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# DIFFERENCES BETWEEN PHENOTYPIC AND GENOTYPE CHARACTERIZATION OF S. AUREUS ISOLATED FROM BOVINE MASTITIS IN EGYPT

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**Received:** 31 December 2020; **Accepted:** 30 April 2021

## ABSTRACT

*S. aureus* is one of the most important causes of nosocomial infections, main contagious pathogens that can play a vital and important role in bovine mastitis in veterinary medicine causing high worldwide economic losses, where the primary reservoir harboring the pathogens is cow, and recently recorded as zoonotic microbe which able to transmitted from human to animal and vice versa, identification of *staphylococcus* species mainly *S. aureus* become more quickly and accurate with molecular technique as phenotypic technique due to multidrug resistance developed some mutation and production of biofilm which interference phenotypic identification, as *nuc* gene, and *coa* gene become the golden standard technique for identification *S. aureus* and the study concerned the differentiation between phenotypic and genotypic characterization of isolates collected from milk samples of bovine mastitis, as directed 157 phenotypic staphylococcus isolates on MSA media to biochemical phenotypic tests as 81/157 (51.6%) isolates of them were phenotypic *S. aureus*, and by molecular technique, 16S universal primer detected 141/157 (89.8%) were confirmed *staphylococcus* about 130/141(92.2%) were CoPS by *coa* gene detection, and 111/141 (78.7%) were typical *S. aureus* detected by *nuc* gene, as most of them produced biofilm that detected by *ica* gene in 81/141(57.4%) was the main cause of interrupting the phenotypic characterization by biochemical tests.

***Keywords:*** bovine mastitis, coagulase positive *staphylococcus* (CoPS), *coa* gene, *ica* gene (biofilm formation gene), *nuc* gene, *S. aureus*.

## INTRODUCTION

Bovine mastitis is multifaceted etio-pathology, as including three main factors: 1.exposure to microorganisms, 2.host defense mechanisms, and 3.environmental conditions (Zadoks *et al.*, 2001).

Among all these factors that cause bovine mastitis, *S. aureus* are the main pathogens that can play a vital and important role in mastitis (Lundberg *et al.*, 2014), also The SCM (sub-clinical mastitis) is a multi-etiologic disease, many microorganisms is implicated as causes. S. aureus and Streptococcus agalactiae as dominant Contagious pathogens of SCM, are transmitted from animal to animal where the

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primary reservoir harboring the pathogens is the cow (Shawky *et al.*, 2013 and Youssif *et al.*, 2020).

*S. aureus* secretes two clotting factors, von Willebrand factor binding protein and coagulase (coa) protein. The coa protein is an important phenotypic determinant and virulence factor of *S. aureus*. The ability of its coa to clot plasma is a defining property of *S. aureus* and distinguished the species from other coa-negative *staphylococci* (Gharib *et al.*, 2013; Abbas *et al.*, 2014 and Foster *et al.*, 2015). The tube coagulase test with diluted rabbit plasma is the accepted confirmation of an identification of Staphylococcus aureus, but either a tube test with human plasma broth or some variant of the slide clumping factor (bound coagulase) test is more often used at the bench. With most human strains there is no difficulty, but it has been suggested that strains of S aureus, which are resistant to methicillin, are deficient in clumping factor and even in Protein A. Lally and Woolfrey, found that some strains of *S. aureus* resistant to methicillin may be particularly deficient, and similar findings were reported at a workshop on the problems caused by some strains resistant to this drug. Other characters such as DNase production, phosphatase, and ability to acidify mannitol salt agar have been used with varying efficacy to confirm or suggest an identification of a strain as S aureus (Saraiva *et al.*, 2018).

The *coa* gene of *S. aureus* isolated as considered the most simple and accurate tool for molecular typing. that also reported that this technique could be used in epidemiological detection of *S. aureus* isolates from cattle mastitis as had a high reproducibility and good discriminatory power, it is the easiest with which to analyze coa gene polymorphism among a large number of bacterial isolates, and it generates multiple distinct polymorphism patterns (Da Silva and Da Silva, 2005).

Phenotypic characterization by conventional bacteriology of Staphylococcus aureus is no longer beneficial in controlling mastitis caused by this organism since inter-strain variations exist in terms of virulence potential (Pilla *et al.*, 2013). This returned to Most of the *S. aureus* strains formed the biofilm in an *ica*-dependent mechanism (Kostaki *et al.*, 2012; Mah 2012; Bridier *et al.*, 2015 and Avila-Novoa *et al.*, 2018), *S. aureus* abled to adhere to the surface of indwelling medical devices and develop biofilm, a multilayered structure comprising of bacterial communities embedded within the extra- cellular hydrated polymeric matrix (Paharik and Horswill, 2016).

Recently, PCR has become a very popular molecular technique, especially for the detection and identification of bacteria in mastitic milk by targeting their specific genes in the DNAs, Molecular diagnostic methods like DNA-based mastitis diagnostic system have already been introduced for routine use in the dairy herds (Koskinen *et al.*, 2009; Taponen *et al.*, 2009; Elsayed *et al.*, 2015 and Hoque *et al*, 2018).

## MATERIALS AND METHODS

***Collected samples tools:***

A total of 400 milk samples from clinical mastitis and apparent healthy (sub-clinical) cows, were collected from different localities and dairy cattle farms in Monofyia Governorate. The samples were collected under complete aseptic condition (5 ml of milk collected in sterile falcon tubes) collected from clinically diseased cows (characterized by abnormal milk secretion containing clots, flocks, blood, some with swelling / hardness of the mammary gland and others with systematic disturbance as fever and loss of appetite) and from apparent healthy cows with no signs on udder tissue or fresh raw milk with some exception for some calves whose refuse to breastfeed without a satisfactory reason and persistent diarrhea in some suckling calves. The collected samples were aseptically transferred to the laboratory in an insulated ice box to be examined immediately with a minimum delay to detect the presence of

staphylococcus species. Using standard techniques recommended by (Collee *et al.*, 1989) and National Mastitis Council of United States (Anonymous, 1990).

