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

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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 etiopathology, 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
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 Monofiya 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

**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 37°C 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 37°C 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 cloting, 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 °c 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**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product size</th>
<th>Primer Nucleotides sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>756 bp</td>
<td>5'-AAC TCT GTT ATT AGG GAA GAA CA-3'</td>
<td>Zhang et al., 2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-CCA CCT TCC TCC GGT TTG TCA CC-3'</td>
<td></td>
</tr>
<tr>
<td>Nuc</td>
<td>279 bp</td>
<td>GCG ATT GAT GGT GAT ACG GTT ACG CAA GCC TTG ACG AAC TAA AGC</td>
<td>Shortle, 1983</td>
</tr>
<tr>
<td>Ica gene</td>
<td>483 bp</td>
<td>GAA CCG CTT GCC ATG TGT TG GCT TGA CCA TGT TGC GTA ACC</td>
<td>Namvar et al., 2013</td>
</tr>
<tr>
<td>Coa Polymorphism</td>
<td>CGA GAC CAA GAT TCA ACA AG AAA GAA AAC CAC TCA CAT CA</td>
<td>Guler et al., 2005</td>
<td></td>
</tr>
</tbody>
</table>

**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 DISCUSSION**

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.

<table>
<thead>
<tr>
<th>Total sample</th>
<th>yellow colony surround by yellow halo on MSA medium</th>
<th>pink colony on MSA medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>271 (100%)</td>
<td>240 (88.6%)</td>
<td>31 (11.4%)</td>
</tr>
</tbody>
</table>

Suspected staphylococcus spp. Causing bovine mastitis

<table>
<thead>
<tr>
<th>S. aureus</th>
<th>S. intermidius, S. xylosus, S. haemolyticus, S. saprophyticus., S. schleiferi subsp coagulans, S. delphini</th>
</tr>
</thead>
</table>

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.

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

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.

<table>
<thead>
<tr>
<th>gene</th>
<th>no. of sample examined</th>
<th>positive</th>
<th>negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S</td>
<td>157</td>
<td>141 (89.8%)</td>
<td>16 (10.2%)</td>
</tr>
<tr>
<td>coa</td>
<td>141</td>
<td>130 (92.2%)</td>
<td>11 (7.8%)</td>
</tr>
<tr>
<td>nuc</td>
<td>141</td>
<td>111 (78.7%)</td>
<td>30 (21.3%)</td>
</tr>
<tr>
<td>ica</td>
<td>141</td>
<td>81 (57.4%)</td>
<td>60 (42.6%)</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Phenotypic tests</th>
<th>Genotypic expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbial test</td>
<td>Gene expression</td>
</tr>
<tr>
<td></td>
<td>positive</td>
</tr>
<tr>
<td></td>
<td>positive</td>
</tr>
<tr>
<td>MSA</td>
<td>157</td>
</tr>
<tr>
<td>typical S. aureus</td>
<td>141/157</td>
</tr>
<tr>
<td>depending on</td>
<td></td>
</tr>
<tr>
<td>biochemical tests</td>
<td>81/141</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Single coa gene band</th>
<th>Double coa gene bands</th>
<th>Triple coa gene bands</th>
<th>Quarter coa gene bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>700</td>
<td>20</td>
<td>200/5000</td>
<td>1</td>
</tr>
<tr>
<td>800</td>
<td>27</td>
<td>300/800/1000</td>
<td>2</td>
</tr>
<tr>
<td>900</td>
<td>10</td>
<td>700/800/1000</td>
<td>1</td>
</tr>
<tr>
<td>1000</td>
<td>7</td>
<td>700/2000</td>
<td>4</td>
</tr>
<tr>
<td>1500</td>
<td>1</td>
<td>700/4000</td>
<td>6</td>
</tr>
<tr>
<td>3000</td>
<td>1</td>
<td>800/1500/3000</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>800/2000</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>900/1500</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>900/4000</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000/1200</td>
<td>6</td>
</tr>
<tr>
<td>66 (50.8%)</td>
<td>47 (36.2%)</td>
<td>14 (10.7%)</td>
<td>3 (2.3%)</td>
</tr>
<tr>
<td>52 of them S. aureus</td>
<td>45 of them S. aureus</td>
<td>13 of them S. aureus</td>
<td>One of them S. aureus</td>
</tr>
<tr>
<td>130 (92.2%)</td>
<td>positive coa gene</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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. intermedius, 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 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 Montaz 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 noticed 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-Satlo 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.

CONCLUSIONS

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 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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المصابة بالتهاب الاختلاف بين التوصيف الظاهري والجيني للمكور العنقودي الذهبى المعزول من الأبقار

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المكورات العنقودية الذهبية أحد أهم أسباب عدوي المستشفيات، وسبب الأمراض المعدية الرئيسية التي تلعب دورًا حيويًا وذريًا في التهاب الضرع البقري في الطب البيطري مما يسبب في خسائر اقتصادية عالية في جميع أنحاء العالم، وتعتبر البقرة هي الحامل الأولى لذلك الميكروب، وموجلاً 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 المعدة طورت بعض الطفرات وانتاج الأغشية الحيوية التي تداخل مع تحديد النمط الظاهري، حيث أصبح جين coa وجين nuc الأسلاك القياسي الذهبي لتحديد S. aureus وتناولت الدراسة التمييز بين التوصيف الظاهري ونظام الجيني للعزلات التي تم جمعها من عينات الليم جمعة من الأبقار المصابة بالتهاب الضرع، 167 عزلة من المكورات العنقودية الظهرياتがありました من العزلات كانت من MSA والاختبارات البيوكيميائية (15/75% × 157/141% × 161/89%, 157/81% × 157/141% × 161/89%, 157/141% × 161/89%, 157/81% × 157/141% × 161/89%).

عزلة مؤكدة للمكور العنقودي coa و coa كان للعزلات الذهبي coa و coa، و nuc coa دخلت على بعض العزلات 141/1111 (1111/141% × 141/1111) أنتجت غشاء حبيبي أو تصلب توصيف وجود جين coa في coa 141/1111 (1111/141% × 141/1111) والظنين الأكبر في أن يكون الميكروب لهذا الغشاء حيوي تصلب في تداخل النتائج للتصويت الظاهري بواسطة الاختبارات البيوكيميائية.