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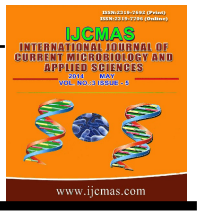

Original Research Article

Evaluation of Phenotypic Methods versus Molecular Methods for Differentiation of Coagulase Positive Staphylococci causing Bovine Mastitis with a Special Reference to atypical *Staphylococcus aureus*

Hanaa A.E. Asfour[1] and Samah F. Darwish[2]*

[1]Mastitis and Neonatal Diseases Department, Animal Reproduction Research Institute (ARRI), Giza, Egypt
[2]Biotechnology Research Unit, Animal Reproduction Research Institute (ARRI), Giza, Egypt
Corresponding author

Keywords

coagulase positive staphylococci-mPCR - atypical *S. aureus* - bovine mastitis

A B S T R A C T

The objective of this study was to determine the suitable phenotypic methods that could differentiate *Staphylococcus aureus* and other coagulase positive staphylococci (CPS). Therefore, 103 staphylococcus isolates were isolated from bovine mastitic milk. They were subjected to several conventional phenotypic tests versus molecular test. Based on phenotypic identification, 84 (81.5%), 8 (7.8%), 4 (3.9%) and 7 (6.8%) strains were identified as *S. aureus, S. intermedius, S. hyicus* and other staphylococci versus 84 (81.6%), 3 (2.9%), 2 (1.9%) and 14 (13.6%) strains identified by molecular methods, respectively. Some discrepancy between phenotypic and genotypic results was found and discussed briefly. Detection of 11 (55%) coagulase negative *S. aureus* versus 8 (40%) as identified by phenotypic and genotypic methods, respectively, was very surprising. This group of strains could be misidentified as coagulase negative staphylococci (CNS) if diagnosis relied only on tube coagulase test and PBA media. The most important tests which must be included in the phenotypic identification scheme of both CPS and coagulase negative *S. aureus* strains are acriflavine sensitivity, acetoin production, maltose and anaerobic mannitol fermentations and RPFA. Finally, when precise identification of CPS is required, numerous phenotypic tests must be adopted in addition to molecular based method. The multiplex PCR assay applied in this study was found to be an ideal way to differentiate CPS to the species level. Additionally, attention must be paid toward detection and identification of atypical tube coagulase negative *S. aureus* strains as a cause of bovine mastitis in dairy herds.

Introduction

Staphylococcus aureus is the major cause of bovine mastitis when compared to other species of the Staphyloccus genus (Vasudevan *et al*., 2003). However, accurate identification of this microorganism is not carried out by the

majority of laboratories. This is due to the high costs of commercial kits used for its identification. Moreover, correct discrimination requires the use of laborious and expensive procedures (Sasaki *et al*., 2007b; Costa *et al*., 2010 and Casanova *et al*., 2011). Therefore, the majority of veterinary laboratories are only able to differentiate isolates into CPS or CNS depending on results of tube coagulase test.

Identification of CPS is required to be performed accurately in veterinary clinical laboratories for two reasons. Firstly, the MIC breakpoints of some antibiotics differ with species. Therefore, such inadequate species identification could lead to inappropriate treatment decisions especially for methicillin-resistant staphylococcal infections (Pottumarthy *et al.*, 2004; Bemis *et al*., 2006; Sasaki *et al.*, 2007a). Secondly, improper species identification may decrease the importance of other CPS species including *S. hyicus, S. intermedius* and *S. pseudointermedius* as agents of bovine mastitis (Roberson *et al*., 1992).

Based on phenotypic differences, it is difficult to discriminate between CPS species because there is a lack of unique biochemical markers for species identification (Freney *et al*., 1999; Sasaki *et al*., 2007b). The routine method for *S. aureus, S. hyicus, S. intermedius* and *S. pseudointermedius* identification, involving inoculation in a selective-differential medium followed by confirmation with a coagulase test (Downes and ITO, 2001), is not sufficiently discriminate these four CPS species. Thus, a simple and precise method for discriminating among CPS species is highly required. Several authors have suggested the use of molecular methods as a valuable alternative to the traditionally used morphological and biochemical methods (Silva *et al*., 2003; Baron *et al*., 2004; Becker *et al*., 2005; Yang *et al*., 2007; Sasaki *et al*., 2010). Therefore, the main objective of this study was to determine the most suitable methods that could be used to differentiate between *S. aureus* and other coagulase positive strains including *S. hyicus, S. intermedius* and *S. pseudointermedius* in comparison to a multiplex PCR assay as a rapid and accurate method. As well, the incidence of different CPS causing bovine mastitis will be determined to highlight the role of each species as an agent of mastitis. Additionally, attention will be paid toward identification of atypical *S. aureus* strains.

