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# Detection of Virulence Genes in *Staphylococcus aureus* and *Streptococcus agalactiae* Isolated from Mastitis in the Middle East

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author AEB designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed the literature searches. Authors ME, EM and YB managed the analyses of the study and literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** The current study was carried out to genotypically characterize *Staphylococcus aureus* (*S. aureus*) and *Streptococcus agalactiae* (*S. agalactiae*) isolated from clinical and subclincal cases of bovine mastitis.
**Study Design:** The *S. aureus* and *S. agalactiae* strains were characterized phenotypically, and were further characterized genotypically by polymerase chain reaction (PCR) using oligonucleotide primers that amplified genes encoding the fibronectin-binding proteins A (*fnbA*), the clumping factors A (*clfA*), the surface protein A (*spa*) and the coagulase (*coa*) genes for *S. aureus* and the surface immunogenic protein (*sip*), hyluronidase (*hyl*), CAMP factor (*cfb*), surface enzyme (*scpB*), β-hemolysin/cytolysin (*cylE*) and alpha-C-protein (*bca*) for *S. agalactiae*.

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**Place and Duration of Study:** A total of 61 *S. aureus* and 43 *S. agalactiae* isolates were obtained from milk samples collected from 160 apparently healthy cows and 54 clinical cases during a survey conducted for six months at different localities in El-Behira province, Egypt and Al-Qassim region, Kingdom of Saudi Arabia.

**Results:** 59 (~97%) of *S. aureus* isolates illustrated a positive coagulase-test and 100% were positive when tested with the BBL™ Staphyloslide™ Latex. All identified strains of *S. aureus* were PCR positive for the *fnbA*, *clfA*, *spa* (x-region) and *spa* (IgG Binding region) genes which displayed a single size amplicon of approximately 1226 bp (73.77%), 1000 bp (88.52%), 500 bp (80.32%) and 300 bp (19.67%), respectively. In contrast, the amplification of the *coa*-gene exhibited three dissimilar size polymorphisms with around 500 bp (57.37%), 600 bp for (8.19%) and 900 bp (8.19%). Furthermore, 42 (~98%) out of 43 *S. agalactiae* isolates were positive by agglutination of the blue latex particles within 20 second in the test circle and 100% demonstrated a positive results in the CAMP test. All confirmed *S. agalactiae* isolates were also PCR positive for the *sip* gene (90.69%), *hyl* gene (81.39%), *cfb* gene (93.02%), *cylE* gene (90.69%), *scpB* gene (67.44%) and *bca* gene (20.93%).

**Conclusion:** The high frequency of virulence genes detected in the current study will help in the understanding of the distribution of infectious *S. aureus* and *S. agalactiae* strains in the Middle East and contribute to the establishment of preventive approaches to reduce the spread of infection.

*Keywords: Staphylococcus aureus; Streptococcus agalactiae; virulence genes; mastitis.*

## 1. INTRODUCTION

Bovine mastitis is considered as one of the most common problematic diseases and continues to have major economic impact on the dairy industry worldwide [1,2]. Among several bacterial pathogens that cause mastitis, *S. aureus* and *S. agalactiae* are probably the most lethal agents that are extremely problematic to be treated [3,4]. Generally, mastitis caused by these two pathogens is chiefly subclinical type, consequently initial recognition of such cases is very important. Although of high incidence of Staphylococci and Streptococci in both clinical and subclinical bovine mastitis, scanty information is available about the virulence factors of both pathogens [5]. For improving the health condition of the animals and decrease economic losses, it is crucial to reduce the incidence of bovine mastitis caused by these pathogens. To successfully achieve this, it is necessary to develop the current information about the virulence factors of *S. aureus* and *S. agalactiae*.

