BACTERIOLOGICAL AND MOLECULAR DETECTION OF *STAPHYLOCOCCUS AUREUS*SUPERANTIGENIC TOXIN GENES IN WOMEN ,COWS AND BUFFALOES MILK

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ABSTRACT

A total of 300 random samples of milk represented by women, cows and buffaloes (100 for each) were examined by conventional bacteriology, serology and molecular biology tests for the presence of *Staphylococcus aureus* and its super antigenic toxin and its relation to methicillin-resistance.Positive bacterial isolation was observed in 82(**27.3**%.) milk samples,including37 (37%) cows, 30 (30%) women and 15 (15%) buffaloes samples. By using latex agglutination test,all buffaloes milk *S. aureus* isolatesexhibitedpositive results(100% ,n=15)followed by isolates of women(**83.1**%,n=25)and cows(**81.1**%,n=30) with an overall percentage**(23.3**%**,n=70**.).

Amplification of *S aureus* species, staphylococcal superantigenic toxin (*sea, seb and sec*) and methicillin-resistant *S. aureus*(*mec*A) gene using Polymerase Chain Reaction (PCR) revealed that, **82**/300 (**27.3**%) of the total examined milk samples were positive for both*species(nuc)*and resistance(*mec*A) genes. While, *sea* positive results exhibited by 5 (38.5)%) cows milkisolates .Both women and buffaloes*milk isolates*showed*seb*positive resultsin a percentage of 55.5% (5 isolates for each),*sec* was not producedby women milk isolates and appeared in cows and buffaloes milk isolates (7.7 and 11.1% respectively).Further confirmation for the *S aureus* diagnosis was performed by sequencing analysis and BLAST comparative analysis, the results of this analysis revealed that partial *nuc*gene sequences of local tested isolates of *S. ureus*showed 100% sequence identity with published data of *S.aureus*(strain 08-02300, coStaphylococcusaureusmplete genome and Staphylococcus aureus isolate 22_LA_562 genome assembly, chromosome: I

INTRODUCTION

Milk serves as an optimum medium for propagation of various pathogenic and spoilage micro-organisms (1). Staphylococcal intoxication is considered the second or the third most common food intoxication of microbiological origin (2).

Among the extracellular protein toxins produced by *S. aureus*, staphylococcal heat stable enterotoxin (SE) and toxic shock syndrome toxin-1 (TSST-1) are the most important virulent factors belonging to the superantigen family (3; 4).Those superantigens can stimulate a high percentage of non-specific T-cells, and consequently they can stimulate the release of pro-inflammatory cytokines, such as interleukin (IL)-1, IL-2, interferon-g (IFN-g) and tumor necrosis factor-a (TNF-a) leading to fever, hypotension and shock (5).

More than 20 distinct superantigenic toxins are known to be produced by *S. aureus*(6; 7). Among these enterotoxins, staphylococcal enterotoxins SEA, SEB and SED are the most common causes of outbreaks of food poisoning (8; 4).

Most methicillin-resistant *S. aureus*(MRSA) strains carry *mec*A encoding low affinity penicillin-binding protein PBP2a (or PBP2') (9). Both MRSA and methicillin-sensitive *S. aureus*(MSSA) can harbor one or more super-antigenic toxin genes (10). MRSA is significantly more associated with mortality than MSSA (11). The pathogenic mechanism and virulence factors are assumed to be different between MRSA and MSSA, but these issues are still controversial (12).The Centers for Disease Controles timatea total number of 240,000 Staphylococcus Food Poisoning (SFP) cases peryearin the US (13). In the EU ,the number of SFP out-breaks is rising ,with 386SFP outbreaks reported in 2014(14). Many different staphylococcal enterotoxins and enterotoxin like super antigen shave been described(15).There is evidence demonstrating emetic activity in humans for all classical enterotoxins SEA-SEE(15) and recently also for some newly described enterotoxins(16).Immunogenic and moleculare methods for detection of *S. aureus* and comparison of the super-antigenic toxin genes in MRSA and MSSA isolated from bovine ,buffaloes and human milk are the main objectives of this study.

