MOLECULAR DETECTION OF TOXINS AND DISINFECTANT RESISTANCE GENES AMONG STAPHYLOCOCCUS AUREUS ISOLATED FROM DAIRY CATTLE IN EGYPT

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ABSTRACT

The objectives of the study were to detect toxins and antiseptic resistance genes in Staphylococcus aureus isolated from cows with subclinical mastitis in Egypt. A total of 400 quarter milk samples (QMS) were collected from different dairy herds in which quaternary ammonium compounds (QAC) had been used as a disinfectant for more than 3 years. The collected samples were subjected to bacterial investigation. S. aureus was successfully isolated confirmed by duplex PCR targeting 16S rRNA and nuc genes. Also determined their antibiogram and sensitivity to disinfectant. Genes of QAC(qacA/B), enterotoxins (Sea, Seb) and exfoliative toxins (ETB) were detected by simplex and multiplex PCR. Results of bacterial investigation revealed 103 (25.75%) S. aureus isolates. Results of antibiogram demonstrate that the most microbial antibiotics resistance were recorded for Penicillin G (85.7%) and Tetracycline (54.2%). While Gentamycin, Neomycin and Amoxicillin+ clavulanic acid show moderate resistance (21.4%, 10% and 7.1%) respectively, although Norfloxacin and Cephradine exhibited seldom resistance with high sensitivity of 95% and 94.3% respectively. Regarding the results of QAC sensitivity, only 8 isolates (7.76%) were resistant to benzalkonium chloride (BC) versus to 13 isolates (12.62%) harbour QAC gene could be detected by PCR with specific amplicon of 220bp corresponding to qacA/B. The results revealed Positive amplification of 102 bp specific for Sea gene in 19(18.44%) isolates and 164bp specific for Seb gene in 13(12.62%) isolates while there is no amplification was detected for etb gene. In conclusion, Antiobiogram, as well as the identification of toxigenic and QAC genes in this study, may open another perspective in planning some alternative therapeutic strategies against multi resistances S. aureus mastitis. Monitoring cross-resistance between antibiotics and antiseptic should be further investigated.

Keywords: Mastitis, quaternary ammonium compounds (QAC), QMS, PCR, S.aureus,

INTRODUCTION

The cumulative extensive use of antiseptic compounds in veterinary applications and food industry as an important controlling of infectious pathogens generates pressure cause of the appearance of antiseptic resistance among S. aureus (Bjorland et al., 2001; Noor et al., 2019). The genetic resistance against antiseptics, principally quaternary ammonium compounds (QACs), is a venerable problem (Russell, 2004) especially in the dairy farms because they are commonly used (QACs), for disinfection of milking tackle, milk tanks and as teat dip disinfection to maintain udder health and prevent infectious mastitis for their rapid bactericidal effect towards wide variety of Gram-positive and Gram-negative microorganisms. (NMC, 1999). Additionally, they are almost used to prevent the colonization of microorganisms thus considered an important key in mastitis control especially infections caused by S.aureus, which is an imperative mastitis pathogen in dairy farms worldwide (Ucuncu, 2015).
However, the infections can be persisting for a long time in mammary glands, thus serve as reservoirs from which the organism may spread to other cows within a herd and sometimes to other herds (Ergun et al., 2017). Resistance against QACs is primarily encoded in the qac genes. The presence of these genes can affect the use of antiseptics (Cervinkova et al., 2013). Different QAC gene has been found including two major groups of resistance genes, the major facilitator superfamily qac/A and qac/B (Jaglic and Cervinkova D. 2012).

The dangerous of pathogenicity of S. aureus is largely associated with a combination of invasive capacity, toxin-mediated virulence and antibiotic resistance (Argudin et al., 2010; Rasha 2018). Toxigenic S. aureus contains large number toxin genes including enterotoxins (SEs), toxic shock syndrome toxin, and exfoliative toxins (eta and etb) (Haveri et al., 2007). Considering S. aureus as a significant cause of zoonotic transmission between animals and humans through close contact, or consumption of infected food of animal origin and the probable transfer of antibiotic or antiseptic resistance has been a matter of great alarm (Song et al., 2015; Pereyra et al., 2016). Also, the number of resistant staphylococci seems to be increasing worldwide (De Jong et al., 2018).