*S. aureus* encodes several protein substances that act as virulence factors, leading to a variety of infections, including intramammary infection (IMI) in cows. Among these virulence factors *spA*, *coA*, *clfA* and *fnbA* are significant for the capability of *S. aureus* to adhere to and invade phagocytic cells as well as to evade the host immune system. *Spa* is a membrane-bound exoprotein illustrated and acknowledged for its capability to adhere to the fragment crystallizable region (Fc region) of immunoglobulins of various animal species [6,7]. This protein is codified by the *Spa* gene with a polymorphic (X) and a conserved region. Coelho et al. [7] and Koreen et al. [8] indicated that the polymorphic region X consists of a different number of repeated 24 base pairs being located in a certain region of cellular wall C-terminal extremity. Palma et al. [9] found that *coa* protein has the ability to convert fibrinogen into fibrin threads by a mechanism varies from natural clotting. In addition, *coa* is considered a virulence factor in the IMI. This protein possesses a conserved and a repeated polymorphic region that can be used to measure the relatedness among *S. aureus* isolates. It is documented that the primary adherence of *S. aureus* of epithelial lining cells of the teat canal depends on the interaction of bacterial surface proteins, such as *clfA* and *fnbA* with host fibrinogen and fibronectin proteins located in the basement membrane, around myoepithelial cells and fibroblasts [10-12].

Furthermore, it is identified that bovine mastitis caused by *S. agalactiae* (Lancefield group B; GBS) is a grave problem next to *S. aureus* that affect on the health condition of animal and farm productivity [13,14]. Despite the high incidence of *S. agalactiae* in both clinical and subclinical cases of bovine mastitis, scanty data is available about its virulence factors [5]. The ability of the bacteria to invade the immune host cells needs a various virulence factors to begin growth and multiplication. *S. agalactiae* possesses several virulence factors including structural components, toxins and enzymes that play an important role in IMI [5]. During the last decade,

several cell-associated and extra-cellular virulence factors of *Streptococcus* species have been detected and these pathogens have the ability to react with numerous plasma and extra-cellular host derived protein like immunoglobulin G, victronectin, fibrinogen, collagen and plasminogen [5]. Such interactions can be facilitated by numerous virulence attributes such as hyluronidase, fibrinolysin and pore-forming protein toxins which play a significant role in the penetration of *S. agalactiae* in the udder tissue, but until now their role in the transmission and pathogenesis of bovine mastitis is not clear [15].

From the previously mentioned data, it was concluded that different isolates of *S. aureus* and *S. agalactiae* have virulence factors that probably develop their ability to colonize and persist in the mammary gland and even on inert surfaces. The detection of virulence factors of *S. aureus* and *S. agalactiae* in the context of clinical and subclinical mastitis in various dairy farms at El-Behira province of Egypt and Al-Qassim region in the Kingdom of Saudi Arabia may be helpful to build up accurate actions, treatments to control bovine mastitis [16].

## 2. MATERIALS AND METHODS

### 2.1 Collection of Milk Samples

Milk samples were collected from 160 apparently healthy cows ((invisible abnormalities with high rise in the total somatic cell count)) and 54 suffered from clinical mastitis (milk with clots) within six months from June to August 2014 in El-Behira province, Egypt and from December 2014 to February 2015 at different locations in Al-Qassim region, Kingdom of Saudi Arabia. To isolate bacteria from milk samples, about 100 µl of sampled milk having total somatic cell count (SCC) more than 5.000.000 cells/ml were firstly streaked onto blood agar plates and incubated at 37ºC for 48 h under 10% $CO_2$ tension to obtain pure cultures free from contamination. Isolation of all isolates was done according to the National Mastitis Council recommendations on examination of quarter-milk samples. The isolates were then stored at -80ºC for further investigation.

### 2.2 Bacterial Isolates

A total of 61 *S. aureus* and 43 *S. agalactiae* field isolates isolated from clinical and subclinical cases of bovine mastitis were used in the current study. The reference *S. aureus* strains used for each test was *S. aureus* ATCC® 25923 as a positive control and *Staphylococcus epidermidis* (*S. epidermidis*) ATCC™ 12228 as negative control while the reference *S. agalactiae* strain was *S. agalactiae* ATCC® 12386.

