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Phenotypic and Genotypic Identification of Typical and Atypical *Staphylococcus aureus* Isolated From Bovine Milk Samples

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Abstract

This study aimed to determine the phenotypic and genotypic identification and differentiation of typical coagulase positive *Staphylococcus aureus* (CPSA) and atypical coagulase negative *Staphylococcus aureus* (CNSA) from other Staphylococci isolated form bovine milk. Out of 111 milk samples, 67 Staphylococcus strains were isolated, phenotypically, based on resistance to Acriflavine; *Staphylococcus aureus* (S. aureus) was detected in 44 isolates of them (30 CPSA strains and 14 CNSA strains). Twenty five from CPSA and 5 from CNSA isolates were positive for slime production on Congo red agar plates. Genotypically, the all five tested typical *Staphylococcus aureus* (CPSA) as well as three atypical *Staphylococcus aureus* (CNSA) strains encoded all investigated genes (*nuc, spa-x* and *clfA*), while the other two atypical CNSA strains, one encoded only nuc gene and the other encoded both *nuc* and *clfA* genes. Acriflavine sensitivity test must be included in the routine of phenotypic identification *S. aureus* as the gold method together with tube coagulase test. PCR analysis is most important confirmation method by detection of *nuc* gene. Genotypically both typical and atypical *S. aureus* isolates are virulent. Typical and atypical *S. aureus* isolated from isolated from bovine milk samples sold in dairy shops were with higher percentage than subclinical mastitic milk samples threating public health hazard. Attention must be paid toward detection and identification of atypical tube coagulase negative *S. aureus* strains.

Keywords: *Typical Staph aureus; Atypical Staph aureus*; Phenotypic; Genotypic; Bovine Milk

Introduction

Staphylococcus aureus (*S. aureus*) is one of the most significant pathogens responsible for contagious mastitis in dairy cattle [1-3]. Also, different food can act as good medium for *S. aureus* such as raw milk and dairy products causing food poisoning [1]. The pathogenicity of *S. aureus* is related to a number of virulence factors that help it to adhere to surface, invade or avoid the immune system and cause harmful toxic effects to the host [4]. These factors include cell surface components e.g., protein A, fibronecting-binding protein, collagen-binding protein, and clumping factor [5]. Moreover, the ability of *S. aureus* to form biofilm is one of the virulence factors that facilitate the adherence and colonization of *Staphylococci spp.* on the mammary gland epithelium [6], result its contributing to the evasion of the immunological defenses and to the difficulty of pathogen eradication with antibiotics [7], leading to recurrent or

persistent infections [3,6].

As coagulase and clumping factor, A and B (*ClfA* and *ClfB*) are species-specific proteins of *S. aureus* were implicated in binding to fibrinogen [4] considered the major virulence factor of bovine mastitis pathogen [6]. The most important typical feature that differentiates the most pathogenic *S. aureus* from less pathogenic *Staphylococcal* strains is the ability to produce coagulase enzyme. Tube – coagulase test (TCT) is still the gold standard in identification of *S. aureus* in clinical laboratory, as *S. aureus* is mainly coagulase positive; but some may be with atypical behavior that does not produce coagulase enzyme [8-10], despite the presence of thermonuclease gene (*nuc* gene) [11]. Although atypical *S. aureus* is not routinely identified as an agent of bovine mastitis, certain studies reported that it may be an etiological agent of mastitis in cows [12], but still less subjected to study [11,13]. Attention must be paid toward accurate detection and identification of atypical tube coagulase negative *S. aureus* or atypical methicillin resistant *Staphylococcus aureus* (MRSA) [14].

Thus, this study aimed to identify CPSA and CNSA strains from other *Staphylococcus* spp. isolated from subclinical mastitic milk and bovine milk samples sold in dairy shops, determination of acriflavine sensitivity test of all *S. aureus* isolates as well as genotypic character of typical and atypical *S. aureus* using PCR for presence of species specific thermonuclease gene (*nuc* gene) and virulence genes as surface protein in X region of protein A (*spa* gene) and clumping factor A (*clfA* gene).

