**Molecular Characterization of *Staphylococcus* species Isolated From Clinical and Subclinical Mastitic Buffaloes In EL-Behera Governorate, Egypt**

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**Abstract**

In the present study, a total of 120 milk samples (50 clinical and 70 subclinical mastitis cases) collected from buffaloes located at different localities at El-Behera Governorate, Egypt were examined phenotypically and genotypically for the presence of *S. aureus* and coagulase-negative staphylococci. Results showed that 42 *S. aureus* and 20 coagulase-negative *Staphylococcus* species isolates were recovered from the examined 120 milk samples. A total of 40 *S. aureus* and coagulase-negative *staphylococci* were further identified based on the amplification of 16S rRNA, *coa*, *clf*A, and *nuc* encoding genes. Results revealed that all *S. aureus* isolated from clinical and subclinical cases were found to harbor 16S rRNA, *coa*, *clf*A, and *nuc* encoding genes. Resistance against 12 antibiotics was determined using disc diffusion methods. All the tested *S. aureus* and coagulase-negative *staphylococcal* isolates exhibited resistance to amoxicillin, ampicillin, cefotaxime, ciprofloxacin, cloxacillin, oxacillin, lincomycin, penicillin, and trimethoprim/sulphamethoxazole. Constitutive production of *mec*Aand *bla*Z encoding resistance against penicillin and cloxacillin was reported in 70% and 20% of *S. aureus* and 80 % and 30% of the coagulase-negative *staphylococcal* isolated from clinical and subclinical mastitis, respectively. In contrast, all isolates found to harbor the specific amplicon of the *sul*1 gene. The amplicon of *aac*-*aph* encoding resistance gene was observed in only 6 *S. aureus* isolates. The specific amplicon of *nor*A gene was detected in 4 *S. aureus* and 2 coagulase-negative *staphylococcal* isolates recovered from clinical mastitis. To conclude, the emergence of multi-drug resistant *S. aureus* and coagulase-negative *staphylococci* as a cause of mastitis in dairy farms is of public health concern requires further investigations.

Keywords: Buffaloes, mastitis, *S. aureus,* coagulase-negative *staphylococci*, antimicrobial resistance genes

**Introduction**

The Egyptian buffaloes form an important part of the animal industry in Egypt, it represents about 47% of the total animal population compared with cattle (Ibrahim 2012). Buffaloe's milk is one of the most favorable dairy products for Egyptian consumers due to its white color and high nutritional value compared with other dairy milk (Arefaine and Kashwa 2015). Mastitis is the inflammation of udder because of various etiological agents in particular bacterial intramammary infections with well-known adverse effects on the health and the productivity of the herd (Ruegg 2017). Mastitis based upon the clinical features can be categorized into symptomatic (clinical) and asymptomatic (sub-clinical) mastitis. The sub-clinical mastitis is the most common type of mastitis with an adverse effect on the efficiency of dairy cows in terms of costs of treatment, decreased milk production and quality and costs of culling and the replacement of cows (Sandgren *et al.,* 2008). Bovine mastitis caused by *S. aureus* is the most widespread type of mastitis amongst ruminants in terms of frequency and severity (Bergonier *et al.,* 2003; de Almeida *et al.,* 2011; Wilson *et al.,* 1997). Intramammary infection with *S. aureus* is the main cause of subclinical mastitis (Turutoglu *et al.,* 2005) and the 2nd major cause of clinical mastitis among other bacteria such as *E. coli* and *S. agalactia* (Amin *et al.,* 2011). The classification of genus *Staphylococcus* into coagulase-positive staphylococci (CoPS) and coagulase-negative staphylococci (CoNS) is not a phylogenetic-based classification it is a simple diagnostic-based practice turned into a clinical attitude to differentiate pathogenic *S. aureus* from non-pathogenic staphylococcal species (Becker *et al.,* 2014). Although, CoNS is not often documented as a main cause of mastitis (Vanderhaeghen *et al.,* 2014), yet, it generally implicated as a foremost cause of subclinical and mild clinical cases of mastitis in many investigated dairy herds worldwide (De Vliegher *et al.,* 2012; Pyorala and Taponen 2009). Mastitis is a multifactorial disease of dairy herds and many bacterial species are involved. Therefore, the accurate identification of the causative bacteria is a crucial step in mastitis prevention and treatment program. Laboratory isolation and identification of staphylococci using the traditional culturing methods is still the gold standard technique, yet it is time-exhausting with numerous other limitations (Maes *et al.,* 2002; Rovai *et al.,* 2014). PCR has been introduced as a fast trustworthy technique for the identification of staphylococcal species isolated from various sources that can be executed in 3-4 h total (Koskinen *et al.,* 2009; Rovai *et al.,* 2014; Tamarapu *et al.,* 2001; Taponen *et al.,* 2009).