### 2.3 Phenotypic Characterization of *S. aureus*

#### 2.3.1 Coagulase, DNase, capsule and slime formation tests

*S. aureus* isolates identified by a series of laboratory methods and their proof through PCR is reported in our earlier study. In brief, coagulase activity was observed in tubes (Tube Coagulase Test, TCT) as illustrated by Quinn et al. [17] and inspected after 4 and 24 h. DNase test was carried out by incubating the samples (isolates) for 24 hours at 37ºC on DNase agar (Thermo Scientific, United States), and pouring approximately 15 ml of 1 N HCl. Excessive amount of acid was detached with a vacuum pipette and DNase positive colonies were demonstrated by clear zones around the bacterial colonies. Presence or absence of bacterial capsule was detected by microscopic examination according to the method described by Turkyilmaz and Kaya [18]. In addition, slime formation was determined using Congo Red Agar (CRA) technique developed by Freeman [18]. Slime-positive isolates were observed as black colonies, while those demonstrating pink colonies were believed to be slime-negative isolates.

#### 2.3.2 BBL™ staphyloslide™ Latex Test

The BBL™ Staphyloslide™ Latex test (Becton, Dickinson and Company, 7 Loveton Circle, Sparks, MD 21152-0999 USA) was used according to the manufacture procedures for characterization of the *S. aureus* by possessing clumping factor and/or Protein A, usually not found in other types of staphylococci.

### 2.4 Phenotypic Characterization of *S. agalactiae*

#### 2.4.1 CAMP reaction

For carrying out this reaction, a β-hemolytic *S. aureus* culture was inoculated across the center of the sheep blood plate and a single colony of the unknown strain (beta hemolytic streptococci) is picked up with an inoculating loop and utilized to make a single streak vertical

but not touching the *S. aureus* streak. A 2-3 mm space should remain between the streaks and a positive reaction was seen after incubation for 18-24 h at 37℃ as a half moon forming zone of complete hemolysis in the zone of incomplete staphylococcal-β- hemolysis. As, commercially available sheep blood agar does not constantly reveal the correct CAMP reaction, group B streptococcus strain SS-617 was used as a positive control on each test plate.

#### 2.4.2 Hemolytic reaction

The hemolytic reaction is predominantly helpful in the differentiation of the Streptococci. This reaction was detected on Trypticase Soy Agar with 5% Sheep Blood. Briefly, all samples were streaked immediately after milking on TSA and subsequently all plates were aerobically incubated in carbon dioxide for 24 h at 37ºC. The suspected colonies of *S. agalactiae* were streaked again on sterilized plates to obtain pure cultures. The use of sheep blood agar plates permit the haemolysis of *S. agalactiae* suspect colonies and allows the growth of *S. agalactiae* but does not support the growth of *Haemophilus haemolyticus* which appears to resemble streptococci on agar containing rabbit, horse, or human blood. ß-hemolytic reaction was observed as complete clearing around the colony. Moreover, α-hemolytic reaction was interpreted as green around the colony and γ-hemolysis was also interpreted as no change in the media surrounding the colony.

#### 2.4.3 BBL ™streptocard™ acid latex test

Acid latex test is considered a Streptococcus grouping test using nitrous acid extraction for the rapid identification of β-hemolytic Streptococci of Lancefield types A, B, C, F and G. Principles and procedures of The BBL™ Streptocard™ Acid Latex Test (Becton, Dickinson and Company, USA) were done according to the instructions of the manufacturer.

### 2.5 Detection of virulence genes encoding for *S. aureus* and *S. agalactiae*

#### 2.5.1 DNA extraction

Extraction of the genomic DNA templates of *S. aureus* and *S. agalactiae* was carried out by DNeasy blood and tissue kit (Qiagen AG, Hombrechtikon, Switzerland).