MATERIALS AND METHODS

Sample and sampling

Milk samples were taken from individual cows, buffaloes and women .The udder and teats were first cleaned with soap and dried using clean towels and then the teats were disinfected with 70% alcohol before sampling. The fore strip milk was then discarded and composite milk samples (10 ml). were taken from each cow and transported to the Microbiology Laboratory of College of Veterinary Medicine of Basrah University, Iraq by keeping in icebox containing icepacks. Upon arrival, the collected samples were immediately stored at 4 °C for a maximum of 24 h until culturing the next day. The samples were collected and processed during November2017toJanuary 2018.

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Isolation and bacteriological identification

The bacteriological culture was performed following the standard microbiological technique (17). A loopful of milk sample using inoculating needle was streaked on sterile5% sheep blood agar (Oxoid, UK) and the plates were incubated aerobically at 37 °C and examined at 24–48 h of incubation. The colonies were identified based on morphological characteristics, hemolytic pattern and Gram's staining reaction. The representative colonies which were positive for Gram's staining and typical grapes like structure under microscope were further sub-cultured on nutrient agar plates (Oxoid, UK) and incubated at 37 °C for 24 h.. Eventually, identification of the agent was done based on biochemical tests such as, mannitol salt agar . Samples were considered positive for S. aureus when the isolates showed fermentation of mannitol (strong yellow discoloration of media).

Serological identification

Following the manufacturer 'sinstructionsThermo Scientific™ Staphaurex™ Plus latex test(UK) was used to identify staphylococci which possess clumping factor, protein A and/or surface antigens, including those characteristic of Methicillin Resistant *Staphylococcus aureus* (MRSA).

Molecular identification

Amplification genesprimers for *S.aureus* ,their super antigens production and their PCR prereaction mix were displayed in table(1,2). The PCR tubes were transferred to the thermalcycler (Techne;UK) to start the amplification reaction according to specifi cprogram for eachgene (Table 3).Amplicons were electrophoresed in 2% agarose gel, stained with ethidium bromide and visualized under a UV transiluminator (VilberLourmal- CE;Taiwan).

Table 1:Primersequence used in PCRdetectionofsuperantigenic*S. aureus*

Primerset	Oligonucleotide sequence	Predicte	References
nuc-F	GCTTGCTATGATTGTGGTAGCC	423bp	(Wongboot. *et al*)[18]
nuc-R	TCTCTAGCAAGTCCCTTTTCCA		
mecA-F	GATGGCTATCGTGTCACAATCG	312bp	Modified from (Vannuffel et al), [19]
mecA-R	ATCTGGAACTTGTTGAGCAGAG		
sea–F	ACCGTTTCCAAAGGTACTGTA	135bp	(Wongboot. *et al*)[18]
sea-R	TGGTACACCAAACAAAACAGC		
seb–F	CCTAAACCAGATGAGTTGCAC	592bp	(Wongboot. *et al*)[18]
seb–R	CAGGCATCATGTCATACCAAA		
sec_F	AGATGAAGTAGTTGATGTGTATGG	454bp	Modified from (Mehrotra*et al*,)[20]
sec_R	CTTCACACTTTTAGAATCAACCG		

Table2: The pre-reaction mix(25µl)for each *nuc*,mecA, *sea* ,*seb*,*sec*

Material	S i
DNAtemplate	5 u
Master mix	1 2
Primer forward	1 u
Primer reverse	1 u
Nuclease free water	5

Table 3:PCR implification condition for *nuc,mecA.,sea, seb* and*sec* genes

Stage	Step	Temperature C°	Time min.	No.of cycle
First	Denaturation1	96	5	1
Second	Denaturation2	94	7	3 5
	Annealing	58	1	
	Extension1	72	1	
Third	Extension2	72	7	1

DNA Sequencingand Sequence Alignment

S aureus Species identification was confirmed by DNA Sequencing and Sequence Alignment. PCR amplicons were sent to Macrogen company labrotary in korea. The assembling of DNA sequences in both directions using forward and reverse of same primers used in PCR yielded a fragment containing 423 bp. The sequence alignments were extensively checked by eye to determine the percentages of similarities and discrepancies. The comparison was done between the sequences alignments of the

currently identified *S aureus* with those of previously published species of *S aureus* (Staphylococcus aureus strain 08-02300,andStaphylococcusaureusisolate 22_LA_562)

Statistical analysis

Data were analyzed by using SPSS statistical software version 11.. Chi-square test and Fischer's exact test were used toanalyze the data. The significance level was set at P = 0.05.