Materials and Methods

Samples and sampling methods

A total of 340 milk samples from cows suffering from mastitis were collected. These samples came from 10 herds from different localities in Egypt. Also, 68 bulk tank milk samples were bacteriologically analyzed. Samples were aseptically collected into sterile vials. Forty milliliters of bulk tank milk samples were collected, after all the animals had been milked and the bulk tank had been agitated for at least 5 min., from the top of the bulk tank using a sterile glass pipette and deposited into a sterile vial. All samples were immediately chilled and then transported to the laboratory on ice bags then incubated at 37°C/6-12 hours for enrichment.

Isolation and phenotypic identification of staphylococcus species

The incubated samples were cultured on blood agar (Blood Agar Base, containing 7% of ovine blood) and incubated at 37°C/24-48 hours, after which they were

evaluated for the presence of bacterial growth. Among the microorganisms primarily identified as staphylococci, 103 isolates were selected for this study. The sorting of microorganisms from family Staphylococcaceae, was performed through assumptive tests, according to Quinn *et al.* (2011). They were based on the macroscopic observation of colonies, verifying the presence of hemolysis, size and pigmentation, observation of microscopic morphology through Gram-stained smears, catalase test and tube coagulase test, employing rabbit plasma. A pure culture of each isolate was kept on soft agar for further phenotypic identification. Then, each isolate was streaked on Mannitol Salt Agar (MSA), Baird Parker Agar supplemented with egg yolk tellurite (BPA) only, and with acriflavine hydrochloride (7µg/ml) and Baird Parker Agar supplemented with rabbit plasma fibrinogen (RPF). The constituents of different media were prepared according to Ollis *et al*. (1995). Plates were incubated at 37°C for 24-48 h. The characteristic growth on each medium was recorded. The isolates were submitted to further identification using anaerobic after mannitol and maltose fermentation tests, as well as acetoin production throught voges-proskauer test (VP). The interpretation of results was done according to Holt *et al*. (1994).

DNA extraction

A rapid boiling procedure was used to prepare crude DNA from bacterial strains according to Reischl *et al*. (1994) and Darwish and Asfour (2013). Briefly, two to 5 loops of staphylococcus isolates taken from the nutrient agar plate were collected and suspended in 200 µl of lysis buffer [1% Triton X-100, 0.5% Tween 20, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA]. After boiling for 10 min, the suspension was centrifuged for 5 min. to sediment bacterial debris. The supernatant was aspirated and from which 5 µl was used directly for PCR amplification.

Molecular identification

Primers

Different primers were used in this study. Their names, sequences, species-specific, PCR products sizes and their references are demonstrated in Table (1).

PCR

Control PCR

To exclude any false negative results, amplification of *16SrRNA* gene of all staphylococcus strains were performed at first using *16SrRNA* control primers of Monday and Bohach (1999). It was established in 25 µl reaction volume contained 5µl of DNA as template, 20 pmol of each primer and 1X of PCR master mix (Dream Taq Green PCR Master Mix, Fermentas Life Science). The amplification cycles were carried out in a PT-100 Thermocycler (MJ Research, USA). Reaction conditions were optimized to be 94°C for 4 min. as initial denaturation, followed by 35 cycles of 94°C for 60 seconds, 55°C for 60 seconds and 72 °C for 60 seconds. A final extension step at 72°C for 10 min. was followed. DNA isolated from *S. aureus* ATCC 25923 was used as positive control. Amplification products were electrophorezed in 1.5% agarose gel containing 0.5X TBE at 70 volts for 70 min. and visualized under ultraviolet light. Amplification of 228 bp bands indicated the isolate to be staphylococcus strain and so amplifiable DNA.

Multiplex Polymerase Chain Reaction (mPCR)

A quadriplex PCR assay modified from the originaly reported by Sasaki *et al.* (2010), targeting different regions in the *nuc* gene, was applied in our study. It was established using a total volume of 25 µl reaction mixtures contained 5µl of DNA as template, 20 pmol of each primer and 1X of PCR master mix. The amplification cycles and reaction conditions were carried out as done in control PCR except for the annealing temperature which was 50°C instead of 55°C. DNA isolated from *S. aureus* ATCC 25923, *S. hyicus*, *S. intermedius* and *S. pseudointermedius* field isolates, previously confirmed by API, was used as positive controls. Water was used as negative control. To assure that the amplification products were of the expected size, a 100 bp DNA ladder was run simultaneously as a DNA marker. Amplification of 359, 430, 793 and 926 bp bands indicated the isolate to be *S. aureus*, *S. intermedius*, *S. hyicus* and *S. pseudointermedius*, respectively.