#### 2.5.2 Virulence gene primers

As can be seen in Table 1, PCR amplifications were carried out by a pair of primers (forward and reverse) specific for several genes such as *spA*, *coa*, *clfA* and *fnbA* for *S. aureus* and *sip*, *hyl*, *cfb*, *scpB*, *cylE* and *bca* for *S. agalactiae* (Table 2). All PCR primers used in the present study were designed by Qiagen AG, Hombrechtikon, Switzerland. A genus-specific, 23S rRNA and 16S rRNA sequences were used as an internal amplification control for

**Table 1. Oligonucleotide sequences and product length of *S. aureus* virulence gene specific primers**

| Gene | Sequence 5´-3´ Forward (F) and Reverse (R) | Amplicon size (bp) | References |
|---|---|---|---|
| 23S rRNA-F | ACGGAGTTACAAAAG GACGAC | 1250 | [20] |
| 23S rRNA-R | AGCTCAGCCTTAACGAGTAC | | |
| *fnbA*-F | CACAACCAGCAAATATAG | 1226 | [21] |
| *fnbA*-R | CTGTGTGGTAATCAATGTC | | |
| *clfA*-F | GGCTTCAGTGCTTGTAGG | 1000 | [22] |
| *cfA*-R | TTTTCAGGGTCAATATAAGC | | |
| *spa* (IgG-binding) -F | CACCTGCTGCAAATGCTGCG | 900 | [23] |
| *spa* (IgG-binding) -R | GGCTTGTTGTTGTCTTCCTC | | |
| *spa* (X-region) -F | CAAGCACCAAAAGAGGAA | 300 | [24] |
| *spa* (X-region) -R | CACCAGGTTTAACGACAT | | |
| *coa1*-F | ATAGAGATGCTGGTACAGG | 500 | [25] |
| *coa1*-R | GCTTCCGATTGTTCGATGC | | |
| *coa2*-F | GCTTCCGATTGTTCGATGC | 600 | [25] |
| *coa2*-R | ATAGAGATGCTGGTACAGG | | |
| *coa3*-F | CCAGACCAAGATTCAATAAQ | 900 | [26] |
| *coa3*-R | AAAGAAAACCACTCACATCGT | | |

**Table 2. Oligonucleotide sequences and product length of *S. agalactiae* virulence gene specific primers**

| Gene | Sequence 5´-3´ Forward (F) and Reverse (R) | Amplicon size (bp) | References |
|---|---|---|---|
| *hyl*-F | TTAACAAAGATATATAACAA | 950 | [27] |
| *hyl*-R | TTTTAGAGAATGAGAAAAAA | | |
| *cfb*-F | CAAAGATAATGTTCAGGGAACAGATTATG | 320 | [5] |
| *cfb*-R | CTTTTGTTCTAATGCCTTTACGTT | | |
| *cylE*-F | TGACATTTACAAGTGACGAAG | 248 | [28] |
| *cylE*-R | TTGCCAGGAGGAGAATAGGA | | |
| *scpB*-F | ACAACGGAAGGCGCTACTGTTC | 255 | [29] |
| *scpB*-R | ACCTGGTGTTTGACCTGAACT | | |
| *bca*-F | TAACAGTTATGATACTTCACAGAC | 535 | [30] |
| *bca*-R | ACGACTTTCTTCCGTCCACTTAG | | |
| *sip*-F | ACTATTGACATCGACAATGGCAGC | 266 | [31] |
| *sip*-R | GTTACTGTCAGTGTTGTCTCA | | |

staphylococcal and streptococcal DNA. The sequence of primers and the sizes of the amplified products are expressed in Tables 1 and 2. The procedures of PCR were performed in a 25 µl reaction volume containing 0.5 µM of each primer, PCR master mix with 3 mm of $MgCl_2$ and 3 µl of template DNA.

The reaction mixtures were amplified with 25 cycles, each consisting of 30 s at 94C, 60 s at 45 C and 90 s at 72C in a T100 thermal cycler manufactured by the company BioRad, USA. After amplification, the PCR products were investigated by gel electrophoresis in 1.5% agarose gel containing ethidium bromide [19]. The products were then photographed with PhotoDoc-It TM 65 Imaging system (Cambridge, UK).

