Journal of Applied Veterinary Sciences, 5 (1): 35-45
ISSN: Online: 2090-3308, Print: 1687-4072
Journal homepage : https://javs.journals.ekb.eg

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 3years. 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, 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 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.

Original Article:
DOI:HTTPS://DX.DOI.ORG/10.21608/AVS.2020.75411

Received :17 December, 2019
Accepted : 22 January, 2020
Published in January 2020

J. Appl. Vet. Sci., 5(1):35 -45

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 3years 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

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) cephradine (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 10^6 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 at4 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.

Table 1: Primers used in PCR assays

Target	Name (strand)	Primer sequence (5 - 3)	References
Staphylococcus	16S rRNA - F 16S rRNA -R	5' - GTA GGT GGC AAG CGT TAT CC -3' 5'- CGC ACA TCA GCG TCA G -3'	Monday and Bohach (1999)
Staph aureus	*nuc* 1 *nuc* 2	5'-GCG ATT GAT GGT GAT ACG GTT-3' 5'-AGC CAA GCC TTG ACG AAC TAA AGC-3'	Brakstad *et al*. (1992)
qacA/B	Forward Reverse	5'-TCCTTTTAATGCTGGCTTATACC-3' 5'-AGCCKTACCTGCTCCAACTA-3'	Martineau *et al.* (2000)
Sea	Forward Reverse	5'-GGTTATCAATGTGCGGGTGG-3' 5'-CGGCACTTTTTTCTCTTCGG-3'	Mehrotra *et al*. (2000)
Seb	Forward Reverse	5'- GTATGGTGGTGTAACTGAGC-3' 5'-CCAAATAGTGACGAGTTAGG-3'	
Etb	Forward Reverse	5'-ACAAGCAAAAGAATACAGCG-3' 5'-GTTTTTGGCTGCTTCTCTTG-3'	

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

Table 2: Cycling protocols of PCR assays

Target	Amplicon size	Cycling program			
		Step	Temp.	Time	No. of cycles
16S rRNA and *nuc* genes	228bp and 279bp	Initial denaturation	94ºC	5min	1 Cycle
		Denaturation	94ºC	45s	35 Cycles
		Annealing	55ºC	45s	
		Extension	72ºC	45s	
Sea, *Seb* and *etb* genes	102, 164 and 226bp	Initial denaturation	94ºC	5min	1 Cycle
		Denaturation	94ºC	The 30s	35 Cycles
		Annealing	50ºC	30s	
		Extension	72ºC	30s	
qacA/B	220bp	Initial denaturation	96ºC	3min	1 Cycle
		Denaturation	95ºC	30s	30 Cycles
		Annealing	56ºC	30s	
		Extension	72ºC	2min	

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

QMS	*S. aureus*	*S. aureus with other M. O*	all *Staphylococci*	other M. O	Neg. Bac.
	No	No	No	No	No
400	103 (25.75%)	37 (9.25%)	140 (35%)	184 (46%)	76 (19%)

Table 4: Antibiotics Susceptibility test

Antibiotics	Resistance		Moderate		Sensitive	
	No	%	No	%	No	%
Penicillin G (P,10)	120	85.7	18	12.9	2	1.4
Gentamycin (CN, 10)	30	21.4	63	45	47	33.6
Tetracycline (TE 30)	76	54.2	6	9.2	68	48.6
Amoxicillin +clavulanic acid (AMC, 30)	10	7.1	21	15	109	77.9
Norfloxacin (NOR, 10)	0	0	7	5	133	95
Neomycin (N, 30)	14	10	0	0	126	90
Cephradine (CE, 30).	0	0	8	5.7	132	94.3

Table 5: Results of detection of enterotoxin and QAC genes in *S. aureus* isolates

Isolates	*S. aureus*	Toxigenic *staph aureus*				Isolates contain QAC gene
		Sea	*Seb*	*etb*	Total toxigenic	qacA/B
Phenotypic	103	-	-	-	-	8 (7.76%)
Genotypic	103 (100%)	19 (18.44%)	13 (12.62%)	- (0%)	32 (31.07%)	13 (12.62%)