***S. aureus* confirmatory PCR**

This PCR was used to confirm atypical *S. aureus* isolates. It was established as above mentioned in control PCR but using *nuc1* and *nuc* 2 primers mentioned in Table (1). Amplification of 279 bp band confirmed the isolate to be *S. aureus.*

Result and Discussion

Phenotypic identification of staphylococcus strains

From 340 individual milk samples and 68 bulk tank milk, 275 and 68 staphylococcus strains were isolated with percentages of 80.9% and100%, respectively. All the isolated strains were subjected to coagulase test. A total of 103 strains of staphylococci were selected for this study on the bases of their coagulase test results and their characteristic growth on MSA. The strains under study were categorized into 3 groups:-

1st group: including 68 strong CPS strains.
2nd group: including 15 weak CPS strains.
3rd group: including 20 CNS strains.

Staphylococcus strains of all groups were subjected to different identification tests. Numbers and percentages of both positive and negative results of each group of strains were recorded for each test in Table (2). Figures 1-3 show the positive and negative result of some tests.

Based on phenotypic identification, out of 103 staphylococcus strains, 84 (81.5%), 8 (7.8%), 4 (3.9%) and 7 (6.8%) were *S. aureus, S. intermedius, S. hyicus* and other staphylococci, respectively. Detailed identification of strains of each group was declared in Table (3).

Molecular identification

All strains successfully amplified the 228 bp fragment of *16S rRNA* gene of genus staphylococci as shown in Figure 4a, therefore, confirmed to be staphylococcus strains. Molecular identification of all strains was performed using multiplex PCR. The modified mPCR successfully amplified the 359, 430, 793 and 926 bp specific PCR products of *S. aureus, S. intermedius, S. hyicus* and *S. pseudointermedius* strains used as positive control, respectively. Figure 4b shows the specific PCR products of positive controls and representative strains of different CPS using mPCR. Based on molecular identification, out of the 103 staphylococcus strains, 84 (81.6%), 3 (2.9%), 2 (1.9%) and 14 (13.6%) were found to be *S. aureus, S. intermedius,*

S.hyicus and other staphylococci, respectively. None of the isolate was identified as *S. pseudointermedius*. Detailed molecular identification of strains of each group was shown in Table (3). Comparison between phenotypic versus molecular identification was cleared in the same table.

A second confirmatory PCR for confirmation of atypical *S. aureus* was used. Figure 4c shows the specific 279 bp PCR product of this PCR. All strains of 3rd group were examined by *S. aureus* confirmatory PCR. Also, strains with conflicting results were also examined. Table (4) shows the staphylococcus strains whose identification was discordant by both phenotypic and molecular methods.

In veterinary clinical laboratories, CPS other than *S. aureus* has frequently been misidentified as being *S. aureus* strains. This is because they have several phenotypic traits in common and there has been no reliable method to distinguish among CPS species (Sasaki *et al.*, 2010).

Furthermore, the biochemical methods traditionally used to identify CPS demand a longer time for analysis which may extend to one week. This is because it is necessary to perform several tests for identification to the species level. Therefore, evaluation of different phenotypic tests used for identification of CPS versus molecular-based method as a gold standard was performed.

Table.1 Primers used in the study, their nucleotide sequences, species specific, references and their PCR products sizes

PCR	Primer name	Sequence 5'-3' (reference)	Species specific	PCR product size in bp
Control PCR	*16S rRNA*F *16S rRNA*R	GTA GGT GGC AAG CGTTAT CC CGC ACA TCA GCG TCA G (Monday & Bohach, 1999)	Staphylococcus species	228
Multiplex PCR	au-F3 au-*nuc*R	TCGCTTGCTATGATTGTGG GCCAATGTTCTACCATAGC (Sasaki *et al*., 2010)	*S. aureus*	359
	in-F in-R3	CATGTCATATTATTGCGAATGA AGGACCATCACCATTGACATAT TGAAACC (Sasaki *et al*., 2010)	*S. intermedius*	430
	hy-F1 hy-R1	CATTATATGATTTGAACGTG GAATCAATATCGTAAAGTTGC (Sasaki *et al*., 2010)	*S. hyicus*	793
	pse-F2 pse-R5	TRGGCAGTAGGATTCGTTAA CTTTTGTGCTYCMTTTTGG (Sasaki *et al*., 2010)	*S. pseudintermedius*	926
S. aureus confirmatory PCR	*nuc 1* *nuc 2*	GCGATTGATGGT GATACGGTT AGCCAAGCCTTGACGAACTAA AGC' (Brakstad *et al*., 1992)	*S. aureus*	279