***Bacteriological examination:***

*Pre enrichment milk sample (APHA., 1992):*
Inoculation 1ml of sample in 5ml of sterile BHI broth (sterilized by autoclaving at 121°C for 15 minutes) incubated at 37°C for 24 hours.

*Mannitol salt agar medium (MSA) (APHA., 1966):*

A selective medium for the isolation of presumptive pathogenic *staphylococci*. And Presumptive coagulase-positive *staphylococci* produce colonies surrounded by bright yellow zones whilst non-pathogenic *staphylococci* produce colonies with reddish purple zones. Suspend 111g in 1 liter of distilled water and bring to the boil to dissolve completely. Sterilized by autoclaving at 121°C for 15 minutes. Poured in sterile petri-dishes and sample material streaked across the plate using sterilized loop and Incubate at 37°C for 24 hours. Examine after 24 hours for yellow colonies surrounded by bright yellow zones (positive result).

***Microscopic appearance:***

Gram Staining (Cruickshank *et al.*, 1975*):* Films were prepared from pure culture of the isolated organisms and stained with Gram's stain and examined microscopically. *S. aureus* are Gram positive cocci arranged in clusters.

***Biochemical identification of Staphylococci*** *according to* (McFadden, 1980; Carter and Cole, 1990 and Quinn *et al.*, 1994):
The following biochemical tests were adopted for identification of isolates:

**A. Oxidase test:**

Applied by using Oxidase detection strips {CODE: MB0266, (Thermo Sci.)®} Touch the colony to be tested with the Oxidase Detection Strip and observe for up to 5 seconds. A deep blue/violet color indicates a positive reaction.

**B. Catalase test:**

Loop full of suspected pure culture growth from nutrient agar slant was mixed with 3 drops of 30% of hydrogen peroxides on a glass slide. The production of gas bubbles immediately constitutes a positive reaction.

**C. Tube Coagulase test:**

Suspect staphylococci colonies were transferred from solid media into small tubes containing 0.2-0.3 ml nutrient broth and emulsified thoroughly. Incubated at 37ºc for 24 hour. Then 0.5ml reconstituted coagulates human plasma with EDTA was added to nutrient broth and mixed thoroughly. The tubes were incubated at 37ºc and examined periodically over 6 hrs. For clot formation. (Koneman *et al.*, 1997). Only firm and complete clot that stay in place when tubes was titled or inverted was considered positive. Partial clotting, formerly 2+ and 3+ coagulase reaction must tested further.

**D. Gelatin hydrolysis test (McDade *et al.*, 1958):**

It distinguishes the gelatinize-positive, pathogenic *S. aureus* from the gelatinize-negative, non-pathogenic *S. epidermidis.* Inoculate a heavy inoculum of test bacteria (18- to 24-hour-old) by stabbing 4-5 times (half inch) on the tube containing nutrient gelatin medium. Incubate the inoculated tube along with an un-inoculated medium at 35°C, or at the test bacterium's optimal growth temperature, for up to 2 weeks. Remove the tubes daily from the incubator and place in ice bath or refrigerator (4°C) for 15-30 minutes (until control is gelled) every day to check for gelatin liquefaction. (Gelatin normally liquefies at 28°C and above, so to confirm that liquefaction was due to gelatinize activity, the tubes are immersed in an ice bath or kept in refrigerator at 4°C). Tilt the tubes to observe if gelatin has been hydrolyzed. Partial or total liquefaction of the inoculated tube even after exposure to cold temperature of ice

bath or refrigerator (4°C) positive *S. aureus*, Complete solidification of the inoculated tube even after exposure to cold temperature of ice bath or refrigerator (4°C).

**Trehalose fermentation test** (Oxoid, 1996)
1% peptone water containing 1% trehalose (sigma®) sterilized tubes by autoclaving at 121°C for 15 minutes. And inoculated by pure colonies and incubated at 37 ºc for 24 hrs. then adding phenol red as indicator for detection a positive result yellow color due to fermentation of sugar and production acid due to change pH to acidic.

**PCR technique:**
**DNA Purification protocol**
Preparing fresh BHI broth from isolates and incubated at 37˚C for 18 hrs. using i-genomic BYF DNA Extraction Mini Kit) (iNtRON Biotechnology) ®for DNA purification.

**PCR techniques:**
**DNA amplification**
PCR reaction were performed, each reaction mixture contained 5 µl of prepared template DNA, 0.5 µl of each primer, 12.5 µl of 2×EasyTaq® PCR SuperMix (transbionovo) ® and the final volume was adjusted to 25ul with distilled water. PCR was performed in rotor gene thermo cycler with the following table data.

**Table 1:** Primer Nucleotides sequences, and PCR was performed in rotor gene thermo cycler with the following steps:

| Gene | Product size | Primer Nucleotides sequence (5'-3') | Reference |
|---|---|---|---|
| 16S rRNA | 756 bp | 5`-AAC TCT GTT ATT AGG GAA GAA CA-3`<br>5`-CCA CCT TCC TCC GGT TTG TCA CC-3` | **Zhang *et al.*, 2004** |
| 94°C for 5 min followed by 10 cycles of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 1.5 min and another 25 cycles of 94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 1.5 min, ending with a final extension step at 72°C for 10 min | | | |
| Nuc | **279 bp** | GCG ATT GAT GGT GAT ACG GTT<br>AGC CAA GCC TTG ACG AAC TAA AGC | **Shortle, 1983** |
| Ica gene | 483 bp | GAA CCG CTT GCC ATG TGT TG<br>GCT TGA CCA TGT TGC GTA ACC | **Namvar *et al.*, 2013** |
| an initial denaturation step at 95°C for 2 min followed by 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 60 s and a final extension step at 72°C for 7 min | | | |
| Coa | Polymorphism | CGA GAC CAA GAT TCA ACA AG<br>AAA GAA AAC CAC TCA CAT CA | **Guler *et al.*, 2005** |
| an initial denaturation step of 2 min at 94°C followed by 30 cycles of 30 s at 94°C, 2 min at 55°C and 4 min at 72°C, and a final extension step of 7 min at 72°C. | | | |

**Separation of PCR amplicons by Gel Electrophoresis:** After the amplification was completed the amplified products was analyzed on agarose gel (consisted of 1.5% agarose in 1×Tris-Acetate EDTA (TAE) buffer or TBE and 1.5µl of ethidium bromide. The samples were electrophoresed at 80 volts for one hour, shown under ultra violet trans-illuminator and photographed.