Material and Methods

Samples

One hundred and eleven milk samples were collected (56 bovine milk samples sold in dairy shops and 55 samples from cows & buffalos suffering from subclinical mastitis, Polled milk sample was collected from every lactating cow, the udders were washed and dried and the teat ends swabbed with 70% alcohol. The first stripping was discarded, and then approximately 10 mL of milk were collected from each quarter into sterile vials. Milk samples were tested by California mastitis test), according to Ollis, et al. (1995) [15]. All milk samples were immediately put in ice container and be chilled to avoid high temperature weather and then transported immediately to the laboratory.

II-Isolation and Phenotypic Identification of *Staphylococcus* Species

According to Quinn, [16] for enrichment only before culturing samples incubated for 6-12 hours at 37°C. Then, a loopful was incubated aerobically into trypticase soy broth with 10% Nacl at 37ºC/24 hours [17]. The incubated samples were cultured onto both nutrient agar medium and blood agar medium containing 7% of sheep blood and then were incubated aerobically at 37°C for 24-48 hours. Purified colonies were morphologically identified by the presence of hemolysis, size and pigmentation, Gram-stained smears, catalase test and tube coagulase test (TCT) with human plasma. Then, the purified colony from primary *Staphylococcal* isolates was streaked onto Mannitol salt agar (MSA), Baird-Parker agar base supplemented with egg yolk tellurite (BPET) only, and with acriflavine hydrochloride (7 µg/ml) (BPRA); [15-18]. Plates were incubated at 37ºC for 24-48 h.

Slime Production Assay

It was performed by cultivation of *S. aureus* strains on Congo Red Agar (CRA) medium consists of brain heart infusion broth 37 g/l, sucrose 50 g/l, agar 10 g/l, and Congo red dye 0.8 g/l. The Congo red dye was prepared as a concentrated aqueous solution and autoclaved separately at 121 ºC for 15 min and was added when the agar had cooled to 55°C. Plates were inoculated and incubated aerobically at 37°C for 24-48 h. Isolates that produced black colonies with dry crystalline consistency were regarded as slime positive, where as those showing pink colonies were slime negative [19].

PCR for Detection of *nuc, clfA* and *spa-x* Genes

Post phenotypically characterization of the isolated *S. aureus,* PCR procedures were performed on ten random selected *S. aureus* isolates (5 strains typical *S. aureus* s and other 5 strains atypical *S. aureus*) as follows:

DNA Extraction: DNA extraction from samples was performed using the QIA amp 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°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.

Oligonucleotide Primer: The primers used (supplied from Metabion - Germany) are listed in Table 1. For PCR amplification, primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), as 1 µl of each primer of 20 pmol concentrations, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in an applied bio-system 2720

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thermal cycler.

Analysis of the PCR Products: The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. A generuler 100 bp ladder (Fermentas, Germany) was used to determine the fragment sizes. The gel was stained ethidium bromide stain and photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software (automatic image capture proteinsimple formerly cell bioscience, USA) [20-22].

Target gene	Primers sequences	Amplified segment (bp)	Primary denatu ration	Secondary denatu ration	Anneal ing	Extension	Final extension	Reference
clfA	GCAAAATCCAGCACAACAGGAAACGA	638	94˚C	94˚C	55˚C	72˚C	72˚C	20
	CTTGATCTCCAGCCATAATTGGTGG		5 min.	30 sec.	40 sec.	45 sec.	10 min.	
nuc	ATATGTATGGCAATCGTTTCAAT	395	94˚C	94˚C	55˚C	72˚C	72˚C	21
	GTAAATGCACTTGCTTCAGGAC		5 min.	30 sec.	40 sec.	40 sec.	10 min.	
Spa (X region)	CAA GCA CCA AAA GAG GAA		94˚C	94˚C	60˚C	72˚C	72˚C	22
	CAC CAG GTT TAA CGA CAT	Variable	5 min.	30 sec.	40 sec.	40 sec.	10 min.	