RESULTS

Prevalence of *S. aureus*

According to conventional bacteriological isolation and identification the overall *S aureus* isolation percentage was **27.3**%(n= 82). Of this, 37(n= 37), 30(n= 30) and 15% (n= 15) cows, women and buffaloes milk isolates percentage respectively. There was statistically significant difference in the number of *S aureus* isolates among the three milk samples sources (χ^2 = 11.517; p = 0.003;df=2) .Further serological identification by latex agglutination test for all *S. aureus* isolates revealed**23.3**% an overall percentage **(n=70**.),Buffaloes milk samples showed higher percentage(100% ,n=15) followed by Women(**83.1**%,n=25)and cows(**81.1**%n=30). (Table 4).

Table 4; Prevalence of *S. aureus* in three milk sources according to identification by conventional bacteriological and latex agglutination tests

Milk samples Sources	examined samples	Bacteriological	Serological	P-value
		Positive n. (%)	Positive n. (%)	
Cowes	**100**	**37(37)**	**30(81.1)**	**<0.05**
Buffaloes	**100**	**15(15)**	**15(100)**	
Women	**100**	**30(30)**	**25(83.1)**	
Total	**300**	**82(27.3)**	**70(23.3)**	

Moleculare detection of superantigenic *S aureus*

The result of PCR amplification was confirmed by electrophoresis as the strands of the DNA which are resulted from successful binding between the extracted DNA and specific primer of *S. aureus(nuc*) ,superantigenic toxin (*sea,seb*,and*sec*), appeared as single band on 2% agarose and under U.V. illuminator using Ethidium

bromide as specific DNA stain. Only single bands with expected size (423,312,135,595and 454 bp) were observed. (Figure. 1,2,3,4) .

Of the 82(**27.3%**)*S. aureus* isolates from buffaloes, cow and women, 9 (**60**%) ,13(**35.1**%)and 9 (30%), respectively, harbored the staphylococcus species and Methicillin resistance genes .Although Buffaloes milk *S. aureus* isolates showed higher percentage in PCR positivity compared to other milk soureces , this difference was considered to be statistically significant($\chi^2 = 17.436$; $p = 0.003$;df=5) (Table 5).

The ***sea,seb, sc*** superantigenic toxin genes present in MRSA isolates were **32.3**% (10/31) ,48.4%(15/31)and **6.5**% (**2**/31), respectively, with *seb* being more frequent(55.5%) in milk isolates of buffaloes and women(5/9 isolates for each) than cow milk isolates(**38.7%**.5/13).While *sea* was more frequent in cow milk(**38.5**%) than buffaloes milk isolates (**33.3**%)and women (**22.2**%) and *sec* was not produced by women isolates and appeared in cows and buffaloes (7.7 and 11.1% respectively). The difference among the sources of milk was statistically significant($\chi^2 = 15.509$; $p = 0.0499$;df=8) (Table 6**)**

Figure (1) PCR product of *S.aureus species (nuc)* gen(423bp) and *mecA*(312 bp)
The product was electrophoresis on 2% agarose

Figure 2; PCR product of *supertoxigenic toxin (sea)* gen with band size 135bp. The product was electrophoresis on 2% agarose