Antimicrobial therapy is the primary choice to treat staphylococcal mastitis in Egypt and is usually done without waiting for the results of the antimicrobial sensitivity assay. These results can help the veterinarian to choose the most effective antimicrobial agent (Moroni *et al.,* 2006). The misuse of antimicrobial chemotherapy significantly increased the development of multi-drug resistant *S. aureus* isolated from animals with mastitis (El-Jakee *et al.,* 2011). The emergence of methicillin-resistant *S. aureus* strains (MRSA) has recently been the primary concern of the public health and food safety authorities (Gharsa *et al.,* 2012). CoNS have shown an extra capability to develop multi-drug resistance compared with *S. aureus* (Taponen *et al.,* 2008). The antibiotic resistance mechanisms of *Staphylococcus* are governed in the main by an array of genetic determinants including *mec*A/*bla*Z, *acc*A-*aph*D, *nor*A, and *sul*1 encoding the resistance against penicillins, aminoglycosides, fluoroquinolones, and sulfonamides, correspondingly (Jensen and Lyon 2009). These resistance mechanisms allow the staphylococcal species to persist in the herd and the environment and resist the effect of antibiotic therapy (Kumar *et al.,* 2011). In Egypt particularly in El-Behera Governorate, there was a dearth of information about the occurrence and the genetic background of coagulase-negative *staphylococci* as a cause of mastitis, especially in buffaloes. This information will help develop accurate preventive and curative measures. Therefore, this study was designed to investigate the occurrence and genetic background of coagulase-positive *S. aureus* and coagulase-negative *staphylococci* in milk samples collected from buffaloes with clinical and subclinical mastitis.

**Materials and methods**

**Ethical Statement**

All procedures of this experiment were approved by the Animal Ethics Committee of Faculty of Veterinary Medicine, University of Sadat City (Local Ethical Approval) and complied with the Guidelines for the Care and Use of Animals in Research (Ethical approval number: VUSC-008-1-20).

**Samples collection and Study area**

The study was conducted on 120 buffaloes milk samples collected from 50 and 70 clinical and sub-clinical mastitis cases, correspondingly, located at different localities at El-Behera Governoate, Egypt. In Brief, the teats were cleaned and disinfected then the first 3 strips were discarded and 10 ml of milk of the infected quarters and quarters showed sub-clinical mastitis signs were aseptically collected by hand milking in a sterile test tube and directly sent to the laboratory for bacteriological examination on the same day and California Mastitis Test (CMT).

**California Mastitis Test (CMT)**

The milk samples from suspected subclinical mastitis cases were tested by CMT according to the procedures previously described (Barth  *et al.,* 2008; Elsayed *et al.,* 2015).

**Isolation and identification of *Staphylococcus* species**

Isolation and identification of *Staphylococcus* species were carried out on Mannitol Salt Agar, Baird-Parker agar, Vogel Johnson agar, and Brain Infusion broth (Oxoid) (Elsayed *et al.,* 2015). Further identification of the typical isolates into coagulase-positive and coagulase-negative strains was conducted using the biochemical tests including oxidase test, catalase test, acetoin production test, deoxyribonuclease test (DNase test), and coagulase test (Quinn *et al.,* 1994). The coagulase-negative *staphylococcal* isolates were further identified to the species level using the API Staph identification system (API Staph ID32 test; bioMérieux, Marcy l’Etoile, France).

**Antimicrobial susceptibility testing of *staphylococcal* species**

A total of 40 confirmed isolates, 20 from coagulase-positive *S. aureus* (10 of each clinical and sub-clinical cases), and 20 from coagulase-negative staphylococci (10 of each clinical and sub-clinical cases) were screened for their antimicrobial susceptibility against 12 commercial antibiotic discs namely, Oxacillin (1 µg), amoxicillin (25 µg), gentamycin (10 µg), penicillin (40 µg), neomycin (10 units), cefotaxime (30 µg), cephradine (30 µg), lincomycin (10 µg), norfloxacin (10 µg), ampicillin (10 µg), ciprofloxacin (5 µg), sulphamethoxazole/trimethoprim (1.25/23.75 µg), using the disc diffusion method according to standards set by the Clinical and Laboratory Standards Institute (CLSI 2015)**.** Media and antibiotic-impregnated discs were obtained from Oxoid, UK. Inhibition zones were measured to assess resistance or susceptibility. Multiple antibiotic resistance (MAR) indexes were calculated for individual isolates by dividing the number of antibiotics to which the isolate was resistant by the total number of screened antibiotics (Tarabees *et al.,* 2017).

**Genotypic identification of *staphylococcal* species**

**DNA Extraction**

The whole genomic DNA of *Staphylococcus* isolates was extracted from 20 coagulase-positive *S. aureus* (10 of each clinical and sub-clinical cases), 20 from coagulase-negative staphylococci (10 of each clinical and sub-clinical cases) of a Muller Hinton broth culture for 24 hrs at 37 °C according to the protocol previously established (Elsayed *et al.,* 2015; Unal *et al.,* 1992).

**Genotypic characterization of isolates**

A total of 40 confirmed isolates were further genotypically characterized using oligonucleotides encoding 16S rRNA gene (Mason *et al.,* 2001). The isolates were further screened for the presence of virulence determinants using oligonucleotides primers included the genes encoding coagulase (*coa*) (Iyer and Kumosani 2011), clumping factor (*clf*A) (Mason *et al.,* 2001), thermonuclease (*nuc*) (Gao *et al.,* 2012), and toxic shock syndrome toxin-1 (*TSST*-1) (Mehrotra *et al.,* 2000). The genotypic characterization of the isolates for the gene encoding antimicrobial resistance targeting *mec*A/*bla*Z (penicillins) (Duran *et al.,* 2012; McClure *et al.,* 2006), *aac*(6”)-*aph*(2”) (aminoglycosides) (Duran  *et al.,* 2012), *nor*A (fluoroquinolones) (Pourmand *et al.,* 2014), and *sul*1 (sulfonamides) (Ibekwe *et al.,* 2011) was further assessed using specific primers oligonucleotides (Table 1). The PCR conditions were performed in 25 µl PCR tubes containing 12.5 µl Emerald Amp GT PCR master mix (Takara Biotechnology, Dalian, Code No. RR310A), forward and reverse primers (1 µl each), 6 µl template DNA, and 4.5 µl PCR grade water. The amplified PCR products were resolved by electrophoresis on 2% agarose and the documentation was carried out using Gel documentation system (Alpha Innotech). The positive control was kindly provided (EL-Sayed et al, 2015) and PCR-RNase free water was used as a negative control.

