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Molecular detection of quaternary ammonium compound resistance genes of *Staphylococcus aureus* from udder surface and mastitis milk of bovines#

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Abstract

Quaternary ammonium compounds (QAC) are widely used biocides in the field of medical and veterinary practice. Resistance to QACs is an emerging problem due to this widespread use. The present study was undertaken to detect the presence of genes that contribute to resistance to QACs in Staphylococcus aureus isolates obtained from udder surface and mastitis milk. A total 22 isolates of S. aureus were obtained from udder washings and milk of bovine mastitis cases. Among these, seventeen isolates were from udder wash samples and five isolates from clinical and subclinical cases of mastitis. Broth microdilution assay was performed to assess the minimum inhibitory concentration (MIC) of the isolates against Cetyltrimethylammonium bromide (CTAB), a QAC. Polymerase chain reaction (PCR) was done targeting qac A/B gene which codes for efflux pump which targets QACs. In the present study qac A/B gene was detected in seven out of 22 isolates of S. aureus. Three of these isolates were obtained from udder washings before milking and four, after milking. None of the S. aureus isolates from mastitis milk possessed the gene. The average minimum inhibitory concentration of qac A/B positive isolates against CTAB was 0.63 ± 0.55 µg/ml. The average MIC between qac A/B positive and negative isolates were statistically insignificant. The study points to the fact that multiple factors could be contributing to biocide resistance in S. aureus.

Keywords: *S. aureus, udder surface, mastitis, CTAB, minimum inhibitory concentration, biocide resistance genes*

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India ranks first in milk production in the world producing 198.4 million tonnes per annum (NDDB, 2021) but the quality of milk is largely unsatisfactory compared to the developed countries. Contagious mastitis is a major reason for poor quality of milk produced and the reason could be linked to various unscientific management practices like lack of care given for udder cleaning and teat disinfection, improper milking methods and poor overall hygiene.

Staphylococcus aureus is one of the commonest bacteria that causes contagious bovine mastitis (Cervinkova *et al.,* 2013). Teat disinfection is considered as important method to prevent mastitis in bovines. Wide variety of disinfectants and antiseptics are available for the purpose. Increased use of antiseptic agents induces selection pressure in *S. aureus* which may lead to development of biocide resistance (Saber *et al.,* 2019). Due to its less corrosiveness, low toxicity and higher stability, QACs are commonly used in medical and food industry (McDonnell and Russell, 1999). The resistance to QACs are usually mediated by a group of efflux pumps with nonspecific target substrate called as Qac proteins. These proteins belong to a group called as small multidrug resistance proteins. Resistance to QACs is believed to be due to the presence of this efflux pump. The present study was conducted to detect *S. aureus* isolates resistant to QACs and to study the role of *qac A/B* gene contribution towards biocide resistance.

Materials and methods

The present study was conducted in 20 organized farms in Wayanad district of Kerala, India. The samples were udder wash and mastitis milk of cows affected with clinical and subclinical mastitis.

Isolation of *Staphylococcus aureus* from udder wash

Udder washings were collected before and after milking from ten percent of total lactating animals in each farm (96 udder wash). Gram positive isolates that were oxidase negative, catalase and coagulase positive were streaked on to Mannitol salt agar (MSA) plates and incubated at 37°C for 24-48 hours. Isolates that produced yellow colonies were identified as *Staphylococcus aureus*.

Isolation of *S. aureus* from milk samples

Mastitis milk samples were aseptically collected from CMT positive animals in a sterile container (34 mastitis milk samples). Loopful of milk sample was streaked on brain heart infusion agar in primary, secondary and tertiary fashion in order to obtain isolated colonies of bacteria. These Petri dishes were incubated for 24 hours 37°C. The growth was examined for the colonial morphology and subsequently different types of colonies were sub cultured on separate plates in order to obtain pure cultures. Gram positive isolates that were oxidase negative, catalase and coagulase positive and produced yellow colonies on MSA plates were identified as *S. aureus* isolates.

Microdilution assay

Minimum inhibitory concentration of CTAB towards each *S. aureus* isolates were assessed by broth microdilution assay (Castilho *et al.,* 2015). A "U" bottom microtitre plate was first filled with 50 µL of CTAB at an initial concentration of 250 µg/ml and was serially double diluted in peptone water, to which 50 µL of the isolate incubated overnight in peptone water and concertation adjusted to 0.5 Mc Farland unit was added. Following overnight incubation at 37°C, one per cent Resazurin dye was added and further incubated for one hour. Presence of bacterial growth was indicated by the dye turning to pink colour. MIC was calculated as the highest dilution where total inhibition of the bacterial growth obtained. The isolates were considered resistant to QAC, if MIC was found to be greater than 4 µg/mL (Bjorland *et al.,* 2001).