## 3. RESULTS

### 3.1 Characterization of *S. aureus* and *S. agalactiae*

Milk samples were collected from 214 subclinical and clinical cases of IMI. 61 specimens (28.50%) and another 43 specimens (20%) of total milk samples in microbiological studies were identified as *S. aureus* and *S. agalactiae*, respectively. Due to simplicity and cost of the phenotypic methods, identification of bacterial pathogens still depends chiefly on these methods. In the present study, 59 (~97%) of staphylococcal isolates illustrated a positive coagulase-test result after 4 h of incubation. In addition, all isolates of staphylococcus tested with the BBL™ Staphyloslide™ Latex were identified as *S. aureus* including the two negative isolates for coagulase test. The 43 streptococcal samples were also examined with the BBL™ Streptocard™ acid latex test and 42 samples were positive by agglutination of the blue latex particles within 20 second in the test circle, with no agglutination in the control circle and therefore were identified as *S. agalactiae*. As well, the 43 streptococcal isolates identified as *S. agalactiae* also demonstrated typical positive results in the CAMP test indicated by an "arrowhead"-shaped enhanced zone of b-hemolysis.

### 3.2 Detection of Virulence Genes for *S. aureus* and *S. agalactiae*

A total of 61 strains of *S. aureus* were additionally examined for different virulence genes. PCR amplification of the gene segment encoding the *fnbA* revealed a size of approximately 1226 bp of 45 isolates (Table 3). The amplification of the gene segments encoding the *clfA* revealed the typical size approximately 1000 bp of 54 isolates (Table 3). Moreover, *S. aureus* coding the *spa* is considered an important gene for typing of *S. aureus*; it was determined in this study in the majority of isolates. As can be noticed in Table 3, PCR amplification of this gene revealed that 59 specimens contained the *spa* gene (x-region) with size about 900 bp in contrast, 12 specimens contained *spa* gene (IgG Binding region) with approximately 300 bp. The *coa* gene amplicon obtained from 49 *S. aureus* isolated from milk of bovine mastitis was about 500 bp in size. Moreover, 43 *S. agalactiae* isolates were examined for various virulence genes and according to the results obtained, 35 specimens of 43 isolates contained the *hyl* gene (Table 4)., 40 specimens contained the *cfb* gene, 39 specimens contained the *cylE* gene, 29 specimens contained *scpB* gene, 9 samples

**Table 3. Frequency of certain virulence genes in the *S. aureus* strains isolated from clinical and subclinical cases of bovine mastitis**

| Specimens | *fnbA* | *clfA* | *spa* (IgG binding) | *spa* (X-region) | *coa* 1 | *coa* 2 | *coa* 3 |
|---|---|---|---|---|---|---|---|
| 61 | 45 | 54 | 12 | 59 | 35 | 5 | 5 |

contained the *bca* gene and 39 samples contained the *sip* gene. From the previous results, various virulence genes were detected in the *S. aureus* and *S. agalactiae* strains isolated from bovine clinical and subclinical mastitis milk in Egypt and Kingdom of Saudi Arabia.

**Table 4. Frequency of certain virulence genes in the *S. agalactiae* strains isolated from clinical and subclinical cases of bovine mastitis**

| Specimens | *hyl* | *cfb* | *cylE* | *scpB* | *bca* |
|---|---|---|---|---|---|
| 43 | 35 | 40 | 39 | 29 | 9 |

## 4. DISCUSSION

The control of bovine mastitis is very important not only in the Middle East but also worldwide as mastitis is considered the main cause among several diseases involved in reduction of milk production [32,33]. Consequently, it is crucial to examine the mastitis causing bacteria using molecular methods as forceful tools to control IMI. Because *S. aureus* and *S. agalactiae* are the most commonly contagious mastitis pathogens worldwide, it is important to reveal subtypes and virulence factors of these agents to develop effective control strategies against mastitis caused by both pathogens [33]. In addition, an effective vaccine against IMI is not available, therefore prevention and control of mastitis needs detection of the principal antigenic determinants for the strategy and progress of more proficient vaccines against mastitis causing bacteria especially *S. aureus* and *S. agalactiae*. Hence, the frequency of certain virulence genes such as *spa*, *coa*, *clfA* and *fnbA* for *S. aureus* and *sip*, *hyl*, *cfb*, *scpB*, *cylE* and *bca* for *S. agalactiae* was evaluated in this study.

