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Phenogenotyping of Closely Related Methicillin Resistant *Staphylococcus aureus* Isolated from Milk and Meat Products

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Abstract

Differentiation of closely related methicillin resistant *staphylococcus aureus* (MRSA) isolated from food chain is required for epidemiological and food safety studies. This study provided evidence for using coagulase (*coa*) gene sequence repeats as a powerful diagnostic tool for differentiation of closely MRSA isolated from different sources. *Staphylococcal* species were isolated from milk, meat, and meat-products. *Staphylococcus aureus* strains with identical antibiograms were differentiated using enterotoxin genotyping, *coa* gene polymorphism, the patterns of polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) of *coa* and nucleotides sequencing of *coa* gene-PCR products. Nine CoPs and MRSA strains were closely related and could not be differentiated by antibiogram. Six strains of them were differentiated by enterotoxin genotyping and PCR-RFLP of *coa* gene and three strains were accurately differentiated by nucleotides sequencing of *coa* gene-PCR products. In conclusion, the above findings highlight the promising role of *coa* repeat region nucleotide sequences in differentiation of closely related MRSA in food chain that can help in their diagnosis and epidemiological studies.

Keywords: MRSA, *coa* gene, *coa* polymorphism, PCR-RFLP.

Introduction

The spread of *Staphylococcus aureus* in milk and meat-products represents global problem especially when these microorganisms are methicillin resistant (MRSA). Regular detection and classification of these isolates are strictly required for control measures in food industry and to limit spread of resistance to antimicrobials. It was estimated that over 60% of the emerging human pathogens come from animals [1] and food products [2]. The minority of *S. aureus* strains obtained from food products are not host specific and consumption of these contaminated feed leads to colonization of *S. aureus* in humans causing community acquired infections [3-5]. In addition, developed resistance to methicillin limits the use of β-lactam antimicrobials and the antibiogram schemes were often uninformative for typing many strains [6].

S. aureus enterotoxins (SEs) and staphylococal-like (*SE*I) proteins are potent gastrointestinal exotoxins synthesized by *S. aureus* throughout the logarithmic phase of growth or during the transition from the exponential to the stationary phase [7-10]. *Staphylococcus aureus* produces a wide variety of toxins including staphylococcal enterotoxins that are responsible for food poisoning, which occurs after ingestion of different foods contaminated with *S. aureus* by improper handling and subsequent storage at elevated temperatures. Enterotoxin genotyping was used recently to differentiate toxogenic from non toxogenic *S. aureus* [7]. They are active in high nanogram to low microgram quantities [11], and are resistant to conditions (heat treatment and low pH) which easily destroy the bacteria, and to proteolytic enzymes, hence retaining their activity in the digestive tract after ingestion [12,13]. All staphylococcus enterotoxin associated genes are located on accessory genetic elements, including plasmids, prophages, *S. aureus* pathogenicity islands (SaPIs), or genomic island next to the staphylococcal cassette chromosome (SCC) elements that can be used for *S. aureus* differentiation. Most of these are mobile genetic elements, and their spread among *S. aureus* isolates can modify their ability to cause disease and contribute to the evolution of this important pathogen [reviewed in 14].

Molecular typing plays an important role in epidemiological studies of nosocomial infection, such as methicillin-resistant MRSA infection. Pulsed-field gel electrophoresis and

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multilocus sequence typing are considered the most discriminatory and reliable methods of typing, although they are technically complex, time consuming, and expensive. On the other hand, it has been reported that PCR restriction fragment length polymorphism (RFLP) typing of the coagulase gene (*coa*) can be used to discriminate *S. aureus* strains on the basis of sequence variation within the 3_ endcoding region of the gene [15-17]. In the C-terminal region of *coa* gene, variability in the number and sequence of 27-amino acid residue (81-bp tandem short sequence repeats), determined by restriction fragment length polymorphism analysis of PCR products, enhanced the differentiation of *S. aureus* isolated from diverse locations. Slow rate of evolution in variable region of *coa* gene was needed in colonial group relation of MRSA collected from geographically diverse locations [18].

The molecular differentiation of closely related MRSA in Egypt needs more studies for epidemiological and diagnostic purposes. This study provides simple, rapid, discriminatory and reproducible methods for typing this pathogen to differentiate the closely related MRSA in milk and meat-products based on the nucleotide sequence variations of *coa* gene repeats in PCR products.