However, studies that discuss the existence of antiseptic resistance genes in Staphylococcus spp. in Egypt have been inadequate. Therefore, the purpose of this study is to detect enterotoxins and antiseptic resistance genes in S. aureus strains isolated from bovine subclinical mastitis in some dairy herds in Egypt.

**MATERIALS AND METHODS**

**Samples**

The present study was done in dairy herds in which QAC had been used for teat dip disinfection for more than 3 years especially benzalkonium chloride (BC). From cows with subclinical mastitis 400 quarter milk samples (QMS) were collected aseptically in sterilized glass bottles with an ice pack, immediately transported to the mastitis microbiology laboratory of the animal reproduction research Institute (ARRI) in Egypt. All samples were subjected to the California mastitis test (CMT), bacteriological identification and PCR amplification.

**Bacteriological examination of QMS**

Wet dots were done according to The National Mastitis Council, NMC(1999), milk samples were plated onto different specific bacteriological media (oxoid UK). Morphological and biochemical characterizations were done on collected isolates. S. aureus which were positively detected in single pure form were conducted to antimicrobial and QAC susceptibility while M.O. other than S. aureus bacteria or mixed infections with S. aureus were excluded.

**Antimicrobial susceptibility tests**

Antimicrobial susceptibility tests were done by Kirby–Bauer disk diffusion method on Muller-Hinton agar (MHA) according to the Clinical and Laboratory Standards Institute (2014). The antibiotics disks were from (Oxoid UK), penicillin G (P, 10 IU), gentamicin (CN, 10 µg), and tetracycline (TE, 30 µg), amoxicillin +clavulanic acid (AMC, 30) norfloxacin (NOR, 10 µg) neomycin (N, 30 µg) cephadrine (CE, 30 µg).

**Susceptibility testing to QAC substances**

According to Bjorland et al., (2001), all S. aureus isolates were initially tested for susceptibility to QAC by studying their growth on Mueller-Hinton (MH) agar containing 10 different concentrations of benzalkonium chloride (BC) ranging from 1 to 10 µg/ml. A control MH agar plate containing no drug was used for the isolate. Overnight MH broth cultures were diluted in 0.9% Na Cl to an inoculum concentration of approximately 10⁶ CFU/ml. Two hundred microliters of the diluted culture were transferred to the surface of an MH agar plate and incubated for 24 h at 37°C. Isolates showing confluent or semi confluent growth on MH agar containing BC at 4 µg/ml were considered resistant to QAC.

**Extraction of DNA**

A boiling procedure was used to extract DNA from bacterial isolates according to Reischl et al. (1994).

**PCR assay**

A duplex PCR assay targeting 16S rRNA gene (staphylococcus genus-specific), nuc gene (S. aureus species-specific), multiplex PCR assay targeting enterotoxins A(Sea) and B(Seb) with exopholative toxin (etb) genes and simplex PCR assay targeting qacA/B gene were performed. All assays were achieved using the total volume of 30ul reaction mix contain 5ul of template DNA, 20 pmol of each primer and 1X of PCR mix (PCR Master Mix, Ferments, Life Science). The PCR cycles were carried out in Eppendorf AG (22331 Hamburg) thermocycler. Detailed sequences of primers and cycling protocols are depicted in (Tables 1, 2). The analysis of PCR products was carried out using 1.5% ethidium bromide-stained agarose gel.