**Results**

**Prevalence of *S. aureus* and CoNs recovered from clinical and subclinical mastitis cases**

Results in Table 2 demonstrated CMT, growth on specific media, and coagulase test of the examined 120 milk samples collected from suspected clinical and sub-clinical mastitis buffaloes. The data showed that among the examined 50 clinical mastitis cases, 35/50 (70%), and 20/35 (57.14%) were positive for growth on specific media and coagulase test, respectively. While, in the case of the examined 70 milk samples collected from sub-clinical mastitis cases, 67/70 (95.71%), 65/67 (97.01%), and 22/65 (33.84%) were positive for the CMT, growth on specific media and coagulase test, correspondingly as shown in Table 2. The suspected 20 coagulase-negative isolates collected from clinical (10 isolates) and subclinical (10 isolates) mastitis cases were further characterized into species level by API Staph commercial system. The obtained results revealed that in the case of clinical mastitis five species were identified as follow, S. *epidermidis*, *S. intermedius*, *S. hominis, S. xylosus, and S. chromogenes.* These species were isolated in percentages of 30% (3/10), 30% (3/10), 20% (2/10), 10% (1/10), and 10% (1/10), respectively. While in the case of subclinical mastitis cases, S. *epidermidis*, *S. intermedius*, *S. hominis, S. xylosus, and S. chromogenes* were isolated in percentages of 30% (3/10), 40% (4/10), 10% (1/10), 10% (1/10), and 10% (1/10), correspondingly.

**Genotypic characterization of the isolates**

Genotypic characterization of 40 isolates obtained from clinical and sub-clinical mastitis cases using oligonucleotides primer included the gene encoding targeting the 16SrRNA as shown in Table 3. The obtained data showed that 100% of the tested staphylococcal isolates isolated from clinical and sub-clinical mastitis cases were found harbor 16SrRNA gene. The isolates were further screened for the presence of *nuc*, *coa*, and *clf*A (specific for *S. aureus*) encoding genes (Table 3). The amplification of the *coa,* *nuc*, and *clfA* in all the tested coagulase-positive *S. aureus* isolates collected from clinical and subclinical mastitis cases. Our results showed that, the successful amplification of *TSST*-1 was only reported in one *S. aureus* isolated from clinical mastitic cases. Regarding the coagulase-negative staphylococcal isolates, the specific amplicons of *coa*, *nuc*, *clf*A, and *TSST*-1 were detected in only 10%, 10%, 20%, and 0% of the tested CoNS isolated obtained from clinical mastitis cases, respectively. While it was detected 20%, 10%, 20%, and 10% of the tested CoNS isolated recovered from sub-clinical mastitic cases, correspondingly as presented in Table 3.

**Antibiotic susceptibility testing of the staphylococcal species**

The data presented in Table 4 demonstrated the antibiotic susceptibility profile of the isolated staphylococcal species. Results showed that all the tested isolates (40) were phenotypically resistant to oxacillin, amoxicillin, penicillin, ampicillin, cefotaxime, cephradine, ciprofloxacin, cloxacillin, and Trimethoprim/Sulphamethoxazole, as shown in Table 4. While only 8/10 (80%) *S. aureus* isolates (clinical mastitis) and 9/10 (90%) *S. aureus* isolates (sub-clinical mastitis) and one isolate coagulase-negative *S. aureus* variant (sub-clinical case) was resistant to gentamicin. Concerning the norfloxacin, only 2/10 (20%) and 10/10 (100%) of *S. aureus* isolated from milk samples collected from clinical mastitis and sub-clinical mastitis buffaloes, respectively were resistant to norfloxacin. In contrast, 10/10 (100%) and 6/10 (60%) of coagulase-negative *staphylococci* isolated from clinical and sub-clinical mastitis cases were phenotypically resistant to norfloxacin, respectively as shown in Table 4 and 6.

**Genotypic characterization of the antibiotic resistance profile of staphylococcal species**

A total of 20 isolates from coagulase-positive *S. aureus* (10 of each clinical and sub-clinical mastitis cases), 20 from coagulase-negative staphylococci (10 of each clinical and sub-clinical mastitis cases) were further screened for the presence of antibiotic resistance genes (*mec*A, *bla*Z, *Sul*1, *nor*A, and *aac*(6”)-*aph*(2”)) encoding resistance against methicillin, penicillin, sulphonamides, norfloxacin and gentamicin, respectively as presented in Table 5 and 6. The data showed that *S. aureus* isolates obtained from clinical mastitis cases were found to have *mec*A, *bla*Z, *Sul*1, *nor*A, and *aac*(6”)-*aph*(2”) in a percentage of 80%, 60%, 100%, 40%, and 40%, respectively. While in the case of sub-clinical mastitis isolates of *S. aureus* were found to have *mec*A, *bla*Z, *Sul*1, *nor*A, and *aac*(6”)-*aph*(2”) in a percentage of 60%, 100%, 100%, 0%, and 20%, of the tested isolates, respectively. Regarding the coagulase-negative staphylococci, in the case of clinical mastitis, the tested isolates were found to have *mec*A, *bla*Z, *Sul*1, *nor*A, and *aac*(6”)-*aph*(2”) in percentages of 20%, 20%, 100%, 20%, and 0%, correspondingly. Whereas in the case of the sub-clinical mastitis cases the percentages were 20%, 40%, 100%, 0%, and 0%, respectively as shown in Table 5 and 6.