Table.2 Phenotypic tests used for identification of staphylococcus isolates and their results

Isolates groups based on coagulase test result	Phenotypic methods used for identification																	
	MSA			BPA		RPFA		BPA with acriflavine hydrochloride		Acetoin production		Anarobic fermentation of mannitol			Maltose fermentation			
	+ve	weak +ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	weak +ve	-ve	+ve	weak +ve	-ve	
1st gp Strong coagulase (No.=68)	58 (85.3%)	4 (5.9%)	6 (8.8%)	68 (100%)	0 0	66 (97.1%)	2 (2.9%)	62 (91.2%)	6 (8.8%)	64 (94.1%)	4 (5.9%)	62 (91.2%)	4 (5.9%)	2 (2.9%)	58 (85.3%)	8 (11.8%)	2 (2.9%)	
2nd gp weak coagulase (No.=15)	13 (86.7%)	-	2 (13.3%)	0 0	15 (100%)	9 (60%)	6 (40%)	10 (66.7%)	5 (33.3%)	12 (80%)	3 (20%)	12 (80%)	2 (13.3%)	1 (6.7%)	12 (80%)	2 (13.3%)	1 (6.7%)	
3rd gp Negative coagulase (No.= 20)	18 (90%)	-	2 (10%)	0 0	20 (100%)	13 (65%)	7 (35%)	11 (55%)	9 (45%)	12 (60%)	8 (40%)	11 (55%)	7 (35%)	2 (10%)	12 (60%)	6 (30%)	2 (10%)	
Suspected species	SA	SI	SH	SA	SH or SI	Co+ve Mainly SA	Co-ve	SA	SH or SI	SA	SH or SI	SA	SH	SI	SA	SI	SH	
Total No.	89	4	10	68	35	88	15	83	20	88	15	85	13	5	82	16	5	

SA-*S. aureus*, SI-*S. intermedius* and SH-*S. hyicus*

Table.3 Comparison between results of phenotypic and genotypic identification of staphylococcus isolates

Tested strains	Phenotypic identification				Genotypic identification			
	S.aureus	*S.intermedius*	*S.hyicus*	Other Staphylococci	*S.aureus*	*S.intermedius*	*S.hyicus*	Other Staphylococci
1st group (68)	61 (89.7%)	5 (7.4%)	2 (2.9%)	0	64 (94.1%)	3 (4.4%)	1 (1.5%)	0
2nd group (15)	12 (80%)	2 (13.3%)	1 (6.7%)	0	12 (80%)	0	1 (6.7%)	2 (13.3%)
3rd group (20)	11 (81.5%)	1 (5%)	1 (5%)	7 (35%)	8 (40%)	0	0	12 (60%)
Total (103)	84 (81.5%)	8 (7.8%)	4 (3.9%)	7 (6.8%)	84 (81.6%)	3 (2.9%)	2 (1.9%)	14 (13.6%)

Table.4 Staphylococcus strains whose identification was discordant by using Phenotypic and Genotypic tests

groups	No. of isolates	Phenotypic identification	Genotypic identification
1st group	2	*S.intermedius*	*S.aureus*
	1	*S.hyicus*	*S.aureus*
2nd group	2	*S.intermedius*	Other Staphylococci
3rd group	3	*S.aureus*	Other Staphylococci
	1	*S.intermedius*	Other Staphylococci
	1	*S.hyicus*	Other Staphylococci

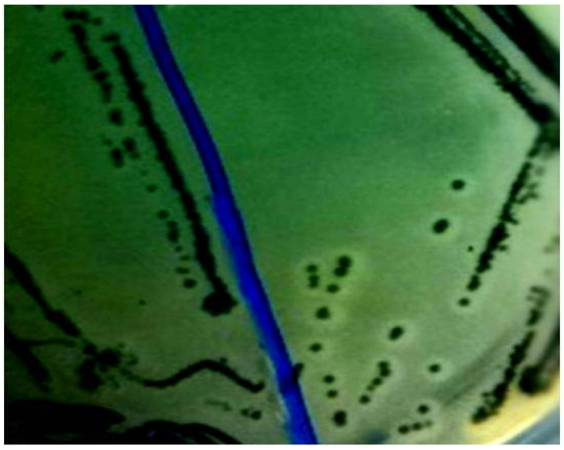

Fig 1 BPA supplemented with egg yolk tellurite showed CPS *S. aureus* with hallo zones around the colonies in the right part while other CPS showed no hallo zone in the left part.