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Table 1: Primers used in PCR assays

<table>
<thead>
<tr>
<th>Target</th>
<th>Name (strand)</th>
<th>Primer sequence (5' - 3')</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus</em></td>
<td>16S rRNA - F</td>
<td>5' - GTA GGT GGC AAG CGT TAT CC -3'</td>
<td>Monday and Bohach (1999)</td>
</tr>
<tr>
<td></td>
<td>16S rRNA - R</td>
<td>5'- CGC ACA TCA GCG TCA G -3'</td>
<td></td>
</tr>
<tr>
<td><em>Staph aureus</em></td>
<td>nuc 1</td>
<td>5' - GCG ATT GAT GGT GAT ACG GTT -3'</td>
<td>Brakstad et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>nuc 2</td>
<td>5'- AGC CAA GCC TTG ACG AAC TAA AGC -3'</td>
<td></td>
</tr>
<tr>
<td><em>qacA/B</em></td>
<td>Forward</td>
<td>5'- TCCTTTTTAATGCTGGCTTTATACC -3'</td>
<td>Martineau et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'- AGCCKTACCTGCTCCAACTA -3'</td>
<td></td>
</tr>
<tr>
<td><em>Sea</em></td>
<td>Forward</td>
<td>5' - GTATGGTGTTGTAACTGAGC -3'</td>
<td>Mehrotra et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'- CCAAATAGTGACGAGTTAGC -3'</td>
<td></td>
</tr>
<tr>
<td><em>Seb</em></td>
<td>Forward</td>
<td>5' - ACAAGCAAAAAGAATACACAGCG -3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'- GTTTTTGCGTGCTTCTTCTG -3'</td>
<td></td>
</tr>
</tbody>
</table>

K means a set of single letter codes have been accepted (K = G or T)

Table 2: Cycling protocols of PCR assays

<table>
<thead>
<tr>
<th>Target</th>
<th>Amplicon size</th>
<th>Cycling program</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Step</td>
</tr>
<tr>
<td><em>16S rRNA and nuc genes</em></td>
<td>228bp and 279bp</td>
<td>Initial denaturation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denaturation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Annealing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Extension</td>
</tr>
<tr>
<td><em>Sea, Seb and etb genes</em></td>
<td>102, 164 and 226bp</td>
<td>Initial denaturation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denaturation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Annealing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Extension</td>
</tr>
<tr>
<td><em>qacA/B</em></td>
<td>220bp</td>
<td>Initial denaturation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denaturation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Annealing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Extension</td>
</tr>
</tbody>
</table>

All PCR programs were ended with a final extension at 72°C for 10 minutes.
RESULTS

Results of CMT revealed 183 out of 400 with a percentage of (45.75%) were subclinical mastitic quarter milk samples (QMS).

Bacterial culture
In this work, 103/ 400 (25.75%) out of QMS were S. aureus in single pure were successfully isolated on specific media. The detailed results of the bacteriological examination were depicted in the table (3).

Antibiotic and QAC sensitivity tests
Results of antibiotic sensitivity test were recorded in the table (4), the most sensitive were Norfloxacin, Cephradine, Neomycin and Amoxicillin +clavulanic acid in contrast higher resistance were recorded for Penicillin G and Tetracycline antibiotics. Regarding the results of QAC sensitivity, 8 isolates (7.76%) out of 103 S. aureus isolates were resistant to benzalkonium chloride (BC) table (5).

Molecular confirmation
All isolates gave successful amplification of both 228and 279bp for 16S rRNA and nuc gene-specific for S. aureus bacteria Fig (3). The results of detection of enterotoxins revealed positive amplification of 102 and 164bp specific for Sea and Seb respectively, while there is no amplification were detected for etb gene Fig. (4). The complete data are shown in table (5). PCR amplification for QAC gene could confirm the presence of qac A/B gene by amplification of specific amplicon of 220bp in 13 isolates were 10 of them are toxigenic strains with a percentage of (76.92%) Fig (5) and Table (5).