**The antimicrobial pattern of coagulase-negative staphylococcal species**

The data presented in Table 3 and 6 showed the antimicrobial susceptibility of coagulase-negative species and the presence of antibiotic resistance encoding genes. The obtained data revealed that, CoNS isolated from clinical and subclinical mastitic cases were resistant to all the tested antimicrobials in particular to penicillin group and sulfonamides, as shown in Table 4 and 6. Concerning the occurrence of resistance encoding gene results showed that none of the CoNS was carrying the *Nor*A and *aac* (6)-*aph* (2) resistance encoding genes only two isolate (*S. epidermidis* and *S. intermedius*) isolated from clinical mastitic as presented in Table 4 and 5. Conversely, all CoNS isolates recovered from clinical and subclinical mastitic cases were found harbor *Sul*1 resistance encoding gene, as shown in Table 4 and 5. Concerning *mec*A and *bla*Z, only two isolates (*S*. *epidermidis*) and (*S. epidermidis* and *S. intermedius*) were found positive for *mec*A and *bla*Z resistance encoding genes, respectively. While, only one isolate of *S. epidermidis* and *S. intermedius* was found harbor *mec*A resistance encoding gene, and four isolates *S. epidermidis* and *S. intermedius* (two of each) were found positive for *bla*Z resistance encoding gene, as presented in Tables 4-6.

**Discussion**

*S. aureus* is considered as an important cause of mastitis in dairy buffaloes (Zaitoun 2008), usually accompanied by enormous economy loses caused to decrease the amount and the quality of milk, increase the costs of treatment and increase the costs of culling and replacement within the diseased herds (Sandgren *et al.,* 2008). There is a dearth of information on the virulence determinants, antimicrobial resistance encoding genes of coagulase-positive *S. aureus* and coagulase-negative staphylococci induced mastitis of buffaloes in El-Behera Governorate, Egypt. Results obtained in the present study showed that all the examined milk samples collected from clinical mastitis (50/50, 100%) were positive by CMT. These results are higher than those previously reported by El Sayed and coauthors, who showed that only 75.1% of the examined buffaloes milk samples were positive by CMT (Elsayed *et al.,* 2015). While in the case of sub-clinical mastitis the percentage of positive CMT was 95.71% . This result is higher than that previously reported by Elsayed et al., who stated that only 45.85% of the examined milk samples collected from sub-clinical mastitis buffaloes were positive by CMT (Elsayed *et al.,* 2015). In Egypt, *S. aureus*-induced mastitis is the predominant type in dairy animals when compared with other bacterial pathogens (Elhaig and Selim 2015; Hamed and Ziatoun 2014). Our results showed that only 57.14% (20/35) and 33.83% (22/65) of the isolates recovered from milk samples collected from clinical and sub-clinical mastitis buffaloes were confirmed as *S. aureus*, respectively. These outcomes are inconsistent with that reported previously by Elsayed et al. (2015) who showed that *S. aureus* was successfully recovered from 72.73% of the examined milk samples collected from sub-clinical mastitis buffaloes in Miunfyia Governorate, Egypt. Awad and coauthors showed that *S. aureus* was recovered from 42% of the examined milk samples collected from dairy herds with mastitis in Damietta and Dakahilya Governorates, Egypt (Awad *et al.,* 2017). These variations in results could be attributed to many factors including, area of sampling, time of sampling, and methods of culturing. Howevere, it is normally accepted that 20%-30% of the examined milk samples collected from dairy cattle exhibited no growth with traditional cultural methods (Bradley *et al.,* 2007; Koskinen *et al.,* 2009). Results also showed that coagulase-negative staphylococci were recovered from clinical and sub-clinical mastitis in a percentage of 42.85% (15/35) and 66.15% (43/65), respectively. The emergence of coagulase-negative staphylococci has previously been reported as a cause of bovine mastitis and may inaccurately diagnosed as coagulase-negative staphylococcal species in routine mastitis diagnosis (Akineden *et al.,* 2011). There is a significant dissimilarity in the phenotypic expression of traits among the isolates belonging to the same species and interpretation of the phenotypic tests is biased (Carretto *et al.,* 2005; Heikens *et al.,* 2005; Zadoks and Watts 2009). PCR has been introduced as fast technique usually take 4 hrs for the identification of different human and animals emerged pathogens. However, caution should be taken because a small amount of DNA in the sample may give a false-positive result compared with the negative results of traditional cultural methods (Rovai *et al.,* 2014). Therefore, contamination of the samples should be avoided and more samples are needed to be examined before considering positive or negative results (Rovai *et al.,* 2014). Herein, the obtained isolates were further genotypically characterized as staphylococci using the 16S rRNA encoding gene and the results demonstrated that all the tested isolates were found to harbor the 16S rRNA gene. These outcomes with others (El-Razik *et al.,* 2017; Elsayed *et al.,* 2015; Monday and Bohach 1999) indicated that the isolated strain are belonged to staphylococcal species.