Materials and Methods

Bacterial isolation

A total of 205 samples including 90 dairy cow milk samples (mastitis milk), and 115 meat-products (broiler, sausage and minced meat) were collected in sterile plastic bags from groceries and dairy farms at different localities in Sharkia Governorate, Egypt. After collection, the samples were placed over ice and transported to the bacteriology lab at the Microbiology Department, Faculty of Veterinary Medicine, Zagazig University for the routine microbiological examination. The samples were processed in PBS under aseptic conditions according to the method previously described [19]. After being processed, each sample was inoculated onto mannitol salt agar and incubated for up to 7 days at 37°C. Pigment production was examined onto milk agar. Micrococci were separated according to bacitracin susceptibility and O-F test as it was previously detailed [20].

Bacterial identification

Coagulase negative staphylococci (CoNs) and coagulase positive staphylococci (CoPs) were biochemically classified by tube coagulase test using fresh sterile rabbit plasma into coagulase positive or coagulase negative isolate according to the protocol of [20]. Further identification of the isolates was confirmed by API kits (BioMerieux, France), and the identification was carried out according to the manufacturer instructions.

Antimicrobial susceptibility testing

All CoNs and CoPs were inoculated onto sterile blood agar base and incubated at 35°C for 24 h. The isolates were tested for antimicrobial sensitivity by qualitative antibiogram using disc diffusion method. Eight antimicrobial agents included: vancomycin (30 µg), methicillin (30 µg), amoxicillin/clavulanic acid (20 µg/10 µg), clindamycin (30 µg), gentamicin (30 µg), ciprofloxacin (30 µg), sulfamethoxazole/trimethoprim (30 µg) and rifampicin (30 µg) were used. The antibiotic discs (Oxoid, UK) were gently pressed onto Muller Hinton agar (MHA, Oxoid, UK) plate containing 4% NaCl to inhibit any contaminants and to provide the suitable growth condition, and then the plate was incubated overnight at 37°C. *S. aureus* ATCC 25923 was used as a quality control. Resistance percentages of staphylococci to antimicrobials were recorded [21] and the antibiotic resistance profile of each isolate was also reported.

Coagulase and enterotoxin genes amplification

Amplification of *S. aureus coa* and species-specific *nuc* genes were amplified by uniplex PCR, while detection of *S. aureus* enterotoxin genes (*sea-see*) were performed by multiplex PCRs with cycling protocols [22]. DNA was extracted using DNA extraction kit (Bio-Fermentus, MD, U.S.A), Catalogue No. # K1081, Lot: 00055548 according to manufacture instructions. Briefly, few colonies of *S. aureus* were harvested in sterile saline in a 1.5 mL micro-centrifuge tube, pletted by centrifugation at 5000 xg, and then the pellets were resuspended in 180 µL of lysis buffer and incubated for 30 min at 37ºC. Consequently,

the lysate was centrifuged at 10,000 rpm for 10 min at 4°C. DNA purification was done using Wizard plus Minipreps SV DNA purification system (Promega, Madison, USA) according to the manufacturer's instructions. The quality of the extracted DNA was confirmed by running the extracts on 0.8% agarose gel and by measuring the concentration of DNA. *Coa* and enterotoxin genes amplification was performed using DNA thermal cycler (Perkin–Elmer, U.S.A) in a total volume of 25 µL. The reaction mixture composed of 6.2 µL of master mix (Promega Inc, Madison, USA) (2.5 µL TAE 10X buffer + 2 µL dNTPs + 1.5 µL $MgCl_2$ + 0.2 µL Taq polymerase), 1.0 µL from each primer, 5 µL of the DNA template and finally water nuclease-free was added up to 25 µL and the mixture was mixed well. Consequently, PCR amplicons electrophoresis was run in 1.5% agarose gel and visualized by ethidium bromide to confirm the the existence of the target products.

