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Prevalence of coagulase positive pathogenic *Staphylococcus aureus* in milk and dairy products

Radwa H.Negm[1], Ashraf A. Abd El Tawab [2] ,Ahmed S. Alfayomy [3], Doaa M.Sharaf[4], and Marwa A.Saad [5]

[1] Bacteriology, Immunology and Mycology Department Faculty Veterinary Medicine, Sadat city University, Egypt. [2] Bacteriology, Immunology and Mycology Department Faculty of Veterinary Medicine, Benha University, Egypt. [3] Anatomy Department Faculty of Veterinary Medicine Shebin El-com Menofia University, Egypt. [4] Animal Health Research Institute, Tanta Branch, Egypt. [5] Food Hygiene and control Shebin El-com Menofia University, Egypt

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*Correspondence to
e-mail@e-mail.com

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ABSTRACT

Coagulase positive *Staphylococcus aureus* (*S. aureus*) is one of the most common etiological pathogens, causing intramammary infection in dairy herds leading to severe economic losses in dairy industry,So this study aimed for the sampling, isolation ,biochemical and genotypic characterization of coagulase positive *Staphylococcus aureus* from raw milk (cow and buffalo)and dairy products . A total of 100 random samples of raw milk(cow and buffalo), kareish cheese, baladi yogurt (25 of each) were collected from different shops and supermarkets , all samples examined bacteriologically for characterization of coagulase positive *Staphylococcus aureus.* A total of 52 isolates of *S.aureus* was recovered 21/25 (84%), 17/25 (68%), 8/25 (32%), 6/25 (24%)from raw cow milk, raw buffalo milk, kareish cheese, baladi yogurt , respectively, then the positive isolates examined for their coagulase activity and for their resistance to different types of antibiotics and results of some strains were highly resistant to penicillin (80%) followed by clindamycin (60%)

, erythromycin (53%) and other strains were highly sensitive for gentamicin(100%) ,norfloxacin (93%) ,vancomycin (86%) and linezolid (60%). Then Polymerase chain reaction (PCR) was applied on positive strains of *S.aureus* to detect their virulence genes *staphylococcal* protein A (*SpA)* and coagulase gene (*coa*) which were detected with percentage (100%)of tested isolates, and beta- lactam resistance gene (*bla*Z) was found in (33%) of examined *s.aureus* strains.

1.INTRODUCTION

Staphylococcus aureus is one of the most common facultative pathogenic bacterium that has long been recognized as a challenge in both human and veterinary medicine[1] , organism of bovine mastitis ,which is a common ,complicated and economically unbearable disease in dairy animals worldwide. *Staphylococci* can have a wide variety of virulence factors which allow the bacteria to avoid the immune system and contribute to increased severity of infections. Most of these factors have been initially founded in *S. aureus* and include surface proteins (Protein A, clumping factor, fibronectin binding proteins or iron regulated surface determinants), capsular polysaccharides, molecules involved in biofilm formation (for example polysaccharide intercellular adhesin) or toxins (pore-forming toxins, toxins that act as superantigens). Cell wall adhesins that recognize extracellular matrix proteins and Some enzymes (coagulase, staphylokinase and proteases) also contribute to immune evasion and host tissue penetration [3],[4],[5],[6].

Coagulase substance is seen as the fundamental danger factor that coagulation plasma and coats the bacterial cell, so prevent phagocytosis[7]through enables staphylococci to got into a fibrin meshwork, spread and go against opsonophagocytic instrument of host safe cells [8]. Production of coagulase is a crucial phenotypic feature and the major determinant factor for identifying *S. aureus* strains, the variability at the 3′ end coding region of the *coa* gene has been used for genotyping of *S. aureus* strains from humans and animals [9]. Staphylococcal protein A (spA) is a layer bound exoprotein of bacterial cell wall that consider a critical destructiveness factor which ruin opsonization by serum supplement and phagocytosis of Polymorphonuclear leukocytes through confining to FC locale of immunoglobulins[10]. *S.aureus* cultivated a high protected against a wide grouping of hostile to contamination specialists which increase their danger and inconvenience in treatment [11]. The most important antibiotic resistant genes of *S.aureus* strains was *blaZ* gene which coded for *β*-lactamase important virulence factor that impair

opsonization by serum complement and phagocytosis of Polymorphonuclear leukocytes through binding to FC region of immunoglobulins[12].