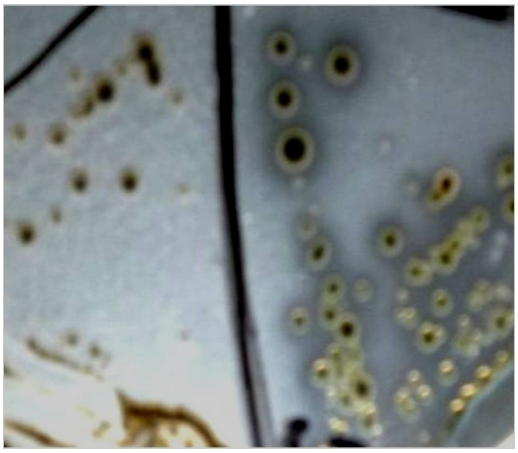

Fig 2 RPFA media, left side showed coagulase negative while right side showed CPS with characteristic opaque hallo zones of fibrin around the colonies.

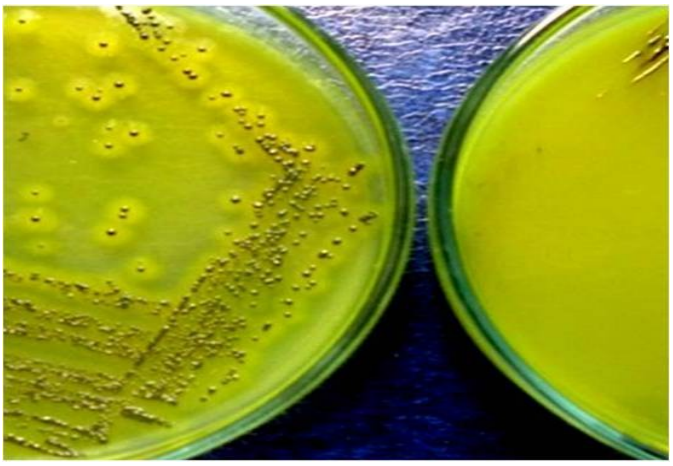

Fig.3 Growth of *S. aureus* on BPA supplemented with egg yolk tellurite and acriflavine hydrochloride (7µg/ml) while the other CPS couldn't grow.

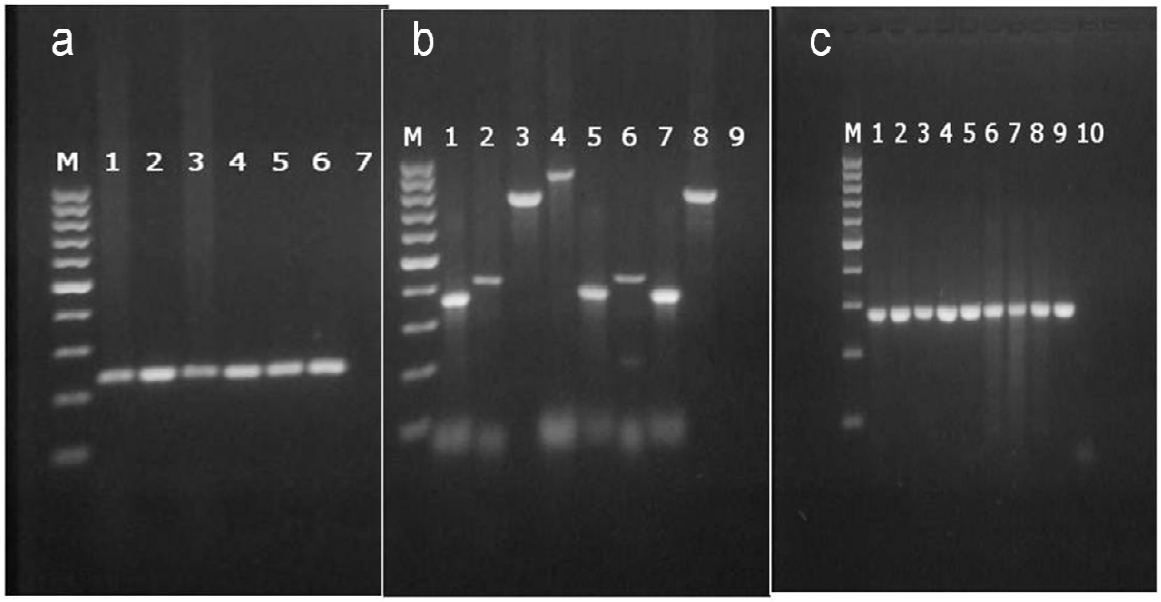

Fig.4a Positive 228 bp PCR products of *16S rRNA* gene of staphylococci. Lane M: 100 bp ladder DNA marker, Lane 1: *S. aureus* (ATCC 29213) used as positive control, Lanes 2-6: representative staphylococcal isolates and Lane 7: negative control.