PCR-restriction fragment length polymorphism (PCR-RFLP) of coagulase gene

Restriction analysis of *coa* gene-PCR products was performed with *AluI* enzyme digestion [23]. Nine *coa* gene products from closely related MRSA isolates from milk, meat and meat products were exposed to restriction digestion. Seven microlitres of *coa*-PCR product was incubated with 5 units of *AluI* in 20 µL reaction mixture at 37°C for 16 h in a water bath. The *AluI* digests were visualized on 2.5% agarose gels stained with ethidium bromide and the restriction patterns were recorded**.** The sizes of the PCR products and restriction DNA digests (RFLPs with respect to the overall number of 81-bp tandem repeats) were estimated by comparison with 100 bp molecular size standard marker. Consequently, the bands were photographed under ultraviolet illumination.

Coa gene PCR product sequencing

The MRSA strains unclassified by restriction typing were subjected to sequencing of *coa* gene PCR products. The amplicons were purified by Wizard RSV Gel and PCR clean-up system (Promega Inc., Madison, WI, USA). Sequencing reaction was performed with fluorescent dideoxy chain termination chemistry using the BigDye Terminator Version 3.1 cycle sequencing kit (Applied Biosystems, USA). Alignments, and sequence identity of *coa* among sequences were analyzed using the MEGA 4 package and tandem repeat finder version 4.07b [24,25].

Results

Percentage of Staphylococci in mik and meat products samples

A total of 25 (27.7%) and 28 (31.1%) of milk samples were contaminated with *S. aureus* and CoNs, respectively, meanwhile, 28 (24.3%) of meat- products specimens were contaminated with *S. aureus*. The CoNs in milk samples were *S. scuri, S. lentus, S. chromogenes, S. xylosus* and *S. simulans* and they represented 50, 10, 15, 15 and 10% of the examined isolates respectively.

Table 1: Resistance percentage of CoNs and *S. aureus* to antimicrobials

Antimicrobial agents	CoNs in milk	*S. aureus*	
		Milk	Meat Products
Methicillin	75.0	40.0	28.6
Amoxycillin/ clavulanic acid	50.0	40.0	53.6
Ciprofloxacin	21.4	12.0	0.7
Clindamycin	53.6	12.0	32.1
Rifampicin	32.1	12.0	0.2
Trimethoprime/ sulphamesoxazole	3.6	0.0	0.0
Gentamycin	3.6	0.4	0.35
Vancomycin	0.0	0.0	0.0

Antimicrobials susceptibility pattern

The qualitative antibiogram was done to determine the existence of MRSA strains in both CoNs and CoPs (*S. aureus*) isolates. As shown in Table 1, most CoNs and *S. aureus* strains were resistant to β-lactam antibiotics. The resistance percentages of CoNs isolates to methicillin and amoxicillin/clavulanic were 75 and 50%, respectively; meanwhile, the resistance percentage was 40% against each of these two antibiotics in *S. aureus* isolates from milk. Of note, CoNs isolates in milk showed 53.6% resistance to clindamycin and 32.1% of *S. aureus* from meat products were resistant to clindamycin. (Table 1). Moreover, CoNs isolates from milk and *S. aureus* isolates from milk and meat products represented lower resistance levels to both gentamicin and trimethoprime/sulphamesoxazole. None of the MRSA strains was vancomycin resistant (Table 1).

Table 2: Percentages of staphylococci resistant to only one or more of antimicrobial agents

No. of Antimicrobials	CoNs in milk	*S. aureus*	
		Milk	Meat products
1	10.7	40.0	7.2
2	21.4	32.0	21.4
3	35.7	12.0	28.6
4	14.3	16.0	35.7
5	3.6	0.0	0.0
6	0.0	0.0	7.2
7	14.3	0.04	0.0
8	0.0	0.0	0.0

Regarding the number of antibiotics, the isolates were resistant to at least one up to 7 antibiotics. We have reported difference in the antibiotic resistant patterns among the isolates (Table 2). The percentages of resistant milk and meat products` isolates to three drugs were 12 and 28.6%, respectively. The highest percent of resistance was reported against *S. aureus* from meat products, where 35.7% of the isolates were resistant to 4 antimicrobials. The percent of isolates resistant to five antibiotics or more was relatively low in all isolates from milk, meat and meat products (Table 2). Based on the qualitative antibiogram, 9 isolates resistant to more than 3 antimicrobials from milk, meat and meat products and with identical antibiogram were randomly selected and confirmed firstly as *S. aureus* by PCR amplification of *nuc* gene. These 9 strains were tested for enterotoxin gene existence and for *coa*-RLFP typing.