Table 3: bacteriological examination

<table>
<thead>
<tr>
<th>QMS</th>
<th>S. aureus</th>
<th>S. aureus with other M. O</th>
<th>all Staphylococci</th>
<th>other M. O</th>
<th>Neg. Bac.</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>103 (25.75%)</td>
<td>37 (9.25%)</td>
<td>140 (35%)</td>
<td>184 (46%)</td>
<td>76 (19%)</td>
</tr>
</tbody>
</table>

Table 4: Antibiotics Susceptibility test

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Resistance</th>
<th>Moderate</th>
<th>Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>%</td>
<td>No</td>
</tr>
<tr>
<td>Penicillin G (P,10)</td>
<td>120</td>
<td>85.7</td>
<td>18</td>
</tr>
<tr>
<td>Gentamycin (CN, 10)</td>
<td>30</td>
<td>21.4</td>
<td>63</td>
</tr>
<tr>
<td>Tetracycline (TE 30)</td>
<td>76</td>
<td>54.2</td>
<td>6</td>
</tr>
<tr>
<td>Amoxicillin +clavulanic acid (AMC, 30)</td>
<td>10</td>
<td>7.1</td>
<td>21</td>
</tr>
<tr>
<td>Norfloxacin (NOR, 10)</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Neomycin (N, 30)</td>
<td>14</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Cephradine (CE, 30).</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
</tbody>
</table>
Table 5: Results of detection of enterotoxin and QAC genes in *S. aureus* isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th><em>S. aureus</em></th>
<th>Toxigenic <em>staph aureus</em></th>
<th>Isolates contain QAC gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>Sea</em></td>
<td><em>Seb</em></td>
</tr>
<tr>
<td>Phenotypic</td>
<td>103</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Genotypic</td>
<td>103 (100%)</td>
<td>19 (18.44%)</td>
<td>13 (12.62%)</td>
</tr>
</tbody>
</table>

Fig. 1: Antibiotic sensitivity test concentration

Fig. 2: QAC sensitivity with different

Fig. 3: Shows ethidium bromide-stained 1.5% agarose gel electrophoresis of duplex PCR assay of *Staphylococcus* spp. Lane M: 100bpDNA ladder, Lane 1: positive control, Lanes 2-12: positive amplicons of 228 and 279bp specific for *S. aureus*, Lane 13: negative control.

Fig. 4: Shows ethidium bromide-stained 1.5% agarose gel electrophoresis of multiplex PCR assay of enterotoxin (*Sea*, *Seb*) and exfoliative toxin (*etb*) of *S. aureus*. Lane M: 100 bp ladder. The number of bands: 6 Size range: 100-600 bp, Lane 1: positive control contains 3 bands (226bp for *etb*, 164bp for *Seb* and 102bp for *Sea*), Lane 2: negative control, Lanes 3, 4 and 8 positives for *Sea* gene, Lanes 6 and 7 positives for *Seb* gene.
Molecular Detection Of Toxins …..

Fig. 5: Shows ethidium bromide-stained 1.5% agarose gel electrophoresis of simplex PCR assay of QAC gene. Lane M: 100bp DNA ladder, Lane 1: positive control, Lanes 2-6 also Lanes 9-12 S. aureus isolates negative to QAC gene, Lanes 8 and 9 positive isolates to QAC gene with specific amplification of 220bp, Lane 13: negative control.

DISCUSSION

In this study, the bacterial investigation was applied on dairy herds in which QAC had been used for teat dip disinfection for more than 3years for isolation of S.aureus and evaluation of their antibiotic and QAC resistance followed by detection of enterotoxin and antiseptic resistance genes of these isolates using the PCR methods.

Subclinical mastitis is hard to detect and consider to be the main form of mastitis in dairy herds. The bacterial investigation revealed S. aureus could be isolated 103 (25.75%), this result is compatible with the results of (Naher et al., 2014) who detected Staphylococi in milk samples of dairy cows with a percentage of 26.71%. and Bedane et al. (2012) who said that nearly 30%-40% of all mastitis cases caused by S. aureus. On the other hand, higher prevalence 75% and 38.50% recorded by Jørgensen et al., (2005) and Ebtsam et al.,(2018)respecitively. lower per cent than recorded by Ebtsam (2001) who found S.aureus 15.8% in subclinical mastitic cows. S. aureus antimicrobial-resistant strains were detected by disc diffusion assay.