A comprehensive understanding of the virulence determinants, antimicrobial resistance patterns of *S. aureus* and coagulase-negative staphylococci may assist improve the measures to control the spread of the bacterium within herds (Haveri *et al.,* 2008). In the current study, 40 coagulase positive and coagulase negative isolates were further screened for the presence of virulence encoding genes included genes targeted *coa*, *clf*A, *nuc*, and *TSST*-1. The coagulase is one of *S. aureus* virulence determinants that enables bacteria to resist phagocytosis and induce chronic infections (Viana *et al.,* 2010). *Coa* gene is considered as an important virulence criterion of pathogenic *S. aureus.* Previous reports stated that, molecular characterization of *coa* gene is a simple and accurate practice for classification of *S. aureus* (Rodrigues da Silva and Silva . 2005) and can be used in the epidemiological investigations (Raimundo *et al.,* 1999; Su  *et al.,* 2000). Results of the present study showed that all the tested *S. aureus* isolated from clinical and subclinical mastitic cases were found harbor *coa* gene. These results are in agreement with that reported by Elsayed et al., (2015) who showed that all the *S. aureus* isolates recovered from clinical and sub-clinical mastitis buffaloes were carrying *coa* encoding gene. While in the case of *clf*A, our results are higher than those formerly reported by Elsayed et al., (2015) and Momtaz et al., (2010). *S. aureus* has been shown to produce a wide variety of proteins that have a significant role in staphylococcal-induced food poisoning in animals and humans including toxic shock syndrome toxin-1 (*TSST*-1) (Argudin *et al.,* 2010). The obtained results showed that one *S. aureus* isolate obtained from clinical mastitis cases was found producing *TSST*-1 gene. This result is consistent with the findings of Awad et al., who demonstrated that *TSST*-1 encoding gene was found in only 11.9% of the confirmed *S. aureus* isolates (Awad *et al.,* 2017). Staphylococcal enterotoxins-like proteins including TSST-1 are involved in human food poisoning (Hennekinne *et al*., 2010). Therefore, regular monitoring of milk and milk products for the presence of pathogenic *S. aureus* in particular those carrying TSST-1 represents an important concern of public health and food authority. Interstingely, our results revealed that one CoNS species (*S. epidermidis*) was found producing TSST-1 encoding gene. This finding highlighted the importance of further investigations on the emergence of CoNS as a potential food posioning inducing agents.

The excessive and insensible use of antibiotics as a sole intervention in the treatment of mastitis in Egypt leads to the development of multidrug-resistant *S. aureus*. Regular monitoring of bacteria recovered from mastitic animals for antimicrobial susceptability profile will help develop accurate preventive measures and select the most effective antibiotic. This approach will in turn minimize the emergence of these multi-drug resistant bacteria into human food chains. The obtained data showed that all the tested *S. aureus* and coagulase-negative staphylococci species isolated from buffaloes with clinical and subclinical mastitis were phenotypically resistant to oxacillin, amoxicillin, penicillin, ampicillin, cefotaxime, cephradine, ciprofloxacin, cloxacillin, and Trimethoprim\Sulphamethoxazole. These outcomes are higher than those previously reported by Awad et al. who showed that 95.2%, 83.3%, and 14.3% of *S. aureus* isolates were resistant to ampicillin, penicillin, and ciprofloxacin, respectively (Awad et al., 2017). Grispoldi et al., showed that *S. aureus* isolates recovered from animals with subclinical mastitis were resistant to penicillin (52.94%) and sensitive to gentamicin and ciprofloxacin (Grispoldi *et al.,* 2019). The emergence of multidrug-resistant *S. aureus* in particular resistance to β-lactamase antibiotics including the powerful class i.e. Methicillin has been the focus of many research studies. The methicillin induced resistance is governed by constitutive production of *mec*A encoding PBP2a (penicillin-binding protein 2a), which has a lower affinity for beta-lactam antibiotics including penicillin, methicillin, oxacillin, and cloxacillin (Hamid *et al.,* 2017). In the present work, all *S. aureus* isolates recovered from clinical mastitis were phenotypically resistant to amoxicillin, ampicillin, cloxacillin, oxacillin, and penicillin, and only 80% and 60% of these isolates were found harbor *mec*A and *bla*Z encoding genes, respectively. While all *S. aureus* (100%) isolates recovered from sub-clinical mastitis were phenotypically resistant to amoxicillin, ampicillin, cloxacillin, oxacillin, and penicillin, and 60% and 100% of these isolates were carrying *mec*A and *bla*Z encoding genes, correspondingly. The variation between the phenotypic and genotypic results was previously reported by Hamid and coauthors who showed that while 55.5% of *S. aureus* isolates were phenotypically resistant to oxacillin, only 16.6% of these isolates were carrying *mec*A gene (Hamid *et al.,* 2017). This difference in results might be attributed to the presence of *mec*A divergent gene *mec*C. Of note, three *S. aureus* isolates were negative for *bla*Z and were found sensitive to penicillins. This outcome could be due to the hyperproduction of β-lactamase (Moon *et al.,* 2007) or the mutations in primers binding sites of resistance genes (Haveri *et al.,* 2005). In contrast, other isolates carrying *bla*Z were resistant to penicillins. This outcome is inconsistent with the finding of Srednik et al. (2015). Concerning the antibiotic susceptability patterns of the isolates against other antimicrobials, results demonstrated that all *S. aureus* (100%) isolates obtained from milk samples of clinical and subclinical mastitis cases were phenotypically resistant to Trimethoprim\Sulphamethoxazole and were found carrying the *sul*1 encoding gene. While in the case of gentamicin, only 20% and 80% of *S. aureus* isolates recovered from clinical and subclinical mastitis were phenotypically resistant to gentamicin, and 40% and 20% of them were found carrying *aac* (6”)-*aph* (2”), respectively. Furthermore, 20% and 100% of *S. aureus* isolates identified from clinical and subclinical mastitis were resistant to enrofloxacin, and only 40% of the isolates from clinical mastitis were harbor *nor*A encoding gene. These outcomes are lower than those previously reported by other researchers. Hamid et al., (2017) reported that 66.6% of *S. aureus* isolates were found resistant to enrofloxacin. In contrast, Hussein et al., (2012) stated that 94.73% of *S. aureus* isolates were sensitive to enrofloxacin. The high multi-drug resistance pattern of *S. aureus* might be attributed to the intensive abuse of antibiotics in the treatment of mastitis, geographical area, and the form of mastitis. Therefore, treatment of mastitis should carry out with extreme caution taking into consideration, the cost of treatment, the price of milk, and the results of sensitivity assay. This practice will probably lessen the public fear of transmission of multi-drug resistant pathogens to humans through food production chains.