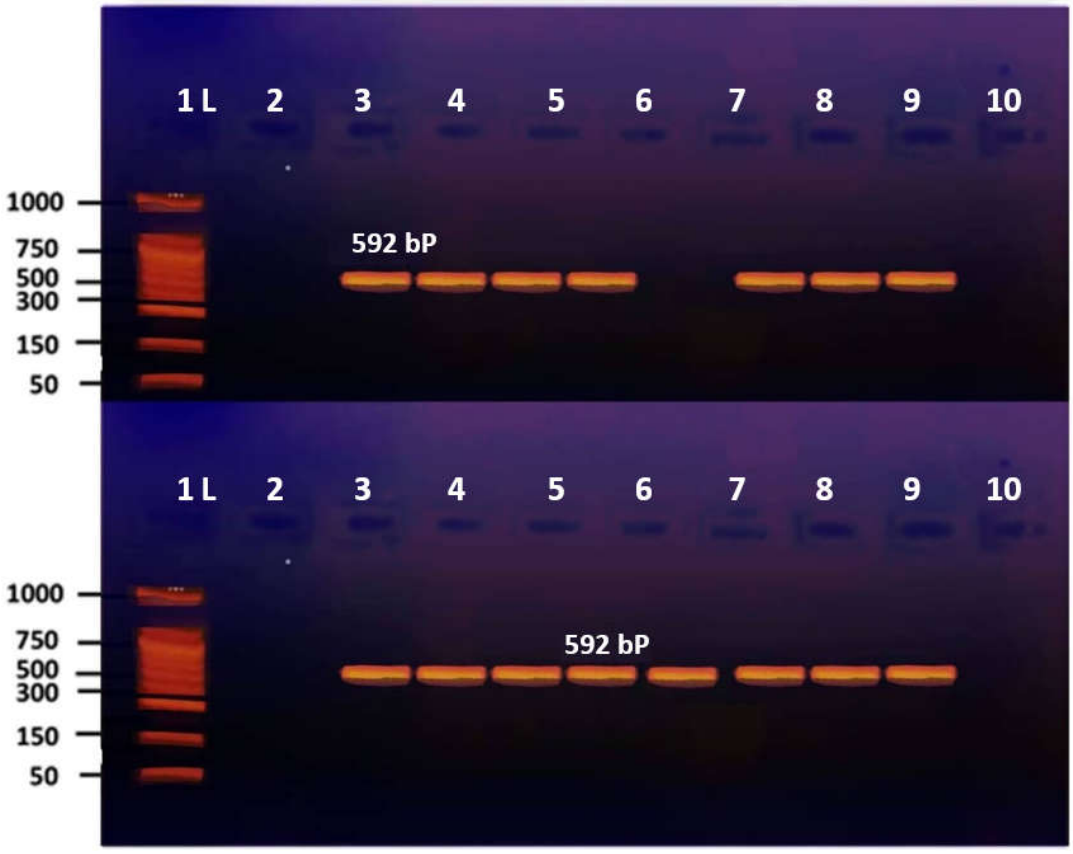

Figure 3;PCR product of *supertoxigenic toxin (seb)* gen with band size 592bp. The product was electrophoresis on 2% agarose

Figure 4; PCR product of *supertoxigenic toxin (sec)* gen with band size 454bp. The product was electrophoresis on 2% agarose

Table 5 ; Prevalence of *S. aureus* in three milk sources according to *nuc* and *mecA* genes

Milk samples Sources	No.of examined samples	Conventional bacteriological Positive n. (%)	*nuc*and*mecA* genes positive n. (%)	P-value
Cowes	100	37(37)	13(35.1)	<0.05
Buffaloes	100	15(15)	9(60)	
Women	100	30(30)	9(30)	
Total	300	82(27.3)	31(37.8)	

Table 6 ;Prevalence of superantigenic toxin genes in *S aureus* ofthree milk sources

Milk samples Sources	MRSA examined Samples n.	seaNo. of positive samples (%)	sebNo. of positive samples (%)	secNo. of positive samples (%)	P-value
Cowes	13	5(38.5)	5(38.7)	1(7.7)	<0.05
Buffaloes	9	3(33.3)	5(55.5)	1(11.1)	
Women	9	2(22.2)	5(55.5)	0(0)	
Total	31	10(32.3)	15(48.4)	2(6.5)	

Sequencing analysis and phylogenetic tree

The results of BLAST comparative analysis, revealed that the partial ***nuc*** gene sequences local tested *S aureus* isolates revealed 100% sequence identity between the current isolate and published data of *Staphylococcus aureus* strain 08-02300,

complete genome and Staphylococcus aureus isolate 22_LA_562 genome assembly, chromosome: I(Fig. 5,6)