Presence of *S. aureus* in milk and dairy items even in low numbers has been viewed as a general wellbeing danger since it has been laid out that *Staphylococcus* enterotoxins continue for longer lengths in the polluted milk and dairy items, even after the microorganisms lose its viability[13].

Improper food handling either in the home or food industry constitutes a major factor leading to *Staphylococcus* food poisoning outbreaks. Only few outbreaks can be traced directly to contamination during food processing [14].

The proper heat treatment followed by the refrigeration can minimize the chance of contamination with *S. aureus*. In our country it is commonly noticed that during heat treatment of milk, the temperature not rise up to the boiling point many a time or even if it reaches, consumers do not boil it enough. [15].

1. MATERIALS AND METHODS

2.1 Samples collection

A total of 100 random samples of raw cow milk, raw buffalo milk, kareish cheese, baladi yogurt (25 of each) were collected from different shops and supermarkets at Menofia and Gharbia Governorate **.** The collected samples were transferred directly to the animal health research laboratory (Tanta branch) in an ice box under complete aseptic conditions for bacteriological examination .

2.2. Bacteriological examination[16],[17].

Samples were homogenized in 0.1% peptone water, maintained for 1 h at 25 ◦C then samples were pre-enriched into nutrient broth and incubated at 37˚C for 24 hrs. It was used for the growth and propagation of isolates before platting under aerobic condition. A loopful from incubated nutrient broth was streaked on Baired parker agar plates (oxoid) and incubated at 37oC for 24-48 hrs Positive samples (showed black shiny colonies with clear halo zone around colonies and opaque zone of precipitation). Also, a loopful from incubated nutrient broth was streaked on Mannitol salt agar (Oxoid) and incubated at 37 oC for 24 -48 hrs Positive samples (yellow colonies and turned media to colorless). These colonies were kept in Brain Heart Infusion broth for biochemical identification and PCR examination

2.2.1. Morphological examination [18].

The suspected *s.aureus* isolates were stained by Gram stain for morphological examination .

2.2.2. Biochemical identification [19].

Indol test, catalase test , urease test, oxidase test ,coagulase test and β – hemolysis test .

2.2.3. Antibiotic sensitivity test:

The obtained bacterial isolates were tested in vitro for their susceptibility to the following antimicrobial discs: pencillin (P) 10 IU, gentamicin (CN) 10 mcg, erythromycin (E) 15 mcg, vancomycin (VA) 30 ml , clindamycin(DA) 2 mcg, norfloxacin (NOR) 10

mcg and linezolid (LNZ) 30 mcg. According to [20] and the level of sensitivitywas deciphered agreeing [21].

2.2.4. Molecular detection of Coagulase Positive *S.aureus* :

DNA was taken out from the bound S.aureus using QIAamp DNA limited scope unit. It was applied on 6 sporadic limits. PCR Master Mix and cycling conditions of the foundations during PCR was prepared according to Emerald Amp GT PCR mastermix (Takara) pack. Oligonucleotide foundations used in PCR have express gathering and improve a specific thing as shown in (table, 1) . DNA tests were upgraded in an amount of 25µl as follows: 12.5 µl of Emerald Amp GT PCR expert mix, 1 µl of each and every preparation of 20 pmol centers, 4.5 µl of water and 6 µl of format DNA. The reaction was acted in a Biometra warm cycler. Temperature and time conditions of the starters during PCR were applied .Aliquots of upgraded PCR things were electrophoresed in 1.5 % agarose gel (ABgene) in 1x TBE pad at room temperature. For gel assessment, 15 µl of PCR things were stacked in each gel opening . A 100 bp DNA ladder (QIAGEN Inc, Valencia, CA, USA) was used to conclude the piece sizes. The gel was caught by a gel documentation structure and the data was inspected through PC programming.