## RESULTS AND DISCUSION

The microbial examination of A total 400 mastitis milk samples on MSA media as 271/400(67.75%) were positive growth on MSA medium out of them 240 sample represented by yellow to orange colonies surround by yellow halo on the other hand 31 isolates appear as pink colony without changing surround medium acc. to JP., 2017; USP, 1995/2018 and EP, 2020.

**Table 2:** Different colonies represented by mannitol salt ager medium (MSA) for positive staphylococcus spp isolates.

| Total sample | yellow colony surround by yellow halo on MSA medium | pink colony on MSA medium |
|---|---|---|
| 271 (100%) | 240(88.6%) | 31(11.4%) |
| Suspected *staphylococcus* spp. Causing bovine mastitis | *S. aureus* *S. intermidius, S. xylosus, S. haemolyticus, S. saprophyticus., S. schleiferi subsp coagulans, S. delphini* | *S. epidermidis , S. lugdunensis, S. hyicus* |

Biochemical characters of 157(100%) suspected *staphylococcus* isolates (according to Procop *et al*., 2017) as all negative oxidase test and positive catalase test ,some of them staining by gram's stain showing gram positive cocci arranged in clusters (grapes like appearance). The tube coagulase test preformed as 81/157 (51.6%) were positive clot formation that may indicate *S. aureus,* Gelatin liquefaction test represented 81/157 (51.6%) positive pathogenic *S. aureus*. In addition to perform a terhalose fermentation test 157/157 (100%) Positive terhalose fermentation may produce by *S. aureus, S. intermidius, S. xyloses, S. haemolyticus, S. saprophyticus, S.hyicus.*

We concluded from this previous result that typical phenotypic *S. aureus* represented by 81/157 (51.6%).

**Table 3**: Biochemical test to classification staphylococcus spp isolates.

| test | no. of sample | poitive | variable | negative | indication |
|---|---|---|---|---|---|
| catalase test | 157 | 157 (100%) | 0 | 0 | All *staphylococci* species catalase positive |
| oxidase test | 157 | 0 | 0 | 157 (100%) | All *staphylococci* species oxidase negative |
| tube coagulase test | 157 | 81 (51.6%) | 0 | 76 (48.4 %) | 81 (51.6%) *S. aureus* |
| gelatin liquefaction test | 157 | 81 (51.6%) | 6 (3.8%) | 70 (44.6 %) | Positive pathogenic *S. aureus* |
| terhalose fermentation test | 157 | 157(95.5%) | 7(4.5%) | 0 | Positive *S. aureus, S. intermidius, S. xylosus, S. haemolyticus, S. saprophyticus, S. hyicus/* Negative *S. epidermidis* |

**Genotypic characterization of randomly selected 157 phenotypic suspected *staphylococcus* isolates by cPCR technique.**

**Table 4:** Genotypic classification of 157 phenotypic suspected *staphylococcus* isolates by different primers used.

| gene | no. of sample examined | positive | negative |
|---|---|---|---|
| *16S* | 157 | 141 (89.8%) | 16 (10.2%) |
| *coa* | 141 | 130(92.2%) | 11(7.8%) |
| *nuc* | 141 | 111(78.7%) | 30(21.3%) |
| *ica* | 141 | 81(57.4%) | 60(42.6%) |

*16S* universal *staphylococcus* primer for detection *staphylococcus* microorganism (756bp).
*coa* gene primer for detection *staphylococcus* coagulase positive microorganism (polymorphic band).
*nuc* primer for detection *S. aureus* positive microorganism (279 bp).
*ica* primer for detection *staphylococcus* biofilm formation positive (483 bp).

***% was calculated according to positive isolates (141)**

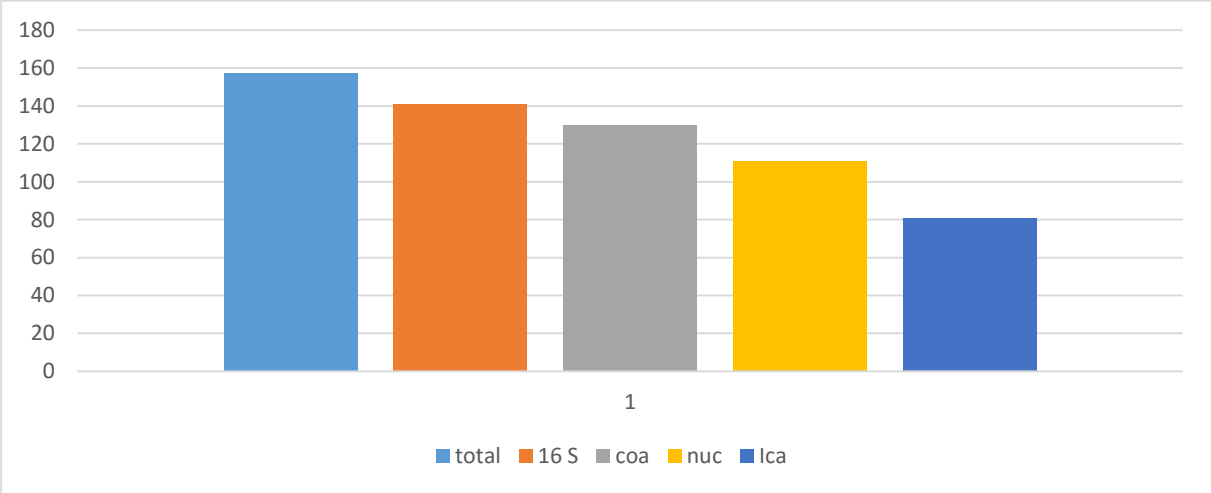


**Chart (1):** represent genotypic classification of 157 phenotypic suspected *staphylococcus* isolates by different primers

Genotypic cPCR of randomly selected 157 phenotypic suspected *staphylococcus* isolates examined by *16S* universal primer for *staphylococcus* as 141/157(89.8%) of phenotypic suspected *staphylococci* isolates were confirmed as *staphylococci*, coagulase gene (Coa gene) detected 130/141 (92.2%) confirmed as coagulase positive *staphylococcus* isolates, *nuc* gene (specific gene for *S. aureus*) were confirmed 111/141(78.7%) *S. aureus* isolates, Also *ica* gene detected in 81/141(57.4%) isolates have the ability to produce biofilm formation as one of resistant action performed by *S. aureus* to resist antibiotics.