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions.

Results and Discussion

S. aureus is a causative agent of mastitis in dairy cattle. When causing mastitis, it can also be found in milk, where high levels of contamination can be rapidly achieved if conditions are favorable [8]. The most important typical feature that differentiates the high pathogenic *S. aureus* from less pathogenic Staphylococcal strains is the ability to produce coagulase enzyme [23] which enzyme implicated to produce biofilm [24]. Tube-coagulase test that detects the free coagulase was the first standard and considered the gold test that differentiates *S. aureus* from other species of staphylococci [18], but some strains of *S. aureus* give negative reaction in TCT are more virulent effects so called atypical free coagulase – negative *S. aureus* strains causing subclinical or clinical forms of mastitis [12]. Lack of coagulase or clumping factor in some *S. aureus* strains is not result of genetic defect or absence but may be due to suppress of expression of coagulase or clumping factor gene [23], subsequently, atypical CNSA can be misidentified as clinically non important organism if diagnosis based only on tube coagulase test [25,26]. Phenotypically atypical MRSA found negative in the tube coagulase and latex coagulase [14].

In the present study, about the characterization phenotypic assays, out of 111 milk samples, 67 *Staphylococcus* strains were isolated (60.4%) showing Gram-positive cocci in clusters and catalase positive reaction, and based on TCT, 34 (50.74%) and 33 (49.25%) strains were coagulase positive staphylococci (CPS) and coagulase negative staphylococci (CNS), respectively, (Table 2). According to the results of TCT and the sensitivity of isolates to Acriflavine, out of 33 tube coagulase negative *Staphylococcal* strains, 14 (20.9%) were atypical coagulase negative *Staph*. *aureus* resistance to Acriflavine (CNSA), and out of 34 tube coagulase positive *Staphylococcal* strains, 30 (44.78%) were typical coagulase positive *Staph. aureus* resistance to Acriflavine (CPSA) as shown in Table 2. Former studies [10] detected *Staphylococcus* strains from individual milk samples and bulk tank milk as 80.9% and 100%, respectively and based on phenotypic identification of *S. aureus* from subclinical were 81.5% and 70% [10,27], respectively. Also, *S. aureus* was isolated from subclinical mastitic cattle and buffaloes were 80% and 72.73%, respectively [2]. *S. aureus* isolated as 45.6% from both clinical and subclinical mastitis [28]. While from raw milk *S. aureus* isolated was 56% [1].

Baird Parker agar contains sodium pyruvate to protect damaged cells and aid in their recovery and egg yolk emulsion as a diagnostic agent as well as Glycine and lithium chloride are the selective agents which suppress the growth of most bacteria, without inhibiting *S. aureus*. Potassium tellurite is reduced to form grey-black shiny colonies and the halo is a result of lipase activity and the clearing zone is due to proteolytic action [29,30]. Nonlipolytic (atypical) strains may be frequent in dairy products or milk samples coming from mastitic animals [12,29,30].

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Milk samples	Slime production from (colony colour)												
	Staph.sp. isolation on MSA and catalase test		Tube coagulase test			CPSA (typical S. aureus) Acriflavine resistant			CNSA (Atypical S. aureus) Acriflavine resistant			Other *Staph.sp.* isolation from	
	+ve	-ve	Total	Black colonies	Red colonies	Total	Black colonies	Red colonies	Total	Black colonies	Red colonies	Coagulase + ve	Coagulase - ve
Dairy shops (Bulk samples) -56	40	16	26	18	8	22	17	5	8	4	4	4	6
Subclinical mastitis milk (55)	27	28	8	7	1	8	8	0	6	1	5	0	13
Total (111)	67	44	34	25	9	30	25	5	14	5	9	4	19

Table 2: Phenotypic identification of Staphylococcal spp. isolates and their sensitivity to acriflavine.
CPSA: coagulase positive *S. aureus.* CNSA: coagulase negative *S. aureus*. Black colonies: Slime producers. Red colonies: Non slime producers. MSA: Mannitol Salt Agar.