Enterotoxin genotyping

Depending on enterotoxin genotyping, three isolates from mastitis milk and meat products were found to contain staphylococcal enterotoxin *sea* and *seb* genes, where the 6 remaining isolates did not contain these common genes. Therefore, these 6 isolates could not be differentiated by genotyping using enterotoxin associated genes.

Coa gene amplification and PCR-RLFP products analysis

The *coa* gene was amplified by PCR and then subjected to *AulI* restriction enzyme digestion to determine the shared fragments among the undifferentiated MRSA isolates via antibiogram and *se* genotyping. PCR analysis revealed the presence of single or three bands with molecular sizes ranging from 648-913 bp with the product of 812 and 648 bp amplicon size being the most frequent (Fig 1A). After restriction enzyme digestion, amplicons of different sizes (Fig 1B) including: 350, 240+350+600, 400+350, 500+380 bp fragments were observed and these PCR products generated 5 different *AluI* restriction patterns or classes (I through V). Types I and II were the most common. The detailed data of the 9 screened isolates regarding their *coa* PCR products and *AluI* RFLP patterns are shown in Table (3).

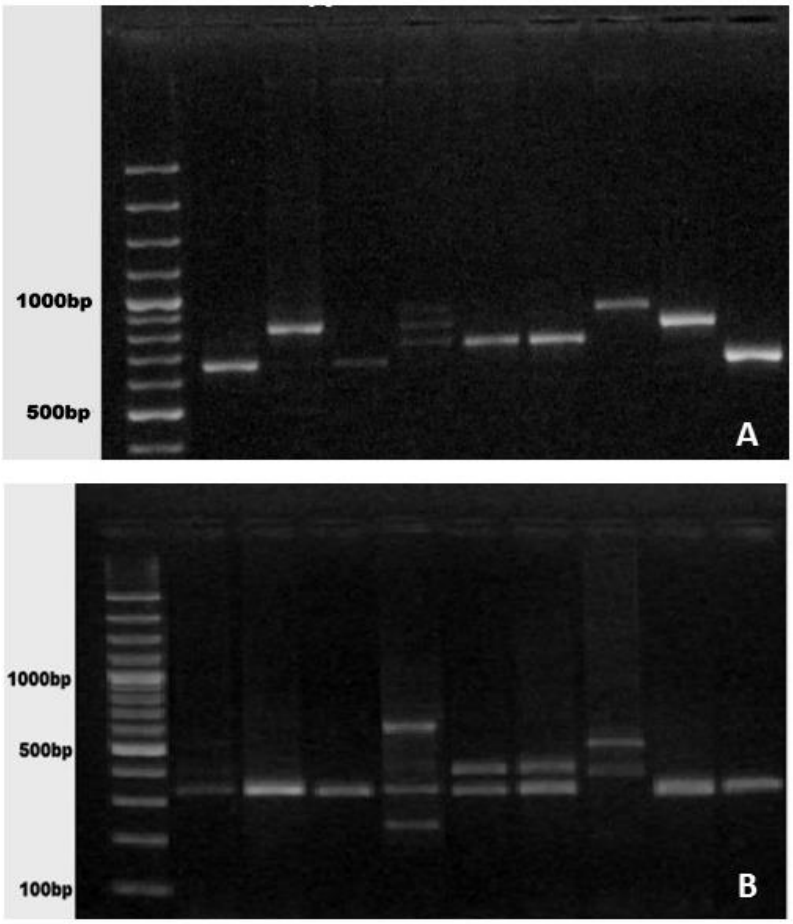

Figure 1: *Coa* gene-PCR products (A) and their corresponding *Alu* restriction pattern (B) of nine *S. aureus* strains (left to right with code numbers 15m ,28m ,18m ,139m, 180m ,242Br, 243Br, 217Mm and 233Sg).

coa nucleotide sequence repeats

The three undifferentiated strains were subjected to nucleotides sequencing of *coa* gene-PCR products. Point mutations in nucleotides of each repeat were different from that of any other *S. aureus* strains. Deduced amino acid sequence in these amplicons could not differentiate these strains, where two pairs of these amplicons have the same sequence of repeats (data not shown). Sequence analysis was carried out and the accession numbers were reported in the GenBank according to nucleotides sequence analysis of *coa* gene PCR products. The GenBank accession numbers for these sequences reported based on the results of this study are: KP728809, KJ081285 and KJ009314.