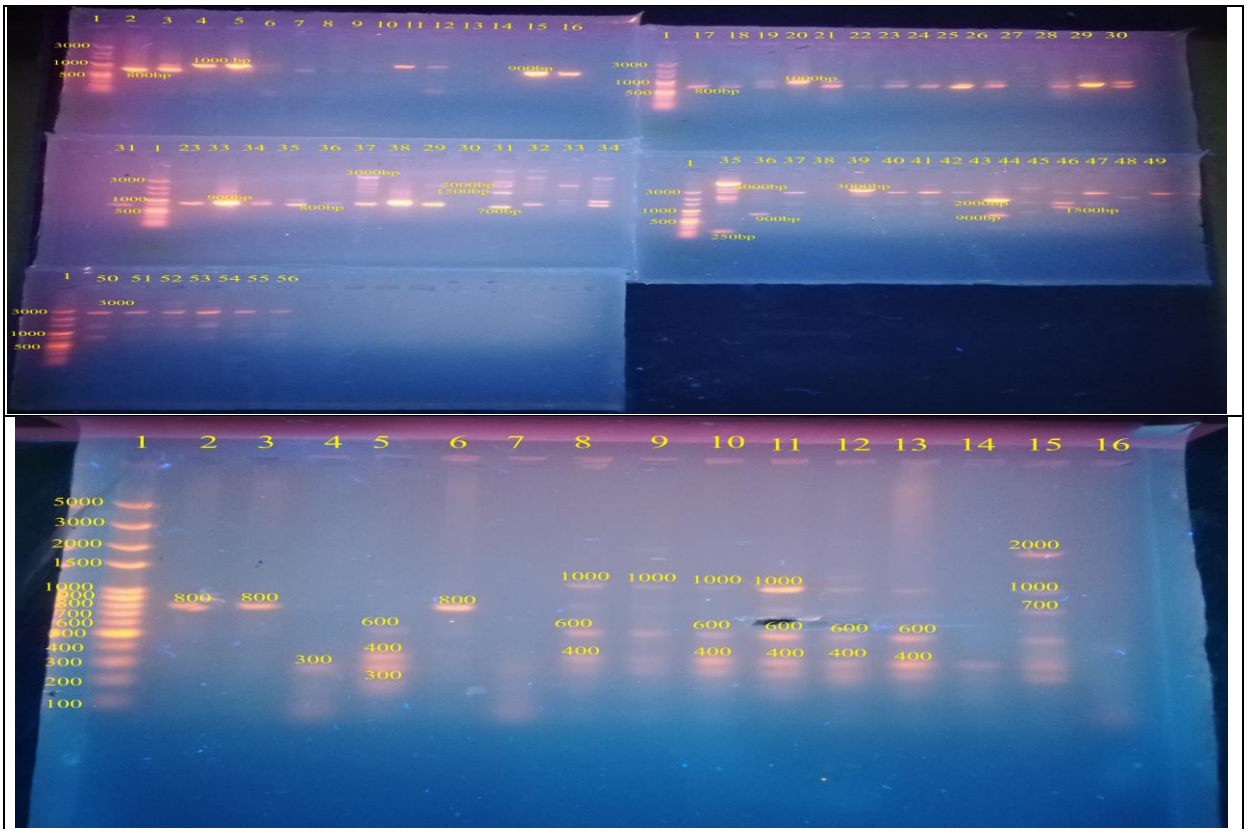


**Fig. (1):** Coagulase *staphylococcus* confirmed with *coa* gene primer, lane (1) ladder 100 bp plus, lane (16) negative control, positive isolates with polymorphic bands, lanes (2, 3, 6 at 800 bp)(4, 5 at 300 bp) (5, 12, 13 at 400,600 bp) (8:11 at 400, 600, 1000 bp) (15 at700, 1000, 2000 bp) positive): coagulase *staphylococcus*, negative isolates (7, 14).


**Fig. (2):** Biofilm formation *staphylococcus* confirmed with *ica* gene primer at 483 bp, lane (1) ladder 100 bp plus, lane (29) control positive, lane (30) negative control, lanes (3:5, 26, 28 faint band, 6:13, 15, 16, 19, 20, 24,27 at 483 bp) positive results.

Comparison between phenotypic *staphylococcus* on different phenotypic test and genotypic expression of *staphylococcus* by different genes, as random selection of 157 isolates positive golden yellow mannitol fermentation on MSA media showing with *16S* universal *staphylococcus* gene 141/157 isolates surly *staphylococcus*, summarized biochemical tests resulted in 81/141 isolates typical phenotypic *S. aureus* in the other hand the *nuc* gene that proved as gene expression to typical *S. aureus* resulted in 111/141 isolates were surely typical *S. aureus*, tube coagulase test resulted in 81/141 isolates, while *coa* gene expressed in 130/141 isolates this may returned to biofilm formation detected in 81/141 of examined isolates by *ica* gene.