CoNS are considered the main cause of subclinical mastitis but it have also been isolated from animals with clinical mastitis (Pyorala and Taponen 2009; Vanderhaeghen *et al.,* 2014). In the current study, coagulase-negative staphylococci recovered from clinical and subclinical mastitic cases were identified into the species level and further tested against 13 commercial antibiotics using disc diffusion method and further genotypically screened for the presence of *mec*A, *bla*Z, *nor*A, and *aac-aph* encoding genes included resistance against corresponding antibiotics. Results demonstrated that five species were identified, *S. epidermidis*, *S. intermidius*, *S. hominis*, *S. xylosus*, and *S. chromogenes*. *S. chromogenes* has been isolated from many herds (Dubois *et al.,* 2010) from the milk, teat skin and orifices, and other body sites of cows and heifers (Gillespie *et al.,* 2009). *S. xylosus* and *S. chromogenes* and *S. intermedius* are frequently isolated from milk samples of mastitic cows and buffaloes in Egypt (Osman *et al.,* 2016). Abd El-Razik et al., (2017) reported the isolation of *S. xylosus* form 25% of the apparently examined healthy and subclinical mastitis milk samples collected form buffaloes. The frequent isolation of *S. xylosus* from milk of animal with clinical and subclinical mastitis highlights the importance of further studies to elucidate the precise role of this microbe in the pathogenesis of bovine mastitis. Herein, CoNS isolates were screened for antimicrobial susceptibility against 12 commercial antimicrobials and further examined for the presence of some antibiotic resistance encoding genes. Our results showed that all the tested CoNS isolates were found resistant to the tested antimicrobial. The resistance of CoNS towards benzylpenicillin and aminopenicillins, tetracycline, macrolides, and aminoglycosides has been reported in many studies (Frey *et al.,* 2013; Persson Waller  *et al.,* 2011; Pitkälä *et al.,* 2004). Results also revealed that, the five species of CoNS (S*. epidermidis*, *S. intermedius*, *S. hominis*, *S. xylosus*, and *S. chromogenes*) were oxacillin and cloxacillin resistant. These outcomes are inconsistent with the findings previously reported by Osman et al., (2016) who stated that among the tested 94 CoNS isolates 42-48% were resistant towards ampicillin, oxacillin, and penicillin. Kaliwal et al. stated that CoNS were resistant to penicillin (76%), ampicillin (69.5%), and gentamicin (57%) (Kaliwal *et al.,* 2011). The emergence of methicillin-resistant CoNS is an issue of special importance, as the growing public health fears from the transmission of the resistance determinants among staphylococcal species and the possibility of transmission to humans (Gindonis *et al.,* 2013; Huber *et al.,* 2011). The resistance against a wide range of antimicrobials observed in the present study could be attributed to the extensive use of antibiotics in the treatment of mastitis and as growth promoters (Osman *et al.,* 2016), as well as the geographical distribution of samples.

Regular monitoring of the resistance pattern of CoNS is important as these species may act as a reservoir for the antibiotic resistance gene and transmit the resistance genes to other staphylococcal species infecting animals and those of humans. In the present study, successful amplification of specific amplicon size of *mec*A was reported in 20% of the isolates recovered from clinical mastitic cases (two *S. epidermidis* isolates) and subclinical mastitic cases (*S*. *epidermidis* and *S. intermedius*, one isolate each). Concerning the *bla*Z gene, only 2 isolates (*S*. *epidermidis* and *S. intermedius*) recovered from clinical mastitic cases and 4 isolates *S*. *epidermidis* and *S. intermedius* (2 isolates of each) recovered from subclinical mastitic cases were found harbor *bla*Z encoding gene. Contrariwise, *S*. *hominis,* *S*. *xylosus*, and *S*. *chromogenes* were found to produce the specific amplicons of *mec*A and *bla*Z. Osman et al., reported the presence of *mec*A and *bla*Z in only 6 and 5 *S*. *hominis* isolates, correspondingly, while no specific amplification was observed in *S*. *chromogenes* isolates (Osman *et al.,* 2016). Regarding *sul*1 gene, results showed that all coagulase-negative staphylococci isolates recovered from clinical and subclinical mastitic cases were found positive for *sul*1 encoding resistance gene. In contrast, the production of specific amplicon size of *nor*A and *aac*(6”)*aph*(2”) was reported in only 2 coagulase-negative staphylococcal isolates (*S*. *epidermidis* and *S*. *intermedius*) recovered from clinical and subclinical mastitis, respectively. CoNS are considered the main reservoir of antibiotic-resistance encoding genes (Osman *et al.,* 2016). During the last few years, CoNS have been received more consideration and became the focus of many studies as its impact on human health and diseases. Many studies have revealed the emergence of enterotoxin-producing strains of CoNS (Oliveira *et al.,* 2011; Oliver *et al.,* 2009; Zell *et al.,* 2008) indicates the possibility of transmission of these strains to humans via animal products (de Mello *et al.,* 2014). The high multidrug resistance pattern of CoNS isolates in the current study reflected the intensive mishandling of antibiotics in the treatment of mastitis at El-Behera Governorate, Egypt. Therefore, caution should be considered to minimize the spread of these strains among herds and localities, and vigilant monitoring programs are crucial to lessen the transmission of these strains to in-contact animals and humans.