Table 3: Genotyping of nine *S. aureus* isolated from milk and meat

Sample source	Code No.	Enterotoxin genotype	*Coa* gene product (bp)	*ALu*I- Restriction pattern	RFLP class
Cow Milk	15m	B	648	350	I
	28 m*	N	648	350	I
	18 m*		812	350	II
	139m		723,812,913	240,350, 600	III
	180 m		723	400,350	IV
Broiler meat	242 Br	A	723	500,380	IV
	243 Br		913	500,380	V
Minced Meat	217 Mm		812	350	II
Sausage	233 Sg*	N	648	350	I

N: negative. m: milk Br: burger Mm: minced meat Sg: sausage *: the undifferentiated isolates subjected for sequencing

Discussion

Preventing cross-infection with epidemic strains of methicillin-resistant *Staphylococcus aureus* (MRSA) requires effective control measures. Wide spread of *S. aureus* in milk and meat-products represent worldwide problem. Detection and accurate diagnosis play a vital role in prevention of MRSA and also can provide effective measures in food industry and in limitation of spreading of *S. aureus* that resist antimicrobials. European Food Safety Authority (EFSA) underlined the increasing concern for Public Health represented by the presence of methicillin-resistant *S. aureus* (MRSA) in food producing animals, and recommended that further work should be performed on sampling, detection and quantification of MRSA carriage in both humans and animals, as well as on the contamination of food and the environment [26]. EFSA recommendation confirm the necessity for accurate technique that can make direct detection and classify of these pathogens that can help in limiting MRSA spreading in food and outbreaks of staphylococcal food poisoning. Recently, genotyping methods are considered the most accurate, reproducible and with discriminatory power compared to the other routine diagnostic methods. In this work, our main objective was to find a discriminatory test for differentiation of closely related MRSA.

Regarding the antimicrobial susceptibility results of staphylococcal species in milk, meat and meat products, we found that 75 and 68.6% of CoNs and CoPs from milk and meat products were respectively methicillin resistant. These isolates were not only resistant to β-lactams, but also were resistant to β-lactamase inhibitors (clavulanic acid), ciprofloxacin and clindamycin. Previously, low level (11.9%) of MRSA was recorded in surveys of European Food Safety Authority [26]. In the current study, *Staphylococcus sciuri* was the predominant identified strain among the detected CoNs to staphylococcal species level and it was previously considered as active player in horizontal transfer of *mecA* and antimicrobial resistance genes among other staphylococci [27]. This could explain the highly developed multiple antibiotic resistance in *S. aureus* isolated from milk [28]. Such MRSA carrying resistance to the other antimicrobial agents, including the aminoglycosides, macrolides, chloramphenicol, tetracycline, and fluoroquinolones were frequently spread [29]. In the current work, the strains under the study showed high resistance to the mostly widely used antimicrobials and they were resistant to three or more of antimicrobials. Meanwhile, lower percentages were previously recorded in other localities [30]. This high resistance in the studied *S. aureus* to antimicrobials may be attributed to random use of antibiotics or the public hygienic conditions.

S. aureus is involved in a wide variety of diseases in humans and animals and its pathogenicity is mainly related to a combination of toxin-mediated virulence, invasive capacity, and antibiotic resistance

[26]. Some strains are able to produce enterotoxins within foodstuff, causing staphylococcal food-poisoning (SFP), and these can be divided into 5 classes: SEA, SEB, SEC, SED, and SEE), and among them SEA and B are the most common [14]. In this study, 3 of the isolates were positive for enterotoxin genes *sea* and *seb*, while 6 of the multiple antibiotic resistant isolates did not contain these most common enterotoxin genes These toxogenic isolates may be due to contamination and unhygienic handling of milk, meat and meat products in farms, slaughter houses or meat processing and manufacturing factory. Enterotoxin genotyping was able to differentiate only 3 MRSA strains with similar antibiogram and could not differentiate the rest of MRSA strains. This may be attributed to the ability of some strains to produce wide variety of enterotoxins under different environmental conditions. Therefore, these unclassified strains were subjected to further typing based on their *coa* gene sequence repeats polymorphism after *coa* gene-PCR amplification.