**Table 5**: Comparison between phenotypic *staphylococcus* on different phenotypic test and genotypic expression of *staphylococcus* by different genes.

| Phenotypic tests | | Genotypic expression | |
|---|---|---|---|
| Microbial test | positive | Gene expression | positive |
| MSA | 157 | *16S* universal *staphylococcus* primer | 141/157 |
| typical *S. aureus* depending on biochemical tests | 81/141 | *nuc* gene | 111/141 |
| | | *coa* gene | 130/141 |

Genotypically *coa* gene was represented by polymorphism bands, as 50.8% (66/130) of positive *coa* gene represented by single band at (700, 800, 900, 1000, 1500, 3000 bp), 36.2% (47/130) had double bands (mainly at 700/1000 bp), 10.7% (14/130) had three bands (mainly at 800/1500/3000 bp) and 2.3% (3/130) had four bands. Also classified into 52/66 of positive *coa* gene carried single bands, 45/47 of positive *coa* gene isolates carried double band, 13/14 of positive *coa* gene carried triple bands and only one isolates of positive *coa* gene isolates carried quarter band classified as *S. aureus* this was referred that most *S. aureus* isolated from milk sample carried mainly one to three fragments to *coa* gene

**Table 6:** *Coa* gene represented by polymorphism bands.

| Single *coa* gene band | | Double *coa* gene bands | | triple *coa* gene bands | | quarter *coa* gene bands | |
|---|---|---|---|---|---|---|---|
| 700 | 20 | 200/5000 | 1 | 200/700/1000 | 1 | 600/800/900/1500 | 1 |
| | | 300/800 | 1 | | | | |
| 800 | 27 | 700/800 | 1 | 600/900/1500 | 2 | 600/1500/2000/3000 | 1 |
| | | 700/1000 | 19 | | | | |
| 900 | 10 | 700/2000 | 1 | 700/1000/1300 | 1 | 700/1000/1500/3000 | 1 |
| | | 700/3000 | 4 | | | | |
| 1000 | 7 | 700/4000 | 1 | 700/1000/2000 | 1 | | |
| | | 800/900 | 3 | | | | |
| 1500 | 1 | 800/1500 | 4 | 700/1000/3000 | 1 | | |
| | | 800/2000 | 1 | | | | |
| 3000 | 1 | 800/3000 | 2 | 800/1000/1200 | 1 | | |
| | | 900/1000 | 1 | | | | |
| | | 900/1500 | 5 | 800/1500/3000 | 6 | | |
| | | 900/4000 | 2 | | | | |
| | | 1000/1200 | 1 | 900/1000/1500 | 1 | | |
| 66 (50.8%) 52 of them *S. aureus* | | 47 (36.2%) 45 of them *S. aureus* | | 14 (10.7%) 13 of them *S. aureus* | | 3 (2.3%) One of them *S. aureus* | |
| 130 (92.2%) positive *coa* gene | | | | | | | |

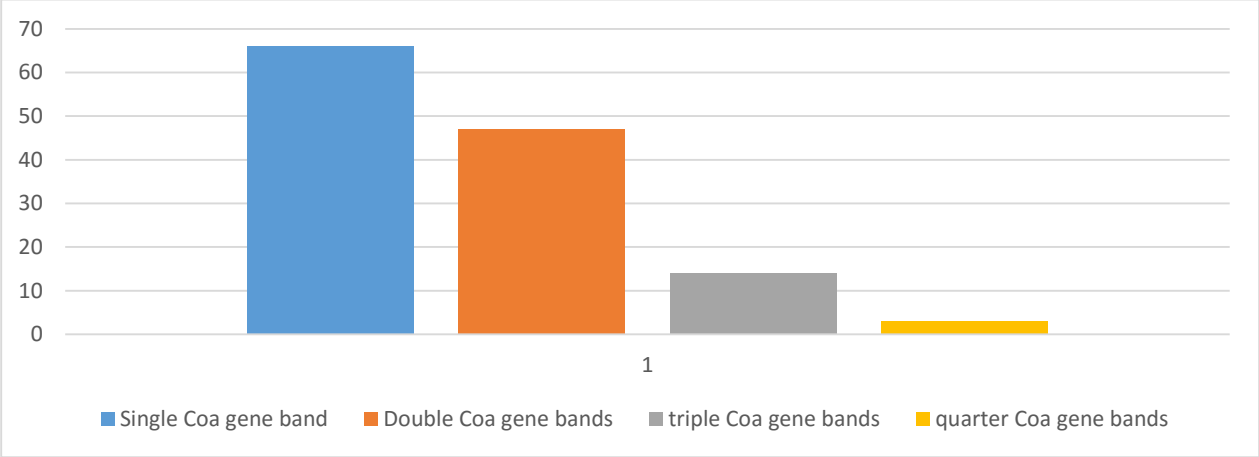


**Chart (2):** illustrated *coa* gene represented by polymorphism bands

*S. aureus* is one of the most important causes of nosocomial infections, (Kasper *et al.*, 2005). Carlton *et al.*, 2010 and Van der Mee-Marquet *et al.*, 2014) Although their reports have highlighted the increasing importance of CoNS species as opportunistic pathogens, but still *S. aureus* remains a major disease-causing agent in veterinary medicine. Furthermore, the increasing role of animals as sources of methicillin-resistant *S. aureus* (MRSA), potentially pathogenic to humans, reinforces the importance of accurately identifying *S. aureus*. (Diederen and Kluytmans, 2006), The increasing rate of CA-MRSA infections in many areas, revealed unique pattern of virulence, clinical picture, and antimicrobial resistance, has important effects on treatment and infection control measures and has a serious challenge for the clinician.