TTACTTATCCATACATACGCCAATGTTCTACCATAGCGATCTTGTTTTTCTTTATCATATTCTAAATAAA
CATCTTGATTTGTTAATGTCTTCTTACTATAATTTGATGCTTCTTTGCCAAATGGTTGTACAGGCGTATT
CGGTTTCACCGTTTCTGGCGTATCAACCCCTATAAGCCTAACTTTAATTTCTTTACCATTTTGATTTGCA
ATAAATGTATCACCATCCACAACTCTTTGAACATGTACTTTATCTTTACCATTTAAATCTTGTACAGTTT
CATGATTTGTTCCCTTTTTAAAAGGACCCGTATGATTCATAAATTGAAATGCTAATACACCTACAATAA
TGATGGCTACCACAATC

Fig.5; Sequence alignment of *nuc* gene for local tested isolates *S aureus*,*Staphylococcusaureus* strain 08-02300, complete genome

TTTACTTATCCATACATACGCCAATGTTCTACCATAGCGGTCTTGCTTTTCTTTATCATATTCTAAATAA
ACATCTTGATTTGTTAATGTCTTCTTACTATAATTTGATGCTTCTTTGCCAAATGGTTGTACAGGCGTA
TTCGGTTTCACCGTTTCTGGCGTATCAACCCCTATAAGCCTAACTTTAATTTCTTTACCATTTTGATTTG
CAATAAATGTATCACCATCCACAACTCTTTGAACATGTACTTTATCTTTACCATTTAAATCTTGTACAGT
TTCATGATTTGTTCCCTTTTTAAAAGGACCCGTATGATTCATAAATTGAAATGCTAATACACCTACAAT
AATGATGGCTACCACAATC

Fig. 6;. Sequence alignment of *nuc* gene for local tested isolates *S. aureus* .Partial sequence.*Staphylococcusaureus* isolate 22_LA_562 genome assembly, chromosome: I

DISCUSSION

*Staphylococcus aureus*can be identified by conventional methods using phenotypic and biochemical properties but because of variability in the results there is uncertainty in its identification., the molecular methods are being used for its identification where a specific segment of DNA (as marker) specific to particular species of bacteria, is detected for confirmation of bacterial species.Molecular methods, especially PCR, are the most promising methods for the rapid and specific diagnosis of many bacteria in women and animals raw milk samples(21,22,)..

The present study was carried out to characterize *S. aureus* from raw milk of cattle. The characterization of *S.aureus* was based on phenotypic properties like cultural characteristics, coagulase production, hemolysis and antibiotic sensitivity. The species

specific identification of *S. aureus*was done by PCR using *nuc* primers. The genotypic characterization of *S. aureus*was done for virulence related genes namely super-antigenic toxin genes (*sea,seb and sec*) and antibiotic resistance gene (*mecA*).

In this study, from 300 cow,buffaloes and women raw milk samples subjected to bacteriological examination, 27.3% (82/300) were found to be positive for *S. aureus*.. This finding is nearly in agreement with the findings observed in other studies, 21.13% (23),29.1% (24)and 27% (25).

However, the result of the present study showed a slight lower contamination rate compared to other works90.4% (26);, 44% (27) and 75% (28)On the other hand the present results were slightly higher than 19.6% (41/209) (29) ,16% (30).And16.2% (31). *Staphylococcus aureus* was isolated from 37 (37%) cow and 15(15%) milk samples. This findings were disagreed in part and agreed in other part with (32) who was previously isolated *S.aureus* from 16% of buffalo's milk and 22.7% of cow milk.This may be attributed to differences in the management practices .

Current PCR results revealed that, uniplex PCR results for *nu c*gene showed that 31/82 (37.8%) of the examined isolates were positive for *nuc* gene. Detection of*nuc*gene by PCR was discussed by several authors as by(18) and, (33).Current PCR results for super-antigen toxin genes revealed that

sea produced by 5 (38.5)%) cows isolates .Both women and buffaloes *milk isolates* produced *seb* in a percentage of 55.5% (5 isolates for each),*sec* was not produced by women isolates and appeared in cows and buffaloes (7.7 and 11.1% respectively)

The present results were supported by other previous studies as (18) who reported that *sea* produced by 5/11(45.45%) clinical samples, *sec* and *sed*produced by4/11(36.36%) samples, *seb*and *see* not produced by any strains.