In the current work table (4) revealed that S. aureus isolates showed high sensitivity to Norfloxacin, Cephradine, Neomycin and Amoxicillin +clavulanic acid 95%,94.3% 90% and 77.9%, respectively. The bacterial investigation revealed S. aureus isolates showed high sensitivity to Norfloxacin, Cephradine, Neomycin and Amoxicillin +clavulanic acid. In contrast, higher resistance was recorded for Penicillin G and Tetracycline with a percentage of 85.7% and 54.2% respectively (Saini et al., 2012; Akindolire et al., 2015). Similarly, the results were near to that reported by Ebtsam(2018) in which the resistance of penicillin; gentamycin and tetracycline were 100%, 43.5% and 58%, respectively. Also, some difference was recorded by Yang et al., (2016) as antimicrobial resistance of S. aureus were (84.09%, 9.09% and 15.91%) for penicillin, gentamycin and tetracycline respectively.
Resistance to penicillin may be attributed to its widespread use in intramammary preparations (Bagcigil et al., 2012). Moreover, a comparable pattern of resistance was documented for penicillin G and tetracycline (Ito et al., 2003; Pesavento et al., 2007; Zmantar et al., 2011; Angeles et al., 2013; Yamamoto et al., 2013).

So determining the antimicrobial susceptibility profiles are required not only for effective therapy but also for monitoring the spread of resistant strains in defined ecological niches. Therefore, early detection of subclinical cases and their causes followed by treatment of mastitic cattle helping in the prevention of new udder infections and reduction of these losses. *Staphylococcus aureus* can possess a serious hazard to human consumers due to higher prevalence or toxins. Particularly enterotoxins (SE), specially SEA-SEE were the greatest discovered genes in cattle and play a role in the development of mastitis, by creating an attractive environment for colonization. The isolated *S. aureus* had enterotoxigenic type A and B (Sea and Seb) genes where detected by PCR sited as 19/103 (18.44%) and 13/103 (12.62%) respectively as reported in the table (5).

Contrary to the previous study of (Abd El Tawab et al., 2016) who revealed that *Sea* gene was produced by 5 (45.45%) while *Seb* was not produced by any strains isolated from milk and milk products. In the same context (El-Jakee et al., 2011) reported that *S. aureus* isolates were positive for both *sea* and *seb* genes. While This result was lower than that was recorded by Ebtsam et al., (2018), that type *Sea* and *Seb* genes distinguished by PCR as 7/69 (10.1%) and 1/69 (1.5%) respectively, Yu-Cheng et al.,(2008), they found *sea* (29.2%) and *seb* (19.7%) from isolated *S. aureus*.

There was no detection of *etb* gene in all isolates. This result is in agreement with the results of (Perez et al., 2009; Akindolire et al., 2015 and Monéstro et al., 2018) showing that *S. aureus* isolates were seldom positive for exfoliative toxins *eta* and *etb*. However, our finding disagrees with the previous finding of Abbasi et al., (2017) who obtained the *etb* gene in 6.8% of their isolates.

The extensive application of products containing commonly used biocides, such as phenolics and quaternary ammonium compounds (QACs), increases the subject of their efficacy and it additionally increases alarms about the probable rise of microbial resistance (Vijayakumar and Sandle 2018). The presence of QAC genes in staphylococci has been reported in several studies performed in Hong Kong (Zhang et al., 2011), in Japan Alam et al., 2003) North America. (Vali et al., 2008; Longtin et al., 2011). This study revealed the presence of QAC gene in 13 isolates (12.62%) out of 103 isolates this result was in agreement with the finding of Damavandi et al., (2017) ; Ignac et al., (2017) who recorded the existence of QAC gene in 15 /120 (12.50%) and 3/29 (10.3%) clinical *S. aureus* isolates respectively.