The amplification of the gene segments encoding *clfA*, *spa* (x-region) and *spa* (IgG Binding region) for *S. aureus* strains revealed typical sizes approximately 1000 bp of 54 isolates, 900 bp for 59 specimens, 300 bp for 12 specimens, respectively. The presence of the *clfA* gene and the gene encoding the X-region of the protein A are believed to be the most common virulence genes help in the development and severity of *S. aureus* mastitis [3,34]. Moreover, the amplicon of the *coa*-gene demonstrated a single size polymorphism with approximately 500 bp for 35 (~55%) strains, 600 bp for 5 (~8%) strains and 900 bp for 5 (~8%) strains. In this study, the most common amplicon for coa gene was 500 bp which displayed in most isolates. These results are similar to the results obtained by Cabral et al. [35] who proposed that an amplicon of approximately 600 bp is common in strains isolated from bovine mastitis in Brazil. Hence, it is essential to note that in the current study, some strains presented more than one amplicon what could be explained by the presence of more than one allelic form of the coa gene [7,36]. According to the results in the present study, 45 strains (80.32%) contained the *coa* gene with different sizes and this result was in agreement with results obtained by Akineden et al. [33] and Karahan and Cetinkaya [37]. In their study from the 200 *S. aureus* strains isolated from the subclinical bovine mastitis, 161 samples (80.6%) contained the *coa* gene. These results show that this is a direct association between the existence of the *coa* gene in *S. aureus* and bovine mastitis.

Because *S. agalactiae* is considered one of the major contagious mastitis pathogen after *S. aureus*, preventive strategy was carried out to eradicate *S. agalactiae* mastitis from numerous countries in Western Europe [38,39] but this type of mastitis is still causing a severe problem in developing countries. Therefore, studying the different virulence genes is very crucial for prevention and control of mastitis caused by *S. agalactiae* among dairy herds. In the present study, the gene segments encoding *hyl, cfb, cylE, scpB, bca* and *sip* were amplified in 43 *S. agalactiae* strains. It was noticed that 35 specimens (81.39%) out of 43 isolates contained the *hyl* gene, which is considered an important virulence factor that help in the spreading of bacteria on the host tissues [40]. This result was in accordance with those obtained by Krishnaveni et al. [5]; Cai et al. [41] and Correa et al. [42]. 40 specimens (93.39%) demonstrated the *cfb* gene, which is a cell surface protein that produces a traditional Christie-Atkins-Munch-Petersen (CAMP) phenomenon (the typical half

moon forming hemolytic zones on blood agar plates). This result was in agreement with the previous studies confirming broad frequency of *cfb* gene possessing *S. agalactiae*. In addition, 39 specimens (90.69%) contained the *cylE* and *sip* genes and this result was not in agreement with those obtained by Bergseng et al. [28] and Spellerberg et al. [43] who found 23% and 34,3% isolates positive for *cylE* gene, respectively. In addition, 29 specimens (67.44%) contained *scpB* gene and only 9 samples (20.93%) contained the *bca* gene. In Brief, results obtained from the current study are considered the beginning for more complete experimental study of the genes encoding virulence determinants in developing bovine mastitis caused by *S. aureus* and *S. agalactiae*. Furthermore, cloning of virulence genes in the prokaryotic system and utilize of the recombinant protein is competent in control measures and managing of this economic problem in among dairy herds.

## 5. CONCLUSION

This study reports high frequency of virulence genes in the isolates that help in the understanding of the distribution of infectious *S. aureus* and *S. agalactiae* strains in the Middle East and contribute to the establishment of preventive approaches to reduce the spread of infection. The high percentage of *fnbA*, coa, *clfA*, *spa* (x-region) genes for *S. aureus* strains and *cfb*, *hyl, cylE*, *scpB* and *sip* genes for *S. agalactiae* obtained in this work; suggest an important role of these virulence genes in the pathogenesis of bovine mastitis in the Middle East.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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