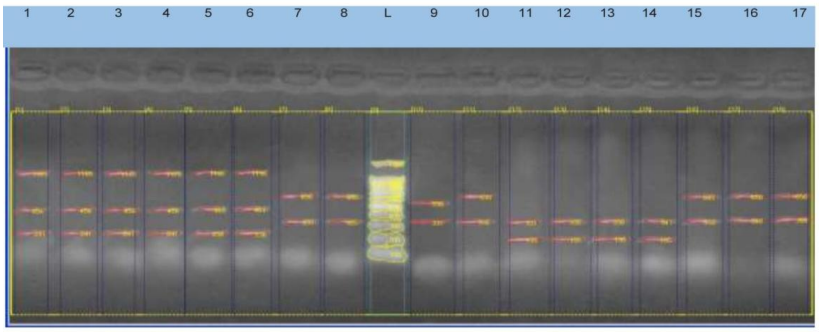

Photo 2. Restricted fragments of *coa* gene by BsuRIenzyme; Lane L: 100bp ladderLanes1-6: represented imported samples isolates, Lanes (7-10), (11-14), (15-17): represented local samples isolates obtained from factories no 1,2, and 3 respectively. The results of *coa* gene typing of *S. aureus* strains by RFLP were mentioned in the table (3).

Table 3. PCR product and restricted fragment size of *coa gene*

Sample no	Source	PCR product (bp)	Restricted fragments (bp)	Genotype
1	Imported pork by-products	498,1164	241,454,1175	
2	Imported pork by-products	498,1183	241,458,1185	
3	Imported pork by-products	500,1164	241,458,1175	
4	Imported pork by-products	498,1183	241,458,1175	I
5	Imported pork by-products	498,1183	238,465,1196	
6	Imported pork by-products	491,1178	236,469,1196	
7	Local pork by-products (LF1)	496,861	338,656	
8	Local pork by-products (LF1)	500,861	335,650	
9	Local pork by-products (LF1)	496,840	331,538	II
10	Local pork by-products (LF1)	504,858	338,650	
11	Local pork by-products (LF2)	491,647,990	195,331	
12	Local pork by-products (LF2)	496,648,990	195,338	
13	Local pork by-products (LF2)	496,654,990	198,338	III
14	Local pork by-products (LF2)	491,654,990	195,341	
15	Local pork by-products (LF3)	504,850	338,643	
16	Local pork by-products (LF3)	917	348,650	IV
17	Local pork by-products (LF3)	514,861	355,656	

LF1=Local factory (1)LF 2= Local factory (2)LF 3= Local factory (3)

Table (3) showed that, samples from (1to 6), (7 to 10), (11to14), (15to 17) are the same respectively.

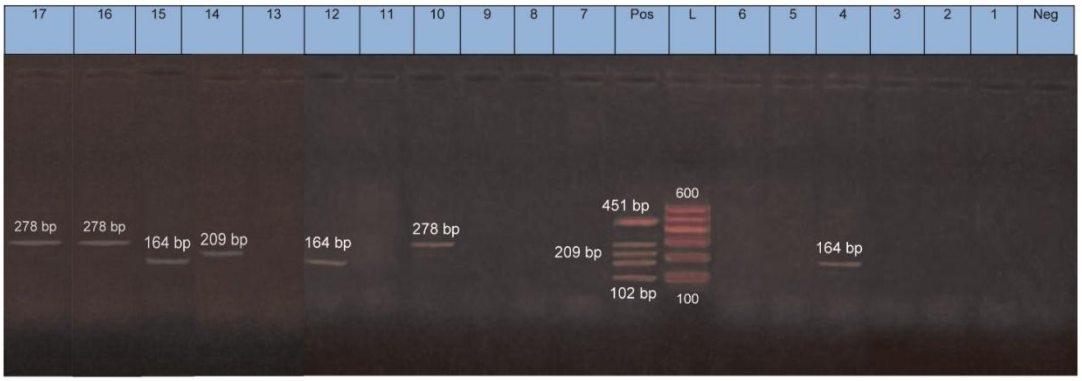

Photo 3: Multiplex PCR of enterotoxins; Lane L: 100bp ladder. Lane Neg: negative control. Lane Pos: positive control.Lanes4,12,15: realized *Seb*. Lanes 10,16,17realized *Sed*and Lane 14: realized *Seb*.

The 17 coagulase positive strains were screened for the presence of enterotoxin genes (*Sea, Seb, Sec, Sed, and See*). The results obtained from (Photo 3) showed that there are 7 strains harbored enterotoxin genes, each strain contains one gene by a ratio (41%) and by total isolated strains 7/27 (26%). One strain was belonged to the imported samples, harboring *Seb* (164 bp) and the other 6 were isolated from the local ones, 3 carry *Sed* (278bp), two carry *Seb*, and one carries *See* (209bp).