Classification based on RFLP of the *coa* gene of *S. aureus* isolates has been considered simple and accurate method for molecular typing. This technique could be used in epidemiological investigations of *S. aureus* isolated from different sources because of its high reproducibility and good discriminatory power on the basis of sequence variation within the 3′ end coding region of the gene and *Alu* restriction typing of variable nucleotide sequence repeats in *coa* gene-PCR products [15, 23]. The sizes of the products ranged from 350 to 917 bp in increments of 81 bp, reflecting the number of 81-bp repeat units contained in the *coa* gene after digestion with *AluI* and this allowed the differentiation of 31 *coa*-RFLP types and was able to distinguish them from each other confirming the sensitivity of *coa*/RFLP in differentiation [23]. This difference between our results and other studies [23,28] might be due to our small sized samples or due to differences in MRSA strains as mentioned in previous studies [6,16,31] who reported that *S. aureus* strains of the same coagulase serotype can be differentiated by *Alu* restriction and nucleotides sequence analysis of *coa* gene. In the current study, nucleotides sequence variability of tandem repeat region in *coa*-gene PCR products was able to discriminate the examined unclassified isolates. However, using the deduced amino acid sequences in repeats (data not shown) of *coa* gene- PCR products could distinguish these strains. Previously; using the whole codon sequence of the C-terminal variable region of *coa* gene could differentiate most of examined strains [28]. Taking together, this data confirmed that the *coa* repeat region nucleotide sequences can be used in differentiation between the closely related MRSA.

Conclusion

Although the number of samples was few, it could be concluded from the data that most *S. aureus* in milk and meat-products undifferentiated by qualitative antibiogram depending on antibiotics with chromosomal markers were subjected to enterotoxin genotyping, *coa* gene amplification and *AluI* restriction patterns. Importantly, closely related strains could be classified only according to the nucleotide sequences of *coa* gene repeats. These findings can help in epidemiological and diagnosis studies that could be useful in controlling MRSA spreading and infection prevention. Further studies are required on more of other closely related MRSA to reveal the classification potential of nucleotide sequence of *coa*-gene repeats.

Conflict of interest

The authors declare no conflict of interest.

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الملخص العربي

التصنيف الظاهري والجيني للميكروبات العنقودية الذهبية شديدة القرابة و المقاومه للميثاسيلين المعزولة من الألبان واللحوم ومنتجاتهما

عادل عطيه احمد و احمد محمد عمار ومحمود بنداري و نورهان خيري و مروه إبراهيم و جمال عبد المنعم المولد*
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يعد تصنيف الميكروب العنقودي الذهبي شديد القرابة والمقاوم للميثاسيلين المعزول من الأطعمه من الضروريات في الدراسات الوبائية ودراسات سلامه الأغذيه لذلك هدفت هذه الدراسة إلي إيجاد طريقه تشخيصيه قويه وفعاله للتصنيف باستخدام التسلسل النيوكليتيدى لمتكررات جين الكواجيولاز. تم عزل الميكروبات العنقودية من عينات من الألبان واللحوم ومنتجاتهما وعمل تصنيف علي أساس حساسيتها للمضادات الحيوية و تواجد جينات السموم المعويه و كذلك تحديد النمط المتعدد لجين الكوأجيولاز وتحديد الجزء الطولي المتغير في جين الاكو أجيولاز باستخدام تفاعل البلمره والتسلسل النيوكليتيدى لمتكررات جين الكواجيولاز و أوضحت النتائج أن تسعه عترات شديدة التقارب و صعب تصنيفها باستخدام اختبار الحساسية للمضادات الحيوية وقد تم تمييز ستة منهم عن طريق تواجد جينات السموم المعويه وكذلك تحديد الجزء الطولي المتغير في جين الاكو أجيولاز باستخدام تفاعل البلمره أما الثلاثة عترات المتبقيه للميكروب العنقودي الذهبي المقاوم للميثيسيلين شديدة التقارب تم تمييزها عن طريق التسلسل النيوكليتيدى لمتكررات جين الكواجيولاز مما يثبت أمكانيه هذا الاختبار في تشخيص الميكروبات العنقودية المعزولة من الأطعمه بدقه وكذلك أمكانيه استخدامه في الدراسات الوبائية لتحديد مدي وكيفيه انتشار هذا الميكروب