**Conclusion**

Results of the current study highlighted the emergence of coagulase-negative staphylococcal species as a cause of mastitis in Egypt requires further investigations. A better understanding of the genetic background of coagulase-negative staphylococciis of immense significance for mastitis prevention and treatment. The multi-drug resistance patterns of *S. aureus* and other coagulase-negative staphylococciisolated from clinical and subclinical mastitis jeopardizing the public health and efficacy of mastitis prevention and treatment programs in Egypt.

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**Table 1** Primers nucleotides sequences (5'-3') for genotypic characterization and antimicrobial resistance of the staphylococcal isolates

|  |  |  |  |
| --- | --- | --- | --- |
| Annealing Temp | Product size (bp) | Primer sequence  (5'-3') | Gene |
| 55 | 791 bp | F-CCTATAAGACTGGGATAACTTCGGG  R-CTTTGAGTTTCAACCTTGCGGTCG | *16SrRNA* |
| 54 | 491 bp | F-GAAGTACGCAGAAGAGA  R-ACATGGCAAGCTCTAGGA | *aac(6')-aph (2'')* |
| 50 | 173 bp | F-ACTTCAACACCTGCTGCTTTC  R-TGACCACTTTTATCAGCAACC | *blaZ* |
| 55 | 638 bp | F-GCAAAATCCAGCACAACAGGAAACGA  R-CTTGATCTCCAGCCATAATTGGTGG | *clfA* |
| 55 | 630 bp | F-ATA GAG ATG CTG GTA CAG G  R-GCT TCC GAT TGT TCG ATG C | *Coa* |
| 50 | 310 bp | F-GTAGAAATGACTGAACGTCCGATAA  R-CCA ATT CCACATTGTTTCGGT CTA A | *mecA* |
| 50 | 620 bp | F-TTCACCAAGCCATCAAAAAG  R-CTTGCCTTTCTCCAGCAATA | *norA* |
| 55 | 395 bp | F-ATATGTATGGCAATCGTTTCAAT  R-GTAAATGCACTTGCTTCAGGAC | *Nuc* |
| 60 | 433 bp | F-CGG CGT GGG CTA CCT GAA CG  R-GCC GAT CGC GTG AAG TTC CG | *Sul1* |
| 50 | 326 bp | F-ACCCCTGTTCCCTTATCATC  R-TTTTCAGTATTTGTAACGCC | *TSST*-1 |

**Table 2** Results of CMT, Growth on media, and coagulase test of the examined milk samples

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Item** | **Clinical mastitis cases** | | **Sub-clinical mastitis cases** | |
| 50 | | 70 | |
| **+ve** | **-ve** | **+ ve** | **- ve** |
| CMT | N/A | | 67/70 (95.71%) | 3/70 (4.29%) |
| Growth on specific media and biochemical tests | 35/50 (70%) | 15/50 (30%) | 65/67 (97.01%) | 2/67 (2.99%) |
| Coagulase test | 20/35 (57.14%) | 15/35 (42.85%) | 22/65 (33.84%) | 43/65 (66.15%) |

CMT; California Mastitis Test

N/A: CMT only carried out in case of subclinical mastitis while in case of clinical mastitis the presence of clinical signs is the indication of mastitis.

**Table 3** Genotypic characterization of isolates based on 16SrRNA, *coa*, and *nuc* genes

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Item** | **Clinical mastitis cases** | | **Sub-clinical mastitis cases** | |
| Coagulase test  (phonotypical) | *S. aureus* (*n*=10) | CoNS (*n*=10) | *S. aureus* (*n*=10) | CoNS (*n*=10) |
| 16S rRNA | 10/10(100%) | 10/10(100%) | 10/10(100%) | 10/10(100%) |
| *Coa* | 10/10(100%) | 1/10(10%) | 10/10(100%) | 2/10(20%) |
| *nuc* | 10/10(100%) | 1/10(10%) | 10/10(100%) | 1/10(10%) |
| *clfA* | 10/10(100%) | 2/10(20%) | 10/10(100%) | 2/10(20%) |
| *Tsst-*1 | 1/10 (10%) | 0 | 0 | 1/10(10%) |

CoNS; Coagulase-negative staphylococci

**Table 4** Antibiotic susceptibility pattern of the tested staphylococcal isolates

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Item | Clinical mastitis cases | | | | | | Sub-clinical mastitis cases | | | | | |
| Coagulase Test  (Phenotypic) | *S. aureus* (*n*=10) | | | CoNS (*n*=10) | | | *S. aureus* (*n*=10) | | | CoNS (*n*=10) | | |
| Antibiotic | R | I | S | R | I | S | R | I | S | R | I | S |
| Amoxicillin | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - |
| Ampicillin | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - |
| Cefotaxime | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - |
| Cephradine | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - |
| Ciprofloxacin | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - |
| Cloxacillin | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - |
| Gentamicin | 2/10 | 6/10 | 2/10 | 10/10 | - | - | 8/10 | 1 | 1 | 8/10 | 1 | 1 |
| Lincomycin | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - |
| Norfloxacin | 2/10 | 8/10 | - | 10/10 | - | - | 10/10 | - | - | 6/10 | 2 | 2 |
| Oxacillin | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - |
| Penicillin | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - |
| Trimethoprim\  Sulphamethoxazole | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - | 10/10 | - | - |