Detection ofstaphylococcal enterotoxins by PCR was discussed by several authors as by Adwan et al.(34) who reported that, out of the 100 *S. aureus* isolates (milk sheep origin= 52, milk cows origin=48) tested for SE-genes by PCR method, 37% were positive. None of these isolates carried more than one toxin gene. The majority of these positive toxingene isolates 20 (54.1%) were *seb* positive andthese results disagreed with the results reported by, (35) at which the multiplex PCR test revealed that enterotoxin A found in 58.3% of *S.Aureus* isolates, both enterotoxins B and C was found in 66.7% of the isolates and enterotoxin Dwas the predominant one found in 75% of the isolates.

The results of PCR for methicillin resistant *S. aureus* showed that31/82 (37.8%)of the examined isolates werecarrying *mec*A gene (MRSA)). Methicillin resistant *S. aureus*by multiplex PCR was also detected by several authors as by Bakeet and Darwish (36);18);Okeand Adewale (37);Mulders et al. (38);.In contrast withWong boot. *et al*[18] who found that11/11(100%) of the examined isolates were carrying *me cA* gene (MRSA). In conclusion the obtained results revealed that contamination of milk with*S. Aureus* which may lead to undesirable changes that render them unfit for human consumption and indicate unpersonal hygiene and unsanitary conditions during processing and handling. Also, MRSA are widely spread among isolates and has been reported to negatively affect the treatment of its associated infections in human and animals.

الكشف الجرثومي والجزيئي لجرثومة *Staphylococcus aureus* و المستضدات الفائقه لذيفاناتها في حليب النساء والابقار والجاموس

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الخلاصة

فحصت 300 عينه عشوائيه من حليب النساء والابقار والجاموس (100 عينه كلا منها) بواسطة الاختبارات البكتريولوجيه التقليديه والمصليه والجزيئيه وذلك للتاكد من وجود جرثومة *Staphylococcus aureus*ومستضداتها الفائقه وعلاقتها بالمقاومه للمثسلين .لوحظت النتائج الموجبه للعزل الجرثومي في 82(**27.3**%.)عية حليب شملت (37%) 37 عينه للابقار و(30%) 30 عينه للنساء و (15%) 15عينه للجاموس(15%). باستعمال اختبارlatex agglutination اظهرت جميع عزلات جرثومة *Staphylococcus aureus* نتائج موجبه (n=15, 100%)تلتها عزلات النساء(n=25,**83.1**%) والابقار (n=30,**81.1**%) مع نسبه مئويه كليه بلغت**23.3**% (**n=70**.).

كشفت نتائج تضخيم الجين (*nuc*) الخاص بنوع جرثومة *Staphylococcus aureus* والجينات الخاصه بالمستضديه الفائقه لذيفاناتها(*sea, seb and sec*) وجين (*mec*A) مقاومتها للمثسلين بواسطة الPCR ان(**27.3**%) **82**/300 عينة من عسنات الحليب الكليه المفحوصه كانت موجبه لكل من جين النوع*nuc* وجين المقاومه*mec*A بينما اظهرت (38.5)% 5عزلات من حليب الابقار نتائج موجبه للجين*sea* وان5عزلات من حليب كلا من النساء والابقار نتائج موجبه للجين *seb* وبنسبه مئويه بلغت55.5%.لم الجين الجين*sec* في عزلات حليب النساء بينما طهر هذا الجين في عزلات حليب الابقار والجاموس وبنسب مئويه بلغت 7.7 % و11.1% على التوالي.استخدم التتابع الجيني ومقارنة نتائجه حسب التحليل المقارن BLAST لزيادة التاكيد من

تشخيص جرثومة*S. aureus* حيث كشفت نتائج هذا التحليل عن وجود نسبة تشابهه بمقدار100% بين التتابع الجزئي للجين*nuc* للعزلة المحليه*S. Aureus* والعلومات المسجله للعترتين

S.aureus(strain 08-02300, coStaphylococcusaureusmplete genomeوStaphylococcus aureus isolate 22_LA_562 genome assembly, chromosome: I

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