our study detection was classified isolates firstly according to Procop *et al.* (2017) and Effendi *et al.* (2019) as classifying isolates in Table (2,3), which classified *staphylococcus* 271 isolates that cultured on MSA media as 240 (88.6%) represented by yellow to orange colonies surround by yellow halo indicate *S. aureus, S. intermidius, S. xylosus, S. haemolyticus, S. saprophyticus., S. schleiferi subsp coagulans, S. delphini*, on the other hand 31(11.4%) appear as pink colony without changing surround medium *S. epidermidis, S. lugdunensis, S. hyicus*, also classify by coagulase test tube 114/271(42%) CoPS species isolates were *S. aureus* (81/114), *S. intermedius, S. pseudintermedius, S. hyicus, S. schleiferi subsp coagulans, S. delphini*, that similler to result of (Carrillo-Casas & Miranda-Morales, 2012; Vanderhaeghen *et al.*, 2015; USP, 1995; Sartori *et al.*, 2018; Wald *et al.*, 2019). Also isolates directed to biochemical test as Horstmann *et al.* (2012) record that differentiation of MRSP (methicillin- resistant *S. pseudintermedius*) from MRSA on MSA media were more complicated because of similar colony color and size as needed more biochemical test to differentiated them. And also identification and subtyping of such strains is very important to apply suitable infection control programs to control MRSA spread (Zecconi and Piccinini, 1999). Both phenotyping and genotyping can be used to identify MRSA, Felten *et al.* (2002) so gene typing identification has significant role and comparing between Table (3) and table (4) as summarized in Table (5) illustrated that 16 S primer resulted in 89.8% sure positive staphylococcus of suspected positive phenotypic staphylococcus isolates on MSA media and 10.2% sure negative staphylococcus (false positive on MSA media), our results refereed to sensitivity of MSA media 89.8%) and this nearly matching sensitivity result of *Kateete et al.* (2010) on MSA media and lower result of Pumipuntu *et al.* (2017) with difference comparing items (molecular detection method with MSA culturing method). The difference in the results of molecular method

still consider more accurate than MSA media as studies of Koskinen *et al.* (2009); Taponen *et al.* (2009); Elsayed *et al.* (2015); Hoque *et al.* (2018) and Ameen *et al.* (2019). This may returned to biofilm bacteria production that detection in 57.4%, as Devriese *et al.* (1985) and Langlois *et al.* (1990) mentioned that Routine bacteriological tests used in the identification of *S. aureus*, like mannitol fermentation, DNAse production, VP, etc., are not enough for definitive characterization, also, Mathews *et al.* (1997), pay attention to the risk of possible diagnostic errors in the identification and differentiation of coagulase-negative *S. aureus* through conventional phenotypic assays. by using arbitrary primers to amplify target genes by PCR, and opinion of Mahmoudi *et al.* (2017) that referred to genotypic variation among different *S .aureus* isolates, which may be considered as an important criterion when treating *staphylococcal* infection.

Our results detection in table (3,4) nuc gene in 78.7% and *coa* gene in 92.2% of isolates this result agree with Saraiva *et al.* (2018) in their result targeting *nuc* genes 78%, but our result with *coa* gene higher than result of their *coa* as 47%, but our *coa* gene result agree with Abdulghany and Khairy, (2014) reported that about 93% of isolates carried typability of *coa* gene amplification, Da Silva and Da Silva, (2005) reported that about 97% of isolates carried typability of *coa* gene amplification.

Madison and Baselski, (1983); Brakstad *et al.* (1992), Sasaki *et al*. (2010) confirmed that the nuc gene has been specific to all *S. aureus*. But also Giannouli *et al*. (2010) mentioned that most of isolates of *S. aureus* has nuc gene, but some isolates were not positive for the gene, as they were suggesting that return to misidentification by PCR could be related to deletions or mutations occurring in the gene, but their recorded also coagulase production is regulated by *coa* gene in *S. aureus*. Saraiva, *et al*. (2018) mentioned that *nuc* gene and *fem* gene considered an accurate method to identify *S. aureus* species from animal sources.

Also result recorded in table (2,3): *coa* gene detected in 92.2%, while tube coagulase test detected only in 57.4 %, which considered by Pourshadi and Klaas, (1984), Bannerman, (2003) a golden standard detection method, but Tiwari *et al. (*2008), Karahan *et al. (*2009) and Hamza *et al.* (2015) were disagreed them and proved in their study that the coagulase (*coa*) gene PCR the gold standard for the identification of *S. aureus*, Da Motta, (2014) and Zapotoczna *et al.*, (2015), referred to that coagulase has an essential role in *Staphylococcus*, by using *coa* gene detection, with a high frequency of *coa* gene in bovine *S. aureus*. and Davis *et al*. (1990), Although the coagulase tube test is the standard phenotypic routine test used to identify *S. aureus* in biological samples, but also Goh *et al*. (1992), Aarestrup *et al*. (1995), Kapur *et al.* (1995), several groups have implemented the molecular analysis of the coagulase gene as an accurate defined test. Moreillon *et al.* (1995) this gene is associated with the capacity of the pathogen to coagulate fibrin and therefore plays a key role in the pathogenesis of the agent by conferring protection against host immune defense mechanisms and in biofilm production. Uses Coa gene primer as coagulase gene typing has proven to be a simple and effective means to identify coagulase-positive *S. aureus* isolates from both human and animal sources, Nada. *et al.* (1996). *S. aureus* isolated Classification based on the *coa* gene which has been considered a simple and accurate method for molecular typing (Da Silva and Da Silva, 2005). Schlegelova *et al.* (2003), all strains of *S. aureus* secrete coagulase enzyme, as a critical virulent factor.

The difference in our result between tube coagulase test and *coa* gene detection by PCR may agree with Bennett and Monday, 2003, that mentioned that tube coagulase test for *S. intermedius* and *S. hyicus* not detected by human plasma and that distinguish as

clumping factor present in *S. aureus* cell binding to fibrinogen or fibrin present in human and rabbit. Also may return to Abdulghany and Khairy, 2014 observation the difference between biochemical test as MRCoNS isolates and 4 isolates, identified as Coagulase positive by coagulase test, were found to be negative with PCR, so molecular detection of *S. aureus* strains is very important and more accurate.

Also this may return to Coagulase is an enzyme produced by *S. aureus* that causes clotting of blood in the human host. *S. aureus* secretes two forms of coagulase enzyme, bound coagulase and free coagulase, Woodford and Sundsfjord, (2005) Friedman and Ratard, (2007), Free coagulase binds with coagulase-reacting factor (CRF) in plasma and creates a complex, staphylo-thrombin, Hosseinpour *et al. (*1992), Schroder *et al. (*2006).