CoNS: Coagulase-negative Staphylococci

**Table 5** genotypic characterization of some antibiotic resistance genes (*mec*A, *tst*, *bla*z, *sul*1, *nor*A, and *aac*(6)-*aph*(2) )

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Item** | **Clinical mastitis cases** | | **Sub-clinical mastitis cases** | |
| Coagulase test  (phenotypical) | *S. aureus* (*n*=10) | CoNS (*n*=10) | *S. aureus* (*n*=10) | CoNS (*n*=10) |
| *mec*A | 8/10 (80%) | 2/10 (20%) | 6/10 (60%) | 2/10(20%) |
| *Bla*z | 6/10(60%) | 2/10(20%) | 10/10 (100%) | 4/10(40%) |
| *Sul*1 | 10/10 (100%) | 10/10 (100%) | 10/10 (100%) | 10/10 (100%) |
| *nor*A | 4/10(40%) | 2/10 (20%) | 0 | 0 |
| *aac*(6”)*aph*(2”) | 4/10 (40%) | 0 | 2/10 (20%) | 0 |

CoNS; Coagulase-negative Staphylococci

**Table 6** Distribution of the resistance genes among the staphylococcal isolate

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **isolate** | Mastitis | **Antibiotics** | | | | **Antibiotic resistance genes** | | | | |
| P | S | Nor | G | *mec*A | *bla*z | *Sul*1 | *nor*A | *aac*(6)-*aph*(2) |
| *S. aureus* | CM | R | R | S | R | **-** | **+** | **+** | **-** | **+** |
| *S. aureus* | R | R | S | S | **-** | **-** | **+** | **-** | **-** |
| *S. aureus* | R | R | R | S | **+** | **+** | **+** | **+** | **-** |
| *S. aureus* | R | R | S | S | **+** | **-** | **+** | **-** | **+** |
| *S. aureus* | R | R | R | S | **+** | **+** | **+** | **+** | **+** |
| *S. aureus* | R | R | S | R | **+** | **+** | **+** | **+** | **+** |
| *S. aureus* | R | R | S | S | **+** | **+** | **+** | **+** | **-** |
| *S. aureus* | R | R | S | S | **+** | **-** | **+** | **-** | **-** |
| *S. aureus* | R | R | S | S | **+** | **+** | **+** | **-** | **-** |
| *S. aureus* | R | R | S | S | **+** | **-** | **+** | **-** | **-** |
| *S. aureus* | SCM | R | R | R | S | **-** | **+** | **+** | **-** | **-** |
| *S. aureus* | R | R | R | R | **+** | **+** | **+** | **-** | **+** |
| *S. aureus* | R | R | R | R | **-** | **+** | **+** | **-** | **+** |
| *S. aureus* | R | R | R | S | **+** | **+** | **+** | **-** | **-** |
| *S. aureus* | R | R | R | R | **+** | **+** | **+** | **-** | **-** |
| *S. aureus* | R | R | R | R | **-** | **+** | **+** | **-** | **-** |
| *S. aureus* | R | R | R | R | **+** | **+** | **+** | **-** | **-** |
| *S. aureus* | R | R | R | R | **+** | **+** | **+** | **-** | **-** |
| *S. aureus* | R | R | R | R | **-** | **+** | **+** | **-** | **-** |
| *S. aureus* | R | R | R | R | **+** | **+** | **+** | **-** | **-** |
| *S. epidermidis* | CM | R | R | R | R | **+** | **-** | **+** | **+** | **-** |
| *S. epidermidis* | R | R | R | R | **+** | **-** | **+** | **-** | **-** |
| *S. epidermidis* | R | R | R | R | **-** | **+** | **+** | **-** | **-** |
| *S. intermedius* | R | R | R | R | **-** | **-** | **+** | **+** | **-** |
| *S. intermedius* | R | R | R | R | **-** | **+** | **+** | **-** | **-** |
| *S. intermedius* | R | R | R | R | **-** | **-** | **+** | **-** | **-** |
| *S. hominis* | R | R | R | R | **-** | **-** | **+** | **-** | **-** |
| *S. hominis* | R | R | R | R | **-** | **-** | **+** | **-** | **-** |
| *S. xylosus* | R | R | R | R | **-** | **-** | **+** | **-** | **-** |
| *S. xylosus* | R | R | R | R | **-** | **-** | **+** | **-** | **-** |
| *S. chromogenes* | R | R | R | R | **-** | **-** | **+** | **-** | **-** |
| *S. epidermidis* | SCM | R | R | R | R | **-** | **-** | **+** | **-** | **-** |
| *S. epidermidis* | R | R | S | R | **+** | **+** | **+** | **-** | **-** |
| *S. epidermidis* | R | R | R | S | **-** | **+** | **+** | **-** | **-** |
| *S. intermedius* | R | R | S | R | **-** | **-** | **+** | **-** | **-** |
| *S. intermedius* | R | R | R | S | **+** | **+** | **+** | **-** | **-** |
| *S. intermedius* | R | R | R | R | **-** | **+** | **+** | **-** | **-** |
| *S. intermedius* | R | R | S | R | **-** | **-** | **+** | **-** | **-** |
| *S. hominis* | R | R | S | R | **-** | **-** | **+** | **-** | **-** |
| *S. xylosus* | R | R | R | R | **-** | **-** | **+** | **-** | **-** |
| *S. chromogenes* | R | R | R | R | **-** | **-** | **+** | **-** | **-** |

P; penicillin group (Amoxicillin, Ampicillin, Cloxacillin, Oxacillin, and Penicillin)

S: Sulfonamides, Nor; Norfloxacin, G; Gentamicin

CM; Clinical Mastitis, SCM; Subclinical Mastitis