Table (1): Sequence and amplicon size of primers used for detection *S. aureus* virulence and resistance genes :

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Target gene	Sequence	Amplified product	Reference
S. aureus 23S rRNA	AC GGAGTTACAAAGGACGAC	1250 bp	**[22]**
	AGCTCAGCCTTAACGAGTAC		
Coa	ATA GAG ATG CTG GTA CAG G	Four different types of bands may be detected 350 bp 430 bp 570 bp 630 bp	**[23]**
	GCT TCC GAT TGT TCG ATG C		
Spa	TCA ACA AAG AAC AAC AAA ATG C	226 bp	**[24]**
	GCT TTC GGT GCT TGA GAT TC		
blaZ	TACAACTGTAATATCGGAGGG	833 bp	**[25]**
	CATTACACTCTTGGCGGTTTC		

Table (2): Cycling conditions of the primers during cPCR:

Target gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
S. aureus 23S rRNA	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 1.2 min.	35	72˚C 12 min.
Coa	94˚C	94˚C	55˚C	72˚C	35	72˚C

	5 min.	30 sec.	40 sec.	45 sec.		10 min.
Spa	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.
blaZ	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec	72˚C 50 sec	35	72˚C 10 in.

3.RESULTS

3.1. Incidence of *S. aureus* isolated from examined samples.

1. A total of 52 isolates of *S. aureus* were recovered from 100 Samples represented as 21/25 (84%), 17/25 (68%), 8/25 (32%), 6/25 (24%) from of raw cow milk, raw buffalo milk, kareish cheese, baladi yogurt , respectively, (Table 3).

2. **Table (3): Incidence *S. aureus* isolated from examined samples:**

Samples	Number of examined samples	Number of positive samples	% of positive samples
Raw cow milk	25	21	84%
Raw buffalo milk	25	17	68%
Kareish cheese	25	8	32%
Baladi yogurt	25	6	24%
Total No	100	52	52%

2.3.Phenotypic Identification of *Staphylococcus aureus*:

In this study *Staphylococcus aureus* were confirmed through Gram staining, *S.aureus* is non-motile facultative anaerobic Gram-positive cocci.graps likr cluster (Figure 1).

The isolated strains were cultured, on Baired-Parker agar: showed black shiny colonies with clear halo zone around colonies and opaque zone of precipitation figure (2), on Mannitol salt agar: showed (yellow colonies and turned media to colorless) figure (3).

Biochemically, Negative results were recorded on Indol test, Oxidase test and positive results were recorded on coagulase test (fig 4) and β – hemolysis test as show in (fig5)

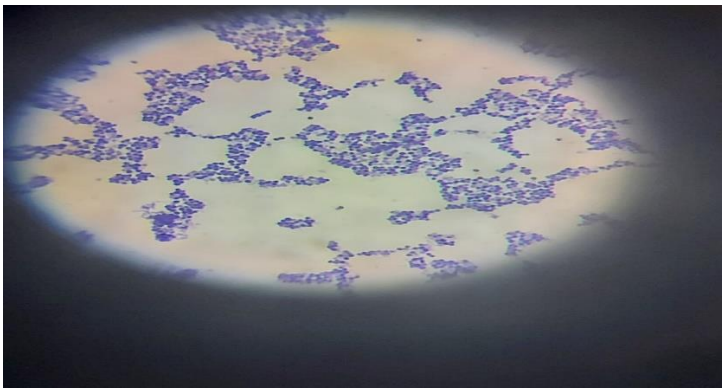

Fig (1) *S.aureus* under microscope after Gram‘s staining appear as grape like cluster

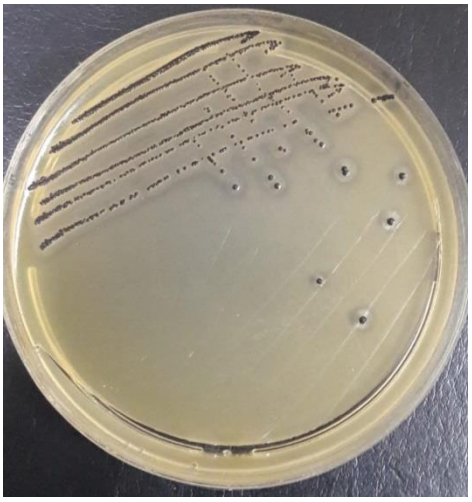

Fig (2) Colonies of *S.aureus* on baired parker agar media.