Additionally, many studies discussed the detection of QAC gene in staphylococci isolated from goat and bovine herds in different countries. Bjorland et al., (2005) identified QAC resistance genes in 10% of the goat herds in Norway and reported that there was a wide prevalence in Staphylococci spp. in goat herds. In the study of Cantekin et al., (2019), (6.3%) were positive for qac genes in goat herds in Turkey. In the same region, Ergun et al., (2017) reported 40% QAC positivity in bovine subclinical staphylococci isolates.

Results presented in this work revealed 8 isolates were considered phenotypically resistant to QAC as showing confluent growth on MH agar containing BC at4 μg/mL. However, 13 (12.62%) isolates were harbour QAC gene could be detected genotypically by PCR. Therefore, this work could overcome the drawback recorded for the study of Loncaric et al., (2019) that antiseptic sensitivity testing; was not performed and was not able to associate between the detection of QAC genes and the phenotypic QAC resistance. Some previous studies that discuss the relation of the presence of qac genes, and their phenotypic resistance in staphylococci, revealed no association between them (Bjorland et al., 2005; Couto et al., 2015; Vali et al., 2017).

However, Ignak et al., (2017) found a significant association between the existence of staphylococci antiseptic resistance genes and the increase of MIC values of BC (>4 μg/mL). It was obvious from (Table 5) that there was no strict correspondence between presence of QAC gene (genotype) and its phenotypic expression as 8 isolates (7.76%) were resistant to benzalkonium chloride (BC) versus to 13 isolates (12.62%) harbour QAC gene could be detected by PCR. This is possibly due to the expression of QAC gene require the participation of other genes, or maybe silenced and are not expressed. The same result was previously reported by Wang et al., (2011) for the hemolysin gene. Also, it probably due to the explanation of Čevinkova et al., (2013) who concluded that the phase of bacterial growth is more important for QAC gene expression of BC resistance than the ability to adapt to this antiseptic.

The worth mentioning, in this study that 10 (76.92%) isolates of all 13 QAC positive isolates were of toxigenic strains. This observation seems to
highlight the presence of the prevalence of QAC genes and the enterotoxins with the upgrading resistance of S. aureus isolates. Similarly, Nakipoglu et al., (2012); Prag et al., (2014); Wassenaar et al., (2015); Ignak et al., (2017) concluded that antiseptic resistance genes together with antibiotic resistance genes contribute to the development of resistance in pathogens. Furthermore, Zhang et al., (2011) found an association between the presence of antiseptic resistance genes, and resistance to some antibiotics e.g. (penicillin and tetracycline) and reduced susceptibility to antiseptics.

This result comes in agreement with the result obtained in this study for the resistance of penicillin and tetracycline. In the same context, several investigations have implied that there is disinfectant cross-resistance with antibiotics (Chapman 2003 and Wang et al., 2008). Additionally, Ho et al., (2015) reported that enterotoxins (SEs genes) were more commonly detected in qacA/B positive isolates, comparing to qacA/B negative strains. They also added in the same work that the resistance to antibiotics especially tetracycline was significantly associated with the existence of qacA/B gene.

CONCLUSION

This study presented a considerable prevalence of S. aureus in bovine quarter milk samples in Egypt. The long-term use of quaternary ammonium compounds may exhibit S. aureus organisms harbouring QAC genes occasionally associated with antibiotic resistance genes. Antibiogram, as well as the identification of toxigenic and QAC genes in this study, may open another perspective in planning some alternative therapeutic strategies against multi-resistance S. aureus that may be transmitted from mastitic cows to human avoiding potential risks for food security and public health. Monitoring cross-resistance between antibiotics and antiseptic should be further investigated.

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