Fig.4b Quadriplex PCR assay detecting the *S. aureus* (359bp), *S. intermedius* (430 bp), *S. hyicus* (793bp) and *S. pseudointermedius* (926 bp), simultaneously. Lane M: 100 bp ladder DNA marker, Lanes 1-4: *S. aureus*, *S. intermedius*, *S. hyicus* and *S. pseudointermedius*, used as positive controls, respectively. Lanes 5 and 7 representative *S. aureus* isolates, Lane 6: *S. intermedius* isolate, Lane 8: *S. hyicus* isolate and Lane 9: negative control.

Fig.4c Positive 279 bp PCR products of *nuc* gene of *S. aureus*. Lane M: 100 bp ladder DNA marker, Lane 1: *S. aureus* (ATCC 29213) used as positive control, Lanes 2-9: coagulase negative *S. aureus* isolates and Lane 10: negative control.

To fulfill the objectives of this study, a total of 103 staphylococcus strains were selected on the basis of their coagulase test results and their characteristic growth on MSA as the most commonly used tests for identification of staphylococci. The tested strains were classified according to coagulase test into 3 groups because a controversy concerning the correct interpretation of results of the coagulase test was reported by Silva *et al*. (2000).

Benett and Lancette (1995) reported that 1+, 2+ and 3+ of coagulase results rarely correlate with results of other criteria for *S. aureus*. However, AOAC (1990) and Lancet and Tatini (1992) protocols were reported to be more flexible: AOAC considered all degree of positivity (1+ to 4+) to be a positive result, while Lancet and Tatini (1992) considers only 3+ and 4+ as positive results. However, coagulase production was still reported to be the most traditional test used to identify *S. aureus* (Schwarszkopf and Karch, 1994). Moreover, Bannerman (2003) reported it to be the gold standard for *S. aureus* identification. Therefore, the strains were classified into strong coagulase, weak coagulase and negative coagulase groups. The last group of strains motivated us to include them in the study because they showed the characteritics of *S. aureus* on MSA while being coagulase negative in two independent trials.

In the 1st group, 58 isolates (85.3%) were positive on MSA with golden yellow colonies, 68 isolates (100%) were positive on BPA with typical characteristic growth of colonies (fig 1), 66 isolates (97.1%) were positive on RPFA (fig 2), 62 isolates (91.2%) showed growth on BPA supplemented with acriflavine hydrochloride (fig 3), 64 isolates (94.1%) were positive for acetoin production, 62 isolates (91.2%) were anaerobic mannitol fermenters while 58 isolates (85.3%) were maltose fermenters.

In the 2nd group, 13 isolates (86.7%) were positive on MSA, no isolate (0%) was positive on BPA (absence of hallo zone around the colonies), 9 isolates (60%) were positive on RPFA, 10 isolates (66.7%) showed growth on BPA supplemented with acriflavine hydrochloride, 12 isolates (80%) were positive for acetoin production, 12 isolates (80%) were anaerobic mannitol fermenters while 12 isolates (80%) were maltose fermenters.

In the 3rd group, 18 (90%) isolates were positive on MSA, no isolate (0%) was positive on BPA, 13 isolates (65%) were positive on RPFA, 11 isolates (55%) showed growth on BPA supplemented with acriflavine hydrochloride, 12 isolates (60%) were positive for acetoin production, 11 isolates (55%) were anaerobic mannitol fermenters while 12 isolates (60%) were maltose fermenters.

Positive results for each test pointed to great extent to *S. aureus*, weak positive results revealed mainly to *S. intermeduis* and negative results revealed mainly to *S. hyicus* especially in 1st and 2nd groups. Positive results in the 3rd group revealed mainly to *S. aureus*. The results of the 3rd group were surprising. There was a high percentage of atypical *S. aureus* (coagulase negative *S.aureus*) in this group. These atypical strains could be escaped from detection if we relied only on coagulase test or on the growth criteria on BPA media.

Based on phenotypic identification, the outcome of all the adopted phenotypic tests resulted in identification of 84

(81.5%), 8 (7.8%), 4 (3.9%) and 7 (6.8%) as *S. aureus, S. intermedius, S. hyicus* and other staphylococci, respectively. In this respect, Capurro *et al*. (1999) found 97% of 177 strains of CPS isolated from bovine mastitic cases to be *S. aureus*, 2% as *S. intermedius* and 1% as *S. hyicus*. Also, Costa *et al*. (2010) recovered 344 CPS from bovine mastitis cases, 98.25% of them were phenotypically identified to be *S. aureus*, 0.86% as *S. intermedius* and 0.86% as *S. hyicus*. While Arslan *et al*. (2009) reported a lower percentage of *S. aureus* strains (67.5%) among CPS isolated from bovine mastitic cases and a higher percentage of *S. intermedius* (32.5%) identified by conventional bacteriological methods.