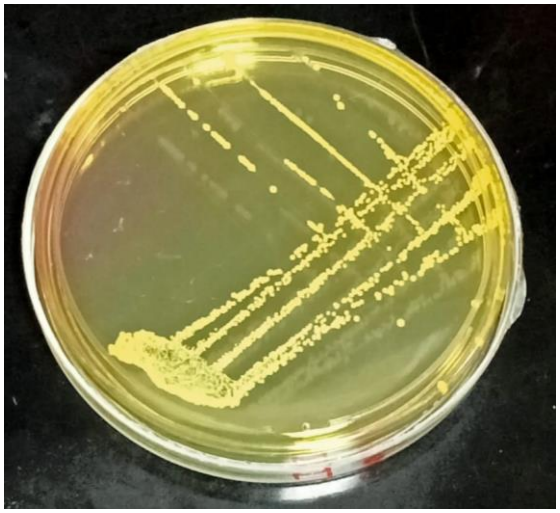

Fig (3) *S.aureus* on mannitol salt agar.

3.3.Prevalence of Coagulase-positive

Samples that showed phenotypically positive growth on Baird–Parker medium investigated for their coagulase activity ,The prevalence of Coagulase-positive *S.aureus* was 14/21(66%) ,7/17(41%) ,6/8(75%) ,3/6(50%) from raw cow milk, raw buffalo milk, kareish cheese, baladi yogurt , respectively.

Table(4) :results of coagulase test:

samples	No, of examined samples	No. of s.aureus isolates	No. of coagulase positive isolates	% of coagulase positive isolates	No. of coagulase negative isolates	% of coagulase negative isolates
Raw cow milk	**25**	**1**	**4**	**66%**	**7**	**34%**
Raw buffalo milk	**25**	**7**		**41%**	**10**	**59%**
Kareish cheese	**25**			**75%**	**2**	**25%**
Baladi yogurt	**25**			**50%**	**3**	**50%**
Total	**100**	**2**	**0**	**–**	**22**	**–**

Fig (4) coagulase test show that clot form when bacterial cell are incubated with plasma.

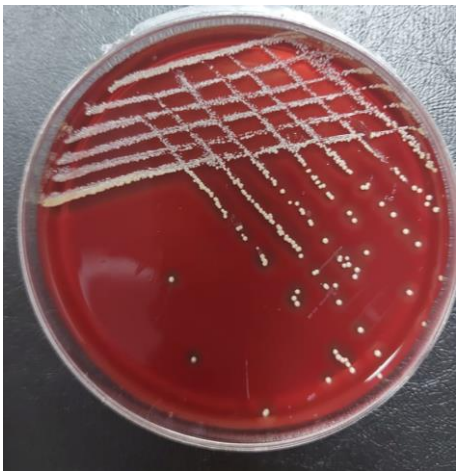

Fig (5) *S.aureus* showed β – hemolysis on blood agar media.

3.4. Antibiotic sensitivity test results:

Based on CLSI breakpoints, Results of antibiotic sensitivity test on 15 isolates of *S. aureus* recovered from raw cow milk, raw buffalo milk, kareish cheese, baladi yogurt were highly resistant for penicillin (80%) followed by clindamycin (60%) , erythromycin (53%) and highly sensitive for gentamicin(100%) ,norfloxacin (93%) ,vancomycin (86%) and linezolid(60%). Figure (6).

Fig (6) Antibiotic sensitivity test of *S.aureus*.

Table (5): Invitro anti-microbial sensitivity test for isolated *S.aureus*

Antimicrobial disc	Code	Disk concentration	Sensitive		Intermediate		Resistant		AA
			No.	%	No.	%	No.	%	
Penicillin	P	10 IU	3	20	0	0.0	12	80	R
Clindamycin	DA	20 µg	6	40	0	0.0	9	60	R
Linezolid	LNZ	30 µg	9	60	0	0.0	6	40	S
Erythromycin	E	15 µg	6	40	1	6.6	8	53	R
Gentamicin	CN	10 µg	15	100	0	0.0	0	0.0	S
Vancomycin	VA	30 µg	13	86	0	0.0	2	14	S
Norfloxacin	NOR	10 µg	14	93	1	6.6	0	0.0	S

AA: Antibiogram activity

% Percentage in relation to isolates of *S. aureus* no=15.