Fig. 1: Antibiotic sensitivity test concentration

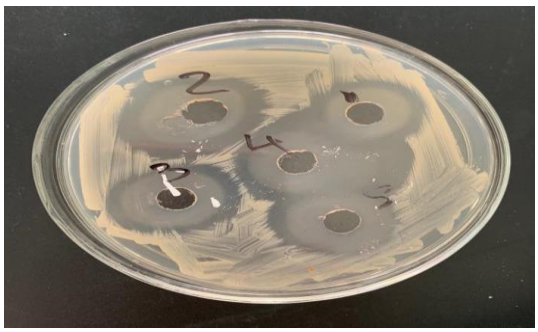

Fig. 2: QAC sensitivity with different

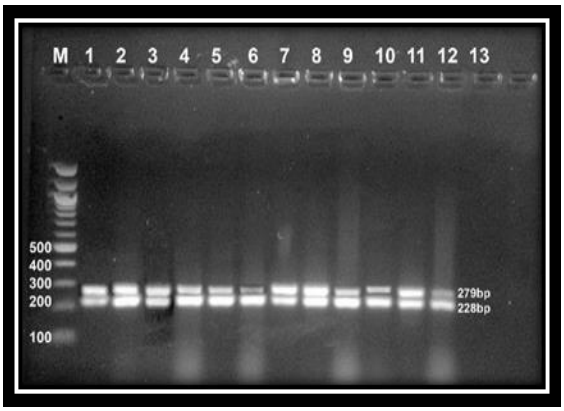

Fig. 3:Shows ethidium bromide-stained 1.5% agarose gel electrophoresis of duplex PCR assay of Staphylococcus spp. Lane M: 100bpDNA ladder, Lane 1: positive control, Lanes 2- 12: positive amplicons of 228 and 279bp specific for *S. aureus*, Lane 13: negative control.

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.

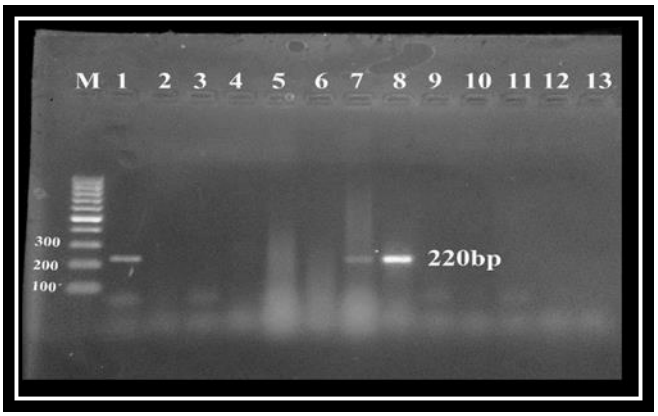

Fig. 5: Shows ethidium bromide-stained 1.5% agarose gel electrophoresis of simplex PCR assay of QAC gene. Lane M: 100bp DNA ladder, Lane 1: positive control, Lanes 2-6 also Lanes 9-12 *S. aureus* isolates negative to QAC gene, Lanes 8 and 9 positive isolates to QAC gene with specific amplification of 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. In the table (3)The results of CMT performed in this study revealed 183 out of 400 with a percentage of (45.75%) were subclinical mastitic quarter milk samples (QMS). This percentage is well supported by earlier studies conducted in India, by **(Kalorey *et al.*, 2007; Banger *et al.*, 2015)** who recorded the presence of subclinical mastitis in the range of 10%–70%. While bacteriological examination revealed that 19% of the samples had no bacterial growth, this result was nearly to that recorded by **Ebtsam (2001)** who recorded that 17.28% while it was lower than (**Ashraf *et al.*, 2017**) who reported 30% and higher than **Ebtsam *et al.*,(2018)**, that indicated the need for another specific pathogen media or spontaneous cure or intermittent shedding of M.O. as *Staphylococcus aureus* which is record as communal contagious pathogen of bovine mastitis possessive many virulent factors as toxins, multidrug and antiseptic resistant, make the disease difficult to cure, increasing global problem which has become critical for dairy industry worldwide.

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 *Staphylococci* 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)**respectively. 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. 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 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. S*taphylococcus. 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)* gens 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 Monistero *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 **Cevinkova *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 importance of the presence of QAC genes and the enterotoxins with the upgrading resistance of *S. aureus* isolates. Similarly, **Nakipoğlu *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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How to cite this article:

Ebtsam E.Z.Kotband Jehan A. Gafer. 2020. Molecular Detection Of Toxins And Disinfectant Resistance Genes Among *Staphylococcus Aureus* Isolated From Dairy Cattle In Egypt**.** Journal of Applied Veterinary Sciences, 5(1): 35- 45.

DOI:HTTPS://DX.DOI.ORG/10.21608/JAVS.2020.75411