To evaluate the results of phenotypic tests, the strains of all groups were subjected to two different PCR assays. The first assay was utilizing a control primer pair targeting 228 bp fragment of *16S rRNA* gene of genus staphylococci. All the strains were confirmed to be staphylococci. This PCR assay was performed mainly to exclude any false negative results in the multiplex PCR. The second PCR assay was a modified multiplex PCR that was designed to detect specifically *S. aureus*, *S. intermedius, S. hyicus* and *S. pseudointermedius* by amplification of 359, 430, 793 and 926 bp specific PCR products, respectively. By using this multiplex PCR assay, out of the 103 staphylococcus strains, 84 (81.6%), 3 (2.9%), 2 (1.9%) and 14 (13.6%) were found to be *S. aureus, S. intermedius, S. hyicus* and other staphylococci, respectively. By comparison between phenotypic and genotypic results (Table 3), in 1st group: 61(89.7%), 5 (7.4%) and 2 (2.9%) out 68 CPS strains were phenotypicaly identified as *S. aureus, S. intermedius* and *S. hyicus* versus 64(94.1%), 3(4.4%) and 1 (1.5%) respectively, by genotypic method. In the 2nd group, 12 (80%), 2 (13.3%) and 1 (6.7%) out 15 weak CPS strains identified by phenotypic method as *S. aureus, S. intermedius* and *S. hyicus*, versus 12 (80%), 0 % and 1(6.7%) respectively, by genotypic identification. In the 3rd group, 11(55%), 1(5%) and 1(5%) phenotypically identified as *S. aureus, S. intermedius* and *S. hyicus* versus 8(40%), 0% and 0% genotypically identified as *S. aureus, S. intermedius* and *S. hyicus*, respectively. Some difference was found between both phenotypic and genotypic methods. This can be explained by variability in results of the majority of biochemical tests which can lead to the occurrence of false negative results due to effect of environmental factors on gene expression (Downes and Ito, 2001).

Results of phenotypic identification of staphylococci were reported to be often incorrect because of the phenotypic variation. Therefore, their diagnostic accuracy has been reported to be 36.7-93.6%, although many conventional tests are applied. So, genotypic analysis was reported to be necessary for definitive species identification (Hiekens *et al*., 2005; Layer *et al*., 2006 and Hirotaki *et al*., 2011).

Sasaki *et al*. (2010) using their original mPCR assay successfully distinguished between *S. aureus, S. hyicus, S. schleiferi, S. intermedius, S. pseudintermedius,* and *S. delphini*. Their method was reported to be both sensitive (99.8%) and specific (100%). Also, it allowed the routine species identification of CPS isolates from various animal species for clinical veterinary diagnosis. This encouraged us to depend on results of mPCR as a gold standard.

These results revealed that, numerous conventional tests must be used for differentiation between CPS as each test used harbored the risk of false negative or false positive result. In this concern, our results agreed with Devriese *et al*. (2005) who reported that extensive phenotypic testing or molecular identification methods are needed in order to identify CPS strains adequately. Recently, Johler *et al*. (2012) found some *S. aureus* strains which exhibited alpha, beta, and delta hemolysis, however, had no visible opaque zone on RPFA. Considering these findings, screening for *S. aureus* by RPFA only harbors the risk of false negative results, potentially leading to severe therapeutic mistakes. This was observed in our results as RPFA gave false negative where there was growth but without opaque hallo zone around the colonies, especially in the strains of the 1st group with a percentage of 2.9%. Additionally, Koluman *et al*. (2011) found that 5.4% of their samples were coagulase negative in the tube test but positive on BP-RPFA.