Molecular detection of *qac A/B* gene

For detection of *qac A/B* gene, PCR of whole DNA extract was carried out. The total DNA was extracted from overnight broth culture using HiPurA® Bacterial Genomic DNA Purification Kit (MB505-HIMEDIA). The PCR reaction mix was prepared with five µL of total DNA, one microliter each of forward

and reverse of primers, 12.5 µL Master mix (Takara) and final volume was adjusted to 25 µL. The primers used were *qac A/B* F (5'-TCC TTTTAATGCTGGCTTATACC-3') and *qac A/B* R (5'-AGCCKTACCTGCTCCAACTA-3') (Martineau *et al.,* 2000). The reaction was carried out in Biorad thermocycler equipment. Reaction protocol was set up as initial denaturation at 96°C for three minutes, followed 30 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for two minutes. A final extension for 10 minutes at 72°C. The amplified product was visualised by agar gel electrophoresis using 1.2 per cent agarose gel stained in ethidium bromide.

Results and discussion

Out of 96 samples of udder wash, *S. aureus* could be isolated from 17 samples (17.70 per cent). The findings are in contrast with Bhati *et al.* (2018), who recovered *S. aureus* form of 54 per cent of the udder surfaces studied. The low prevalence could be due to the presence of other microorganisms that were colonising teat surface. Among 34 culture positive milk samples, *S. aureus* could be isolated from five samples. These results were in accordance with Jena *et al*. (2015). Average minimum inhibitory concentration (MIC) of CTAB towards 22 isolates of *S. aureus* was 0.58 ± 0.24 µg/ml. The MIC of CTAB for *S. aureus* ranged from 0.004 µg/ml to 3.91 µg/ml. The findings are in accordance with Bjorland *et al.* (2001).

The isolates of *S. aureus* obtained from udder wash had average MIC of 0.73 ± 0.30 µg/ml. Isolates from udder wash obtained before milking had average MIC of 0.78 ± 0.54 µg/ml, while the isolates from udder wash after milking had average MIC of 0.7 ± 0.37 µg/ml. There was no significant difference in MIC values between udder wash samples. The average MIC of *S. aureus* isolated from udder wash in farms that did not follow teat disinfection practices was 1.10 ± 0.48 µg/ml. The average MIC of *S. aureus* isolated from udder wash in farms where teat disinfection followed was found to be 0.20 ± 0.13 µg/ml. Statistical analysis using Mann-Whitney U test showed no significant difference between these two groups. The isolates of *S. aureus* from mastitis milk had average MIC of 0.05 ± 0.01µg/ml. Statistical analysis using Mann-Whitney U test showed significant difference ($p<0.05$) between MIC values of *S. aureus* isolates obtained from udder wash and cases of mastitis.

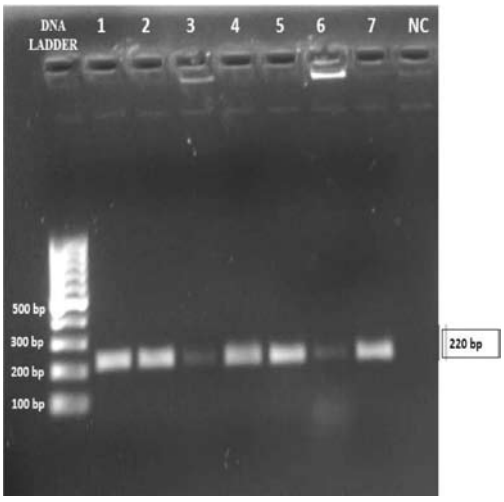

Fig. 1. Amplicons of *qac A/B gene*

Lane 1 to 7 -	Positive samples
NC -	Negative control

In the present study, while conducting PCR, *qac A/B* gene was detected in seven (31.82 per cent) out of 22 isolates (Fig.1), while the remaining 15 (68.18 per cent) isolates were negative for *qac A/B* gene. The gene could be detected only from udder wash isolates, out of which three were obtained before milking and four were obtained after milking. The gene could not be amplified from any of the isolates from mastitis cases. The *qac A/B* gene located in the plasmids (Littlejohn *et al.,* 1991) regulates multidrug transport system in the bacterial cell along with other efflux pumps coded by genes such as *smr* and *nor A* (Noguchi *et al.,* 2005). Kotb and Gafer (2020) could detect presence of *qac* gene in 12.62 percent isolates from milk samples. Shafi *et al.* (2021) also found that 3.33 percent of *S. aureus* isolates form milk samples had *qac A/B* gene. The average MIC of *qac A/B* negative isolates against CTAB was 0.55 ± 0.26 µg/ml and was 0.63 ± 0.55 µg/ml for *qac A/B* positive isolates. No statistically significant difference (Mann-Whitney U test) could be noted between the groups. It was concluded that genes coding for biocide resistance existed in *S. aureus* found on udder surface, even when there was no apparent exposure to the biocide. High MIC was noted both in

isolates with and without the *qac A/B* gene. This indicated that other genetic and environmental factors could be playing a role in high MIC in the isolates that did not possess the *qac A/B* gene. Further studies are warranted to obtain deeper understanding on biocide resistance mechanisms of udder surface *S. aureus* in order to aid in better control of bovine mastitis.

Conclusion

The MIC of *S. aureus* isolated from udder wash was significantly higher than isolates from mastitis milk. Quaternary ammonium compound resistance gene was detected in this study. There was no significant difference between MIC of *qac A/B* positive and negative isolates against CTAB. Hence, other mechanisms involved in biocide sensitivity and resistance need to be studied.

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Conflict of interest

The authors have no conflicts of interest to declare.

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