The present finding achieved public hazard where dairy shop's milk revealed more prevalence as [22 (32.84%) & 8 (11.94%)], while subclinical mastitis milk showed [8 (11.94%) & 6 (8.96%)] of typical and atypical *S. aureus*, respectively Table 2. Since, the TCT is an essential test for diagnosis of *S. aureus*; the emergence of atypical coagulase negative *S. aureus* (CNSA) strains is a warning about the misdiagnosis which was detected in about 7.2 – 20% of all *S. aureus* isolated [18,23,25,31-33] but was detected with higher percentages as 58.6% [34]. In the present study those atypical free coagulase negative *S. aureus* (CNSA), on MSA media the colonies appeared mucoid yellow colour surrounded by yellow halo zone with yellow colour medium and on Baired Parker agar with egg yolk telluride (BPET), atypical *S. aureus* (CNSA) colonies were black, large in size and not surrounded by halo or clear zone, Figure 1, while typical *S. aureus* (CPSA) colonies on BPET medium were 1-1.5mm diameter, surrounded by a 2-5 mm halo zone Figure 2. Meanwhile, on Baired Parker agar with egg yolk telluride supplemented with Acriflavine hydrochloride (BPRA), both typical Figure 3&4A and atypical Figure 4B *S. aureus* strains were acriflavine resistance and had ability to be grown showing black colonies, but the other *Staph. spp.* can't grow, Figure 4. The same results were found also by da Silva, WP [8,18]. Both BPET and BPRA are effective media for suppressing or inhibiting most environmental organisms present in bulk tank milk without damage the recovery of *S. aureus* [15]. The sensitivity and specificity for detecting *S. aureus* in bulk tank milk were 94.8% and 100%, respectively, using Baird-Parker agar base supplemented with egg yolk tellurite emulsion and Acriflavine (BPRA) [15]. Acriflavine sensitivity test is the most be included in the phenotypic identification of both CPSA and CNSA where its result was the closest to that of mPCR assay results in confirmation of CPSA and CNSA through detection of thermonuclease gene (*nuc* gene) [10,18].

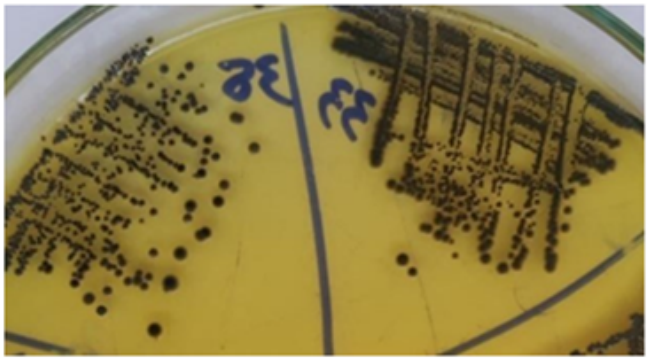

Figure 1: CNSA isolates on BPET showing large black colonies.