The disagree between result of tube coagulase test and *coa* gene molecular detection by PCR may return to many comments, beginning from accuracy of tube coagulase test as Cowan and Steel, (1974) that a proved that the rabbit plasma was accepted in a tube coagulase test as an affirmation of the identification of *staphylococcus* aureus, as it was common to use human plasma broth in the bench, with tube testing and slide clumping factor, these with most human strains there is no difficulty, but were deficient in clumping factor and even in free Protein with strains of *S. aureus*, which are resistant to methicillin, according to Lally and Woolfrey, (1984), and Marples and Cooke, (1985). Also Sperber and Tatini (1974): reported that human plasma or a mixture of it and rabbit coagulase plasma EDTA is more suitable for use in the coagulase test than rabbit coagulase plasma EDTA alone. also, Sperber and Tatini, (1975) proved that a mixture of pig and rabbit plasma in the tube coagulase test is the best choice. Followed by Dickson and Marples, (1986) that proved the human plasma is better than rabbit plasma for human *S. aureus* strains, but rabbit plasma is necessary for animal strains. Also interpretive that epidemic methicillin resistant *S. aureus*, other resistant *S. aureus* and other *S. aureus* gave consistently strong positive results with the tube test, on the other hand recording that reference strains, not all true *S. aureus* were positive results in the tube test found. Also with Aarestrup *et al.* (1995); Su *et al.* (1999), as they were interpretive the variation is relevant to the bacterial pool, hard management, and environmental conditions in each geographical region.

But another opinion taken in consideration as reported with Silva *et al. (*2000): proved that their result of tube coagulase test for classifying *Staphylococci* could change as all *S. aureus* strains were coagulant positive but intensity of the test varied according to the source: environmental *S. aureus* strains gave 3+ to 4+, while of milk *S. aureus* strains isolated gave only 1+ to 2+ score. Also proved that 2.2% of *S. aureus* strains were thermo-nuclease negative and Sixteen thermo-nuclease positive and coagulase positive strains were identified as *S. hyicus,* and misdiagnosis may also taken by Karahan and Cetinkaya, (2007), in Turkey, from phenotypic 200 strains of *S. aureus* isolated from 700 milk samples of cows bovine mastitis milk samples, only 16 positive samples contained *coa* gene.

In our study, 92.2% of phenotypic *Staphylococcus* isolates had *coa* gene genotypically as recorded polymorphism model of the *coa* gene, as individual bands measuring 200 to 5000 bp fragments, These results indicate a considerable heterogeneity in the *coa* gene of the isolated strains as in Table (6) Genotypically *coa* gene represented by polymorphism fragment, as 50.8% (66/130) of positive *coa* gene represented by single band at (700, 800, 900, 1000, 1500, 3000 bp), 36.2% (47/130) had double bands (mainly at 700/1000 bp), 10.7% (14/130) had three bands (mainly at 800/1500/3000 bp) and 2.3% (3/130) had four bands. As classified into 52/66 of positive Coa gene carried single bands,

45/47 of positive Coa gene isolates carried double band, 13/14 of positive *coa* gene carried triple bands and only one isolates of positive *coa* gene isolates carried quarter band classified as *S. aureus*, and this somewhat, agreement with results of Karahan and Cetinkaya, (2009) which found that their most isolates (83.9%) produced a single band for coa after PCR amplification, with sizes of 500 to 1,400 bp, whereas a small number of isolates (16.1%) yielded two amplification products. as higher than those shown in the study of Momtaz *et al.* (2011), only 27.9% of phenotypic *S. aureus* had coa gene genotypically. 73.8% of them contain 970 bp fragment and 26.1% contain 730 bp fragments revalent to *coa* gene. Saei, *et al.* (2009), recorded *S. aureus* strains isolated from bovine mastitis milk samples reported 490 to 850 bp fragment. And also observed (Goh *et al*., 1992; Da Silva and Da Silva, 2005 in Brazil and Aslantas *et al.*, 2007) and agreement also with those of Kursat, *et al., (*2011) that observed in a number of isolates their coa gene resulted insize polymorphisms (four to eight repeats) beside some of isolates showing a single amplicon. also Katsuda, *et al.* (2005), reported observation of three to nine tandem repeats in the coa gene; five tandem repeats was the most common form in bovine *S. aureus* strains. Also agree with Goh *et al.* (1992), that mentioned DNA sequence analysis of the Coagulase (*coa*) gene revealed heterogeneity region. PCR amplification of this region showed DNA bands of different size and number and we come out of all these results and observations and understand that most *S. aureus* have single, double, triple and quarter fragment /bands.

The *coa* gene, coding for the coagulase enzyme, can be used for DNA-based diagnosis of S. aureus. The *coa* gene is highly polymorphic because of differences in the sequence of the 30 variable region. Analysis of the *coa* gene in a variety of *staphylococcal* species has shown diversity in the amino acid sequence and the number of tandem repeats at the 30 end. There is heterogeneity in this domain, including the number of 81-bp tandem short sequence repeats encoding repeated 27 amino acid sequences in the C-terminal region. Detecting the coagulase enzyme in *staphylococci* infections is important because it is considered as one of the pathogenic factors of this bacterium (Goh *et al.,* 1992, Talebi-Satlou *et al.,* 2012), and also we can consider the inaccuracy of result of the tube coagulase test return to geographical differences as Smole *et al.,* 1998, proved that geographical differences can correlate with antigenic variation of capsular polysaccharides and surface glycol polysaccharides of *S. aureus* and can affect the identification test for *S. aureus*, a study has been carried out in three different centers in three European countries. Pérez-Roth *et al.* (2001) referred to the low frequency of this gene might be associated with Polymorphisms of the coa gene have been reported in *S. aureus* from different sources and animal species.

Vieira-da-Motta *et al.* (2001), Previous molecular population genetic analysis of *S. aureus* strains recovered from cows showed an alarming heterogeneity of circulating strains, even within a given herd or hospital. Regardless a phenotypic association, we believe that the heterogeneity observed for the coa gene has a potential discriminatory power for future epidemiological studies of veterinary and medical importance.

Sajadi *et al.* (2017): mentioned that their result of molecular coagulase-positive samples with polymorphic sizes reports emphasized that samples were100% resistant to penicillin and higher resistance towards most antibiotics. Talebi-Satlou *et al.* (2012): proved that Coagulase-positive samples showed more resistance to antibiotics, which confirms the virulence of *S. aureus*.