3.5. PCR results.

Detection of virulence and resistant genes of *S.aureus.* PCR using primers fragments for detection of two virulence genes ,These genes were *staphylococcal spA* and coagulase (*coa*). It was applied on six isolates of *S. aureus*. all tested isolates harbored these genes(100%),Also these isolates were tested for detection of one resistance gene beta- lactam resistance gene (*bla*Z) by uniplex PCR.the results revealed that (33%)of tested isolates harbored this gene **.**

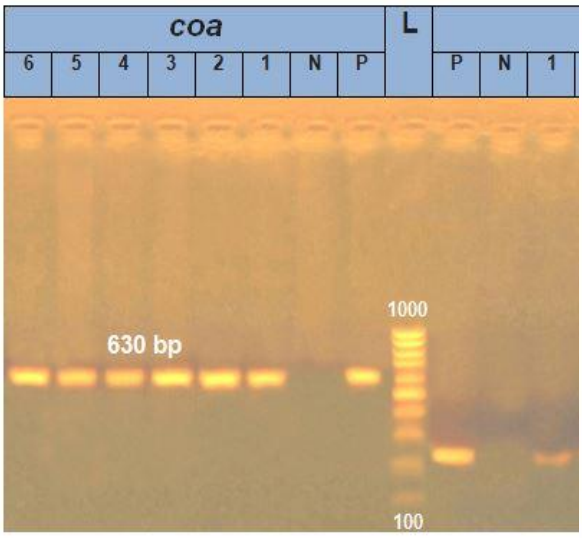

Fig (7): Agarose gel electrophoresis pattern of PCR for detection of s*taphylococcal* spa and coagulase (coa) genes at226 and 630bp respectively.

L: Ladder from 100 bp to 1000 bp

Pos: Positive control: *S.aureus* ATCC 25923.

Neg: Negative control: Field withdraw that were attempted and attested to be negative by PCR for the associated characteristics in R.L.Q.P

Way 1, 2, 3, 4, 5,6: positive upgrade of staphylococcal virulance characteristics (spa and coagulase characteristics).

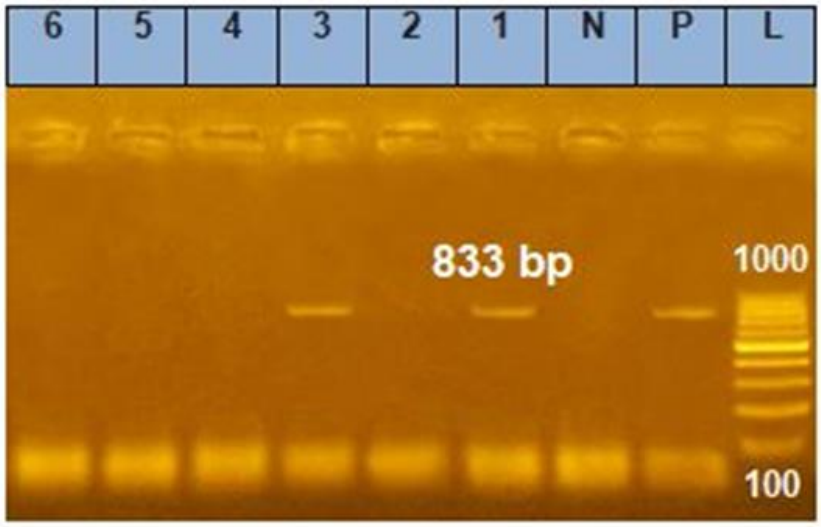

Fig. (8): Agarose gel electrophoresis pattern of PCR for detection of *blaZ* gene of *S. aureus* at 833 bp.

L: Stepping stool from 100 bp to 1000 bp.

Pos: Positive control: *S.aureus* ATCC 25923.