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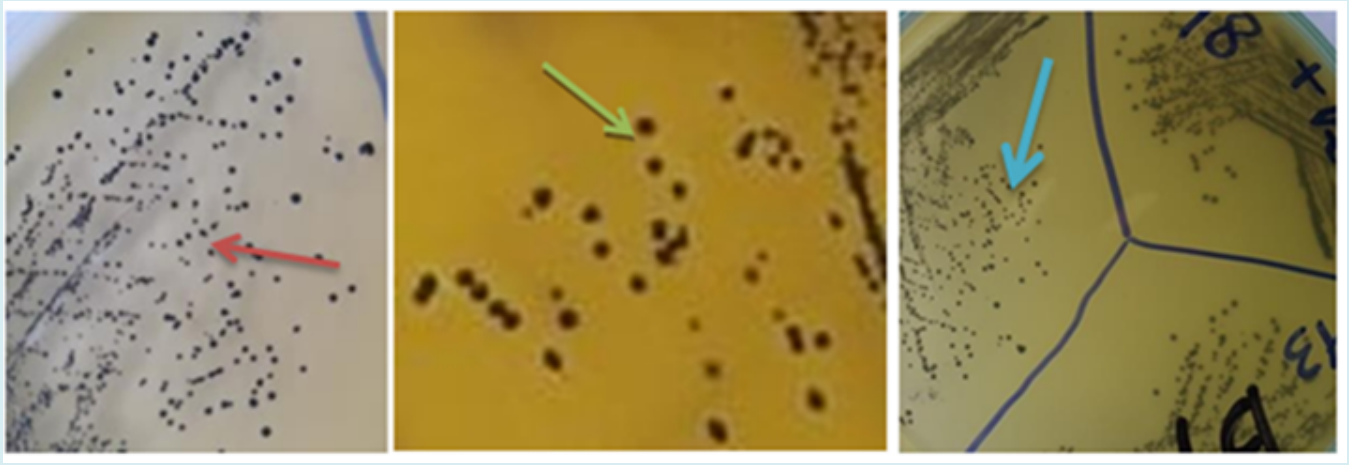

Figure 2: CPSA isolates on BPET showing black colonies surrounded with halo zone (arrows).

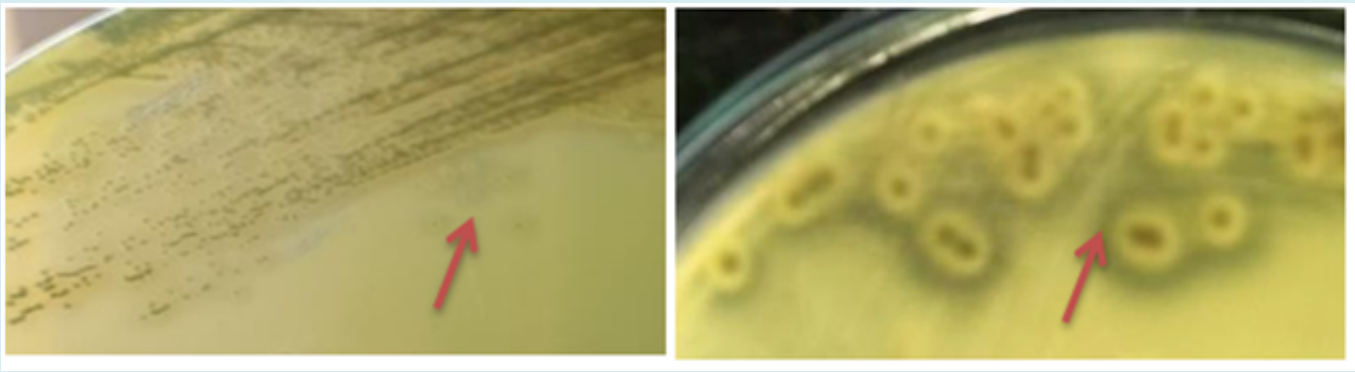

Figure 3: CPSA on BPET with acriflavine showing growing of black colonies with halo zone (arrows).