The polymorphic bands to Coa gene may return to Coagulase gene has variance of amplicon which is in line with the reports of (Hookey *et al.,* 1998, Talebi-Satlou *et al.,* 2012, Afrough *et al.,* 2013, Osmonov *et al.,*

2013, Sajadi S.N. *et al.,* 2017). As Goh *et al.* (1992), Schwarzkopf and Karch, 1994, Da Silva and Da Silva, 2005 mentioned that previously, coa gene amplification was reported to produce single-banded PCR products in *S. aureus* strains isolated from human and animal samples, but thier studies notcied double-branded products for coa gene, but this was a rare finding. After that, Tiwari *et al.* (2008) recorded double and triple bands from human samples were observed. Gharib *et al.* (2013) reported Triple bands in Egypt from human and animal samples.

Abdulghany and Khairy, 2014 reported a completely different finding: multiple bands amplification products (1, 2, 3, 4, 5, and eight bands) were detected for Coa gene which classifying studied strains as 15 coa PCR types, and this agreed partly with da Silva R. E. and da Silva N., 2005 (27 types), and disagreed with the most of other researchers: Janwithayanuchit *et al.* (2006), who determined 4 different patterns of coa gene in 129 MRSA isolates, Himabindu *et al.* (2009), who reported 3 classes among 85 isolates, Demir *et al.* (2011), who reported 4 patterns in 120 isolates, and Talebi-Satlou *et al.* (2012), who reported 4 products in 26 isolates as Goh *et al.* (1992), suggest That most studies that the variability in size and number of coa bands detected may be due to the presence of different allelic coa gene forms in MRSA, allowing one strain to form multiple amplicons.

Also, coa gene polymorphism result in some studies Attributed to Kav *et al*. (2011) that suggested that milking personnel may play a role in the transmission of *S. aureus,* and coa restriction fragment length polymorphism.

*S. aureus* has the ability to produce biofilm to facilitate them to withstand the host immune response recognized as one factor contributing to chronic or persistent infections, represented by biosynthesis of polysaccharide intercellular adhesion (PIA) molecules, Different studies have shown the decisive role of the ica gene as virulence factors in staphylococcal infections Namvar, *et al.* (2013). As ica gene detected in the study represented in 81 (57.4%). As (Kostaki *et al.,* 2012; Mah 2012; Bridier *et al.,* 2015 and Avila-Novoa *et al.,* 2018) proven that most of the *S. aureus* strains formed the biofilm in an *ica*-dependent mechanism as 74 biofilm-positive strains, including about 76% were *ica*. As biofilms, increase bacterial resistance to environmental stresses (including cleaning, disinfection and inhibition) enabling these microorganisms to persist on surfaces and processing equipment, compared to planktonic cells. Namvar *et al.* (2013), proven that the ica-encoded gene (biofilm) one of virulence factors produced by staphylococcus that facilitates them to withstand the host immune response and is recognized as one factor contributing to chronic or persistent infections.

## CONCOLUSION

The *nuc* gene still considered as the main golden diagnostic slandered technique in identification *S. aureus* as quickly and accurate tool than the MSA media and and the *coa* gene more accurate in the face of the tube coagulase test which became old tools in detection coagulase virulent gene due to fast developing of mutation by *S. aureus* to protect themselves from most antibiotic forming biofilm (*ica* gene) surrounding them (which protected *staphylococcus* from phagocytosis by immune cells and persist infection due to resistance to environmental stresses (including cleaning, disinfection and inhibition mechanism).

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## الاختلاف بين التوصيف الظاهرى والجينى للمكور العنقودى الذهبى المعزول من الابقار المصابة بالتهاب الضرع فى مصر

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المكورات العنقودية الذهبية أحد أهم أسباب عدوي المستشفيات ، ومسببات الأمراض المعدية الرئيسية التي تلعب دورًا حيويًا وهامًا في التهاب الضرع البقري في الطب البيطري مما يتسبب في خسائر اقتصادية عالية في جميع أنحاء العالم ، وتعتبر البقرة هي الحامل الاولي لذلك الميكروب، ومؤخراً تم تسجيله على أنه ميكروب حيواني المنشأ قادر على الانتقال من الحيوان إلى الإنسان والعكس صحيح، وأصبح التعرف على أنواع المكورات العنقودية بشكل رئيسي المكورات العنقودية الذهبية أكثر سرعة ودقة مع التقنية الجزيئية حيث أن تقنية النمط الظاهري بسبب مقاومة الأدوية المتعددة طورت بعض الطفرات وإنتاج الأغشية الحيوية التي تتداخل مع تحديد النمط الظاهري ، حيث أصبح جين *nuc* وجين *coa* الأسلوب القياسي الذهبي لتحديد *S. aureus* وتناولت الدراسة التمييز بين التوصيف الظاهري والنمط الجيني للعزلات التي تم جمعها من عينات اللبن المجمة من الابقار المصابة بالتهاب الضرع، ١٥٧ عزلة من المكورات العنقودية المظهرية الايجابية للنمط الظاهري على وسط MSA والاختبارات البيوكيميائية، ١٥٧/٨١ (٥١,٦٪) من العزلات كانت من المكورات العنقودية الذهبية المظهرية ، وبالطريقة الجزيئية ، أظهر الكشف بالجين العام (16S) ١٥٧/١٤١(٨٩,٨٪) عزلة مؤكدة للمكور العنقودي و ١٤١/١٣٠ (٩٢,٢٪) كانت للمكور العنقودي ايجابي التجلط بالكشف عن جين *coa* ، و ١٤١/١١١ (٧٨,٧٪) من المكورات العنقودية الذهبية التي تم توصيفها بواسطة جين *nuc* ، وحيث أن معظم العزلات أنتجت غشاء حيوي تم توصيفه بواسطة جين *ica* في ١٤١/٨١ (٥٧,٤٪) والظن الاكبر في ان تكوين الميكروب لهذا الغشاء حوله تسبب في تداخل النتايج للتوصيف الظاهري بواسطة الاختبارات البيوكيميائية.