Neg: Negative control: Field confine that were tried and affirmed to be negative by PCR for the connected qualities in R.L.Q.P

Path 1, 3: Positive enhancement of blaZ quality at 833 bp

4.DISCUSSION

S. aureus can convey heat stable enterotoxins achieving food intoxication in human with fluctuating power El-Makarem and Amer[26] along these lines, the ongoing audit was planned to perceive and depict coagulase positive S. aureus.

In this study 52 bounds of *S. aureus* (52 %) were recovered from 100 models from unrefined milk and its things, which nearly agreed with the eventual outcomes of Lílian et al., (2011) who reported higher event of *S.aureus* in rough milk (68%) But it went against Ahmadi [27] and El-Sayed [29] who bound *S.aureus* at recurrence rate (14%). Moreover,this is higher prevalence at whatever point diverged from results of Zeinhom [30] who uncovered that *S. aureus* was isolated from the rough milk tests with an overall inescapability of 12.5% (25/200) and saw that as 13% of unrefined milk tests outperformed beyond what many would consider possible as demonstrated by the Egyptian Standards [31].

The high incidence in raw milk could be attributed to environmental pollution, crosscontamination between the milk and each other and poor handling during transportation or in milk collection centers, besides, shedding of *S. aureus* from infected animals is another cause of contamination of milk and dairy food.

Positive isolates tested for their coagulase activity and the results were 56% ,28%,24%,12% for raw cow milk, raw buffalo milk, kareish cheese and baladi yogurt respectively.

According to microbiological standards set out by the Egyptian authority, w cheese must be free from *S. aureus* .

In this study the regularity of *S. aureus* in Karish chese was (32%).which nearly agreed with the eventual outcomes of Mousa [32] and Elmaghraby [33] who nitty gritty that inescapability of 30% and 24% in Karish cheese in Egypt. these results were seen as low when appeared differently in relation to other Egyptian examinations of El - Malt [15] and Sallam [34] that uncovered S. aureus transcendence speeds of 93% and 90% from Karish cheese .

Variable reported incidence rates could be influenced by many factors such as different techniques of cheese production, storage conditions, type of cheese either manufactured from raw or pasteurized milk, and also related to personal hygiene of workers and unhygienic measures during production.

The bacterium (*S. aureus*) is liable to inactivation and elimination by heating, but as the cheese manufacturer in Egypt use raw milk which is most likely not subject to heating [35].

The prevalence of *s.aureus* in baladi yogurt was 24% then the positive isolates tested for coagulase activity and the coagulase positive isolates was 12% .

This consider low recurrence when diverged from delayed consequences of [36]

who reported that % of positive *s.aureus* isolates was 72% and 48% for plain and fruit yogurt respectively with Prevalence of Coagulase-positive *S. aureus* in the examined fermented dairy product samples 35% and 20% for plain and fruit yogurt respectively.

The spread of antibiotic-resistant pathogens continues to challenge sustainable treatment options, with severe public health consequences.

The most common mechanism of resistance to antibiotics of coagulase-positive *staphylococci* is the synthesis of beta lactamases that inactivate penicillinase sensitive antibiotics. Resistance to beta-lactam antibiotics in coagulase- positive staphylococci is increasing and following resistance in human isolates. Some studies show that penicillin disk diffusion method showes deviation in the case of sensitive beta-lactam antibiotics in relation to the dilution method. [37].

Fifteen *S. aureus* detaches were moreover investigated for their shortcoming to antimicrobials ,concerning the results of serum poison weakness testing, by far most of *S.aureus* strains separated were impenetrable to penicillin G (80%),clindamycin(60%) ,erythromycin (53%) sensitive showed most raised responsiveness towards gentamicin (100%) ,followed by norfloxacin (93%) ,vancomycin (80%) and linezolid (60%) .

higher security from penicillin scraped by Abera [38] and Shi [39] in a degree of (94.4%) and (87.6%) independently.