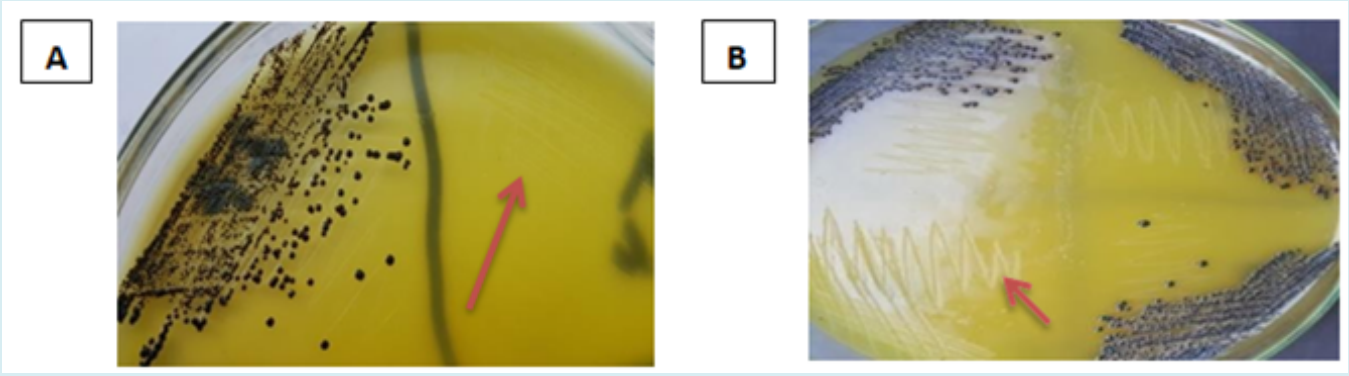

Figure 4: CPSA (A) and CNSA (B) on BPET with acriflavine showing growing of black colonies, while other staph. spp. can't grow (arrows).

Slime producing *S. aureus* strains are more resistant to antibiotics than non-slime producing strains [3,24], where coagulase enzyme is implicated in the formation of biofilm-like aggregates [24]. Biofilm formation, mediated by a polysaccharide intercellular adhesin (PIA) is considered to be an important virulence factor in *S. aureus*. The Congo red dye directly interacts with certain polysaccharides, forming colored complexes or more likely some metabolic changes of the dye to form a secondary product could play a more important part in the formation of dark colonies [35,36].

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About the biofilm production study, all over the present work, out of 67 *Staphylococcus* spp. strains, 34 were CPS and 33 were CNS, where on CRA 25 (37.3%) and 9 (13.4%) were in *vitro* positive biofilm producers, respectively according to the phenotype of the colonies on CRA. While among all *S. aureus* strains, out of the 30 CPSA and 14 CNSA isolates, 25 (37.3%) and 5 (7.46%) isolates were *in vitro* positive for slime production, respectively Table 2 and Figure 5 A&B.

Figure 5: CPSA and CNSA on CRD medium, slime producer (A) showing black colonies and non-slime producer (B) showing red colonies.

Slime-producing *S. aureus* isolates from different clinical origins such as bovine mastitis has been detected *in vitro* using Congo Red Agar plate in percentage as 60, 35.18, 37.2 and 91.4%, [3,37-39], respectively. Phenotypic characteristics can be defined for recognition *S. aureus*; but for precise identification it is necessary to use molecular methods for detection of *S. aureus* species-specific thermonuclease gene (*nuc* gene), since identification and differentiation of CNSA from other coagulase – negative *Staphylococci spp.* depends on detection *nuc* gene [10,18]. In present work, molecular identification of *S. aureus* a primer pairs for the gene (*nuc* gene) were designed for all the tested selected random 10 isolates of *S. aureus* (5 typical and 5 atypical *S. aureus* strains), the amplicon size of the examined gene (*nuc* gene) at 395 bp, where all those tested typical and atypical *S. aureus* strains encoded species-specific thermonuclease *nuc* gene confirmed (100%) the phenotypic characterization Table 3 & Figure 6. Some studies [26,331,32] detected *nuc* gene in all (100%) tested atypical *S. aureus strains, while nuc* gene was found among 97.3, 85% and 66.7 % of the *S. aureus* isolates; [28,40,41], respectively. Out of 35 *staphylococcus* strains, 16 isolates were possessed *nuc* gene specific of *S. aureus*, [18].