IV. Discussion

Using culture, biochemical and API methods, the present study identified a number of 27 *S. aureus* isolates out of 50 imported and locally manufactured pork by-products samples collected from great Cairo territory, with an incidence of 54%.This high recovery ratio was found in other previous studies as, Among 155 'Nham' (a traditional Thai fermented pork product) samples tested, 39.35% of the samples were positive for *S. aureus* [13], another study inOklahoma 43/99 (43.3%)[14].

Our study detected *coa* gene in 17 / 27 *S. aureus* isolates (imported pork by-products, no=6 and local, no=11) with percentage (62.9%) as presented in photo (1). This percentage was lower than the previous reported percentage 80.5% [15]. The amplified *coa* gene PCR products of 17 *S. aureus* strains produced different size fragments which ranged from 195 bp to 1196bp approximately. A near similar range of PCR amplicon products has also been stated by other researchers, 579 to 1442 bp [16], 484 to 1080 bp [17] and 500 to 1400 bp [15]. Instead of the present perception for *coa* amplicons in the present examination, only one type of amplicon was obtained i.e. 500 bp *S. aureus* subclinical mastitis strains in Nigeria[18]. This variance may be attributed to a C-terminal region of The coagulase gene, which composed of 81-bp tandem repeated units, each encode 27amino acid residues, comprising four, five, six, seven and eight units of the 81-tandem repeat, therefore, the size of 3′region of the *coa* gene is variable [19].

According to table (3), and photo (2): The PCR products of *coa* gene were subjected to restriction digestion using RFLP with BsuRI enzyme. The obtained results represented 4 different restriction patterns (I, II, III, and IV). This variation in restriction patterns was referred to the variable number tandem repeat (VNTRs) present in each isolates. The present study emphasizes the genotypic variation among different *S.aureus* isolates discussed in many previous reports. Pulsed-field gel electrophoresis patterns showed a wider variety (discriminatory index 0.83) among 94 strains of *S. aureus* isolated from cow's milk, raw cheese, and a milking machine in 12 dairy farms in northeast Brazil [20]**.** Another study in Iran; fifty eight *S. aureus* isolates were recovered from milk samples of cows with clinical and subclinical mastitis were analyzed using RFLP of *coa* gene with restriction endonuclease HaeIII, resulting in nine patterns [21]. There is a higher *coa* gene polymorphism of *S. aureus* isolated from native cattle breed (eight *coa* types) as compared to that in H-F crossbred cattle (three *coa* types) with the Alu restriction endonuclease [22]. As appeared in table (3), the *S. aureus coa* gene patterns were similar according to the source of samples, this finding in harmony with that study conducted in Iran [23], and proved that, there is no variation between the *coa* genes from the same source.

Due to their public health importance the 17 positive *coa* gene isolates were screened for the presence of enterotoxigenic genes (A-E). The photo (3) PCR analysis showed amplification of one gene type in 7 isolates out of total 27 (25.9%), distributed as one isolate from imported samples, carrying *Seb*., the other six present in the local ones, 3 carry *Sed*, two carry *Seb*, and one carries *See*. The apparently low presence of enterotoxin carrying genes in *S. aureus* isolates is not a surprise as other investigations seem to illustrate these data;one isolate (0.9%) from pork was positive for enterotoxin gene [14],12.8%[24], 18% [25], and15%[26]. Although the classical Staph Enterotoxines (SE) are considered to be major etiological factors in Staphylococcal food

poisoning (SFP), the newly described *SE* or *SE* like genes (*seg* to *ser*, and *seu*) were more frequently detected than the classical SE genes [27]. Also the storage of pork meat and pork byproducts in low temperature plays an important role not only the decreasing enterotoxigenic *S. aureus* detection, but also the total *S. aureus* isolation[13]**.**

V. Conclusion

The ready to eat pork by-products, either imported or locally manufactured are seem to be a source of coagulase positive *S. aureus* (CPS)strains which play an important role in the pathogenicity and invasiveness. Detection of *coa* gene is the principal standard for the identification of CPS strains, the restriction fragment length polymorphism (RFLP) is a more useful tool to detect polymorphism in (*coa*) gene for local epidemiologic purposes.Finally, the presence of enterotoxins genes in about 26% of isolates suggests the risk of food poisoning associated with the retail pork products and affirm the need of improving food handlers guidelines, and the obligatory use of cooled display as a public health establishment.

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