It was noticed in our results especially in the 3rd group as all the tested strains were coagulase negative in the tube test while 65% of them were positive on RPFA. It was clear that CPS isolates were more atypical strains in both the 2nd and 3rd groups. Growth of these strains on BPA appeared atypical; black colonies without halo zone surrounding; as shown in fig (1). This was previously reported by Silva *et al*. (2000) where they found the incidence of atypical colonies of *S. aureus* on BPA to be 39.4% of the colonies. Baird and Lee (1995) cleared that the selectivity of BPA is limited because only the lipolytic and proteolytic *S. aureus* strains which produce the double zone can be easily recognized. According to Benett and Lancette (1995), non-lipolytic strains may be frequent in dairy products or milk samples coming from mastitic animals, so, additional diagnostic features are required to confirm *S. aureus*. This fact was also mentioned by Zadoks *et al.* (2004) who found that, in raw milk samples, the number of coagulase positive atypical *S. aureus* isolates is higher than strains with typical characteristic colonies on BPA and blood agar plates. Recently, Sulaj *et al*. (2013) mentioned that, 6 out of 19 (32%) *S. aureus* strains showed typical characteristics of growing colonies while, 13 out 19 (68%) isolates had atypical colonies on BPA. Fabulously, atypical strains of *S. aureus* were reported to be more virulent and able to produce entertoxines types A and B which cause very strong intoxication in humans and calves (Jones *et al*., 2006).

Considering the use of acriflavine sensitivity in the differentiation between CPS, Harmon *et al.* (1991) reported that, 99.3% of *S. aureus* strains grew on P agar supplemented with 7 µg/ml of acriflavine, whereas only one out of 10 of *S. intermedius* strains grew in this medium, but weakly. Differently, Roberson *et al.* (1992) and Capurro *et al.* (1999) illustrated that, 100% of the *S. aureus* strains grew in P agar and BPA supplemented with acriflavine (7 µg/ml) and none of the strains of *S. intermedius* or *S. hyicus* was able to grow in these media, that agreed with our results as *S. aureus* grew on this media while both *S. intermedius* and *S. hyicus* did not. Therefore, the acriflavine sensitivity must be included in the routine tests used for differentiation between *S. aureus* and other CPS where it is not a conventional test in the majority of our veterinary laboratories.

The very interesting result in this study

was the presence of coagulase negative *S. aureus*; 11(55%) versus 8(40%) out of 20 strains identified by phenotypic and genotypic methods respectively, in the 3rd group of work that may be misidentified as CNS if we depend only on the results of tube coagulase test and PBA media. In this respect, worldwide reports of coagulase-negative variants of *S. aureus* in bovine mastitis are still rare because only few researchers worked on coagulase negative variants *S. aureus* strains (atypical tube coagulase negative strains of *S. aureus*) isolated from milk samples derived from subclinical mastitis cases in dairy cattle (Laevens *et al*., 1996; Matthews *et al*., 1997; Malinowski *et al*., 2009; Akineden *et al*., 2011 and Rusenova *et al*., 2013). The fact, coagulase-negative *S. aureus* variants can occur in the context of intra-mammary infections in cattle may result in its misidentification as CNS in routine mastitis diagnostic, at least in some cases.

To fully ensure the correct species identification, a second *S. aureus* confirmatory PCR assay was adopted on all the isolates of group 3 to ensure their species. The results of this confirmatory PCR confirmed the results of the mPCR. Rusenova and others (2013) stated that when routine method based on coagulase activity level alone was used for detection of *S. aureus,* false determination of 14 *S. aureus* isolates occurred; 3 catalase-negative, 10 coagulase-negative that were identified as *S. aureus* subsp. *aureus* with a very high probability (91.9 - 99.9%), and one CPS was identified as *S. schleiferi subsp. coagulans*.

Therefore, a special attention is required when working with atypical *S. aureus* strains in udder health laboratories, where the identification systems and PCR based methods are not currently used as diagnostic approaches. In Table (4), we discussed the differences in phenotypic and genotypic methods. In the 1st group, 2 strains were identified as *S. intermedius* and one was identified as *S. hyicus* phenotypically, while by genotypic methods the three strains were identified as *S. aureus.* Also in the 2nd and 3rd group there were some discrepancies between the applied methods. This discrepancy was also reported by many authors (Schmitz *et al*., 1998; Motta *et al*., 2001). Tenover *et al.* (1994) compared traditional and molecular techniques to identify different strains of *S. aureus* from human origin and insured that the DNA-based techniques and immunoblotting were the most effective in strain grouping. From our study, we can conclude that the most important tests which must be included in the phenotypic identification scheme of both CPS and coagulase negative *S. aureus* strains are acriflavine sensitivity, acetoin production, maltose and anaerobic mannitol fermentations and finally, RPFA instead of tube coagulase test. This is because they are the closest to mPCR assay results.

In conclusion, when precise identification of coagulase positive staphylococci is required, numerous phenotypic tests must be adopted in addition to molecular based method. Multiplex PCR assay applied in this study was found to be the ideal way to differentiate CPS to the species level. Also, attention must be paid toward detection and identification of atypical tube coagulase negative *S. aureus* strains as a cause of bovine mastitis in dairy herds.

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