Phenotypically identified *S. aureus*		**Genotypically identified S. aureus**		
		nuc gene	***clfA gene***	***Spa-x region gene***
Typical (CPSA)	1	+	+	+
	2	+	+	+
	3	+	+	+
	4	+	+	+
	5	+	+	+
Atypical (CNSA)	6	+	+	+
	7	+	+	+
	8	+	+	-
	9	+	+	+
	10	+	-	-

Table 3: Genotypic characteristics and virulence related gene in both typical and atypical *S. aureus* isolates.

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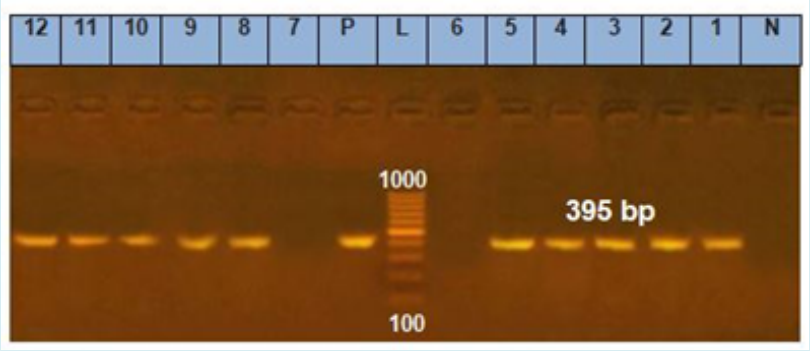

Figure 6: PCR assay for detection of *unc* gene in typical and atypical *S. aureus* isolates. Lane L: 100-1000bp DNA ladder. P: Positive control (at 395 bp) N: Negative control. Lanes: 1- 5 *nuc* gene positive typical *S. aureus* isolates. Lanes: 8-12 *nuc* gene positive atypical *S. aureus* isolates.

As *S. aureus* virulence clumping factor, A (*ClfA*) is one of microbial surface components recognizing adhesive matrix molecules (MSCRAMM) and the ability of intercellular adhesion and biofilm formation of *S. aureus* is contributed to clumping factors A through its ability to bind fibrinogen [42]. The current study was designed to assess the encoding of *ClfA* in the genotyping tested *S. aureus* strains besides the phenotypic biofilm production. The present finding of the two targeted virulence genes clumping factor (*clfA* gene) and surface protein in the X – region of protein A (*spa-X* region) were detected in all (100%) tested typical *S. aureus* strains, as well as *clfA* gene was detected in 4 out 5 isolates and *spa-X* region gene was detected in 3 out 5 of the investigated atypical *S. aureus* isolates (Table 3 and Figure 7&8).

The virulence clumping gene (*clfA*) in previous studies was detected in as 100 & 98 % [9,43] in atypical *S. aureus* tested strains respectively, while few studies [44,45] detected *clfA* in (33.3%) and (19.2%) of the *S. aureus* isolates, respectively. In contrast, a study [18] failed to detect *clfA* gene in all the atypical *S. aureus* isolates, negative reaction for clumping factor test attributed to block up the expression of this gene or too low concentration of the expressed protein or reduced availability on the bacterial surface [46,47], Protein *A* (*spa*) is a bacterial cell wall – associated molecule that binds immunoglobulin (IgG), impairing opsonisation and phagocytosis [48]. In the present work, the *spa-X* gene was detected in all 5 typical *S. aureus* isolates and three isolates out of 5 the atypical *S. aureus*, Table 3 and Figure 8. During previous studies [18,41,49], *spa* gene in X-region binding was detected in all typical and atypical *S. aureus* isolates, while it was detected in 26 (70.3%) *S. aureus* isolates [40]. Atypical *S. aureus* strains seem not to be differed in genetic ability to poses very virulence adhesins genes from typical *S. aureus* strains [46,50].