Practically identical results got by Begum [40] who revealed that S. aureus was 82.86% and 37.14% impenetrable to Penicillin-G and Amoxicillin, independently;

In like manner nearly to eventual outcomes of Abdeen [41] The MRSA strains showed assurance from penicillin (97.1%), cefoxitin (85.7%), and erythromycin (52.8%)

The development of PCR-based methods provides a promising option for the rapid identification of bacteria. With this method, identification of bacterial pathogens can be made in hours, rather than days, as conventional cultural methods require. PCR can also improve the level of detection due to its high sensitivity. Theoretically, only a few cells of pathogen are necessary to yield a positive diagnosis [42]. The molecular identification of the *coa* gene in *S. aureus* strains was performed through amplification of the *coa* gene at (630) bp. The use of the coa gene to detect *S. aureus* strains from milk origin was previously reported in two studies o [15] and [43] which improved the coa quality from mastitic milk and dairy things at 630 and

750 bp. Moreover, Javid [44] perceived the coa quality at 514 bp, 595 bp, 757 bp, and 802 bp.

The findings of the present investigation showed that 100% the examined *S. aureus* isolates (6/6) harbored *coa* gene. Similar result obtained by [37]

who reported that detection of the *coa* gene from milk samples could help to assess the microbiological safety of raw milk intended for direct use in the dairy industry. The *coa* gene was detected in all isolates with the primers used, even though one of them was considered coagulase-negative both by the tube coagulase test and by the clumping factor assay.

The *SpA* gene is a major important surface protein of bacterial cell wall, which binds with FC region of immunoglobulin G, so the decreasing in *SpA* on cell surface of *S. aureus* resulted in increasing number of free receptor sites for complement and enhance phagocytosis [45].

In the ongoing audit, 6/6(100%) of attempted *s.aureus* withdraws clutched SpA quality and it upgraded at 226 bp that like the examination of [43] that showed strengthening of SpA nature of *S.aureus* at 229 bp.and El-Sayed [29] who recognized SpA quality Positive in 90% of isolates and heightened at 226 bp).

[46] reported that *SpA* gene can be used for typing the isolates of *S.aureus*. in addition to [45] point by point that the revelation of genetic polymorphisms in the X district of the SpA quality can be used for making out of *S. aureus.* In like manner Karahan [47] gathered that ID of SpA quality polymorphisms with Coa-PCR is proposed as a fair illustrative method for making out of *S. aureus* limits which gave critical results to practical control of staphylococcal mastitis.

Practically like eventual outcomes of El-Sayed [29] who declared that Spa quality and Coa quality were perceived in (90%) and (80%) of the attempted *S.aureus* strains

The resistance to β-lactam antibiotics occur through hydrolyzing the β-lactam ring and convert to inactive form, so the application of PCR for detection of *blaZ* gene is recommended in veterinary laboratories for detection of the resistant strains of *S. aureus* [48] . In this study, 2/6 (33,3%) limits of *S. aureus* presented to PCR were positive for blaZ quality and gave a single amplicon at 833 bp. At any rate special result got by [49] and [43] recognized a singular amplicon of 173 bp of blaZ quality. [50]

noticed that all positive strains to *mecA* were also positive for gene and the presence of both genes was correlated to phenotypic beta-lactam resistance of S. aureus strains .

In this study, 2\6 of tested *S. aureus* isolates harbored *blaz* gene which disagreed with the result of [51]

who founnd that all S. aureus isolates harbored *blaZ* gene associated with penicillin

5.CONCLUSIONS

Staphylococcal food poisoning is of major concern in public health programs worldwide. S. aureus may be present in milk and milk products as a result of milk collected from the animal suffering from disease condition and excreting S. aureus in milk or due to unhygienic conditions during production, processing, storage and handling of milk products, which are the main causes of food borne diseases. Results clearly indicated that milk and milkbased products available in the market were contaminated with S. aureus, posing a high risk of food poisoning. Thus more hygienic preventive measures are required to reduce the bacterial contamination, so as to increase the wholesomeness of these milk and milk based products.

Study concluded that S. aureus was isolated from milk and milk products, and raw milk was highly contaminated with the bacterium than fermented milk products (cheese and yoghurt). However, none of the dairy products was devoid of the isolates.

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Authors' contributions.
K.M.S: Conceptualization, Formal Analysis, Investigation, Supervision, Resources, Writing – original draft
A.R.S: Data collection, Formal Analysis, Project administration, Resources, Writing – review and editing.
M.M.Z: Conceptualization, Data curation, Formal Analysis, Resources, Supervision, , Writing – review and editing.

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