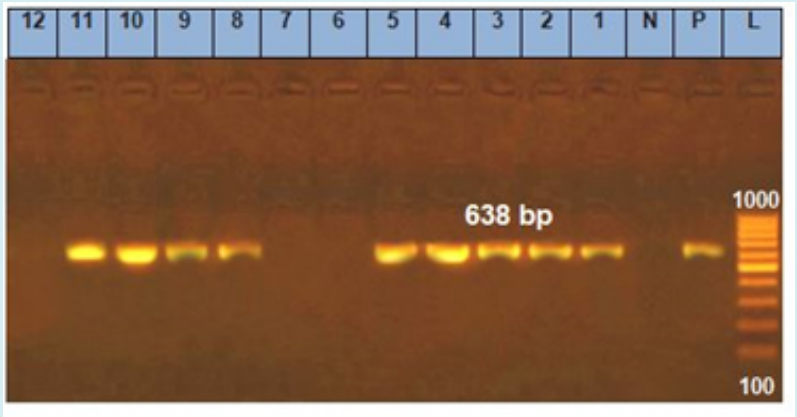

Figure 7: PCR assay for detection of *clfA* gene in typical and atypical *S. aureus* isolates. LAN L: 100-1000bp DNA ladder. P: Positive control (at 638) N: Negative control. Lanes: 1- 5, *clfA* gene positive typical *S. aureus* isolates. Lanes: 8, 9, 10 and 11 *clfA* gene positive atypical *S. aureus*. Lanes 12: *clfA* gene negative atypical *S. aureus* isolates.

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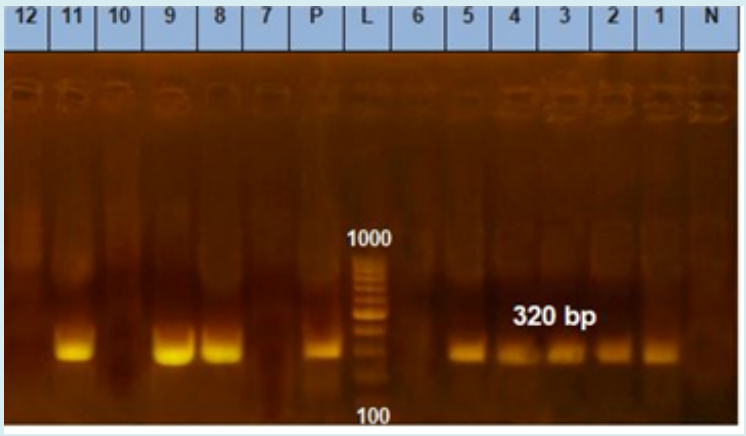

Figure 8: PCR assay for detection of protein A (*spa-X*) gene in typical and atypical *S. aureus* isolates. L: 100-1000 bp Ladder DNA marker. P: Positive control (at 320 bp) N: Negative control. Lanes: 1-5 *spa-X* gene typical *S. aureus* isolates positive isolates. Lines: 8,9,11 *spa-X* gene atypical *S. aureus* isolates positive isolates. Lines: 10 and 12 *spa-X* gene atypical *S. aureus* isolates negative isolates.

Conclusion

It was concluded that for accurate identification and differentiation of typical and atypical *S. aureus* from other Staphylococcal strains, acriflavine sensitivity test must be included in the scheme of phenotypic identification as the gold method together with TCT. PCR analysis is most important confirmation method by detection of thermonuclease gene (*nuc*- gene). Genotypically both typical and atypical *S. aureus* isolates are virulent.

Recommendation

Typical and atypical *S. aureus* isolated from milk resembled high incidence as 44.78% & 20.9% respectively, where dairy shop samples with higher percentage than subclinical mastitis threating public health hazard. So, Attention must be paid toward detection and identification of atypical tube coagulase negative *S. aureus* strains.

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