

Investigation of the presence of slime production, VanA gene and antiseptic resistance genes in *Staphylococci* isolated from bovine mastitis in Algeria



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Abstract

Staphylococcus strains are frequently associated with clinical and subclinical bovine intra-mammary infection. The virulence factors of *staphylococcus* have not been widely studied in Algeria. The objective of this study was to determine the frequency of slime production, VanA gene and antiseptic resistance genes in staphylococci strains isolated from bovine mastitis in Algeria. The study examined 35 *Staphylococci* strains obtained from the inflammatory secretion of mammary glands of cows with mastitis. Slime production was determined by detecting the *icaA* and *icaD* genes using the polymerase chain reaction (PCR) method and Congo red agar (CRA) method. The presence of *qacAB* and *qac C* antiseptic resistance genes and the VanA resistance gene in these isolates was investigated by PCR. The results of the

current study revealed that of the 35 *Staphylococci* isolates, 42.85% (15/35) and 17.14% (6/35) of the isolates harboured the slime production gene by analysing *icaA* and *icaD* genes, respectively and 71.42% (25/35) by the CRA method. However, VanA and antiseptic resistance genes (*qacAB* and *qac C*) were not detected in any of the isolates. Therefore, the majority of *Staphylococcus* strains were capable of producing slime, and the CRA detection rate was higher than the PCR method for the biofilm-producing capacity of *Staphylococcus* strains. Thus, the presence of the *ica* genes in *Staphylococcus* strains confirms its role as a virulence factor in the pathogenesis of bovine mastitis.

Key words: cow; mastitis; resistance genes; slime production; *Staphylococci*; virulence factor; Algeria

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Introduction

Staphylococcus strains are an important cause of bovine mastitis (Hussain et al., 2012, Saidi et al., 2013; Jian-Zhong et al., 2014). *Staphylococcus* strains also cause mastitis and invasive disease in camels, dogs, cats, and hamsters. Its presence is regularly associated with high somatic cell counts in milk and decreased milk production. Staphylococcal infections have major consequences for human health (Saidi et al., 2015).

Treatment with intramammary infusion of antibiotics is the main approach in dealing with infection, and a number of studies using *in vitro* and *in vivo* trials have been conducted to assess antibiotic sensitivity/resistant patterns (Ergün et al., 2012; Bardiau et al., 2013; Saidi et al., 2015). The use of antibiotics by breeders to treat staphylococcal-induced mastitis poses a risk for the development of antibiotic resistance (Virdis et al., 2010). However, there are few reports focusing on the genes involved in resistance, especially concerning *Staphylococcus* isolates of bovine origin.

Encoded by *icaA* and *icaD* genes, slime production is a well-known virulence property that assists *Staphylococci* in udder tissue to evade the host's defence system and antimicrobial agents (Vasudevan et al., 2003; Namvar et al., 2013).

For the first time, antiseptic resistance genes were reported from hospital isolates where antiseptics are used routinely. They are also intensively used in the disinfection of udders (pre-dipping and teat dipping), milking systems and dairy equipment used for producing dairy products (Mayer et al., 2001). Therefore, monitoring resistance genes against different groups of antibiotics and antiseptics can be useful in detecting the development of resistant microorganisms (Merz et al., 2016).

The present study aims to identify antibiotic and antiseptic resistant and

virulence (slime production) genes in *Staphylococcus* strains isolated from subclinical mastitis cases in Algeria.

Identified as causative agents of mastitis in cattle, certain *Staphylococci* strains exhibit the ability of producing a viscous extracellular polysaccharide layer (slime). This is now recognised as a virulence factor, as it promotes bacterial adhesion onto the mammary epithelial cells and protects bacteria from opsonization and phagocytosis.

Material and Methods

Isolates

All 35 *Staphylococci* strains previously isolated from bovine mastitis in Algeria were used in this study. All isolates were activated in Brain Heart Infusion (BHI) Agar incubated at 37 °C for 24 hours aerobically following the standard methods described by Quinn et al. (1994).

Assays for slime production

For the detection of slime production, Congo Red Agar was prepared with 36 g saccharose and 0.8 g Congo red in 1 L brain heart infusion (BHI) agar. One colony from each isolate was taken from BHI agar and inoculated onto Congo Red Agar. For the observation of slime production, agar plates were incubated at 37 °C for 24-48-72 h under aerobic conditions as described previously (Freeman et al., 1989; Yazdani et al., 2006).

Biofilm-associated gene detection (PCR assay)

DNA extraction

For the isolation of template DNA, one colony was taken from BHI agar and mixed with 500 µL TE buffer. The phenol/chloroform nucleic acid extraction method was used for genomic DNA isolation (Sambrook and Russel, 2001). Genomic DNA samples were stored at

Table 1. Propriety of primers pairs used for the PCR analyses in this study

| Target | Primer Name | Primer Sequences (5'- 3') | Size of amplified product | References |
|----------------------|-------------|---------------------------------|---------------------------|-------------------------|
| qacA/qacB | qacA/B f | 5'-TCCTTTTAATGCTGGCTTATACC-3' | 220 bp | Zmantar et al, 2011 |
| | qacA/B r | 5'-AGCCKTACCTGCTCCAACCTA-3' | | |
| qacC | qacCf | 5'-GGCTTTTCAAAATTTATACCATCCT-3' | 249 bp | Zmantar et al, 2011 |
| | qacCr | 5'-ATGCGATGTTCCGAAAATGT-3' | | |
| icaA | ICAAF | 5'-CCTAACTAACGAAAGGTAG-3' | 1315bp | Vasudevan et al. (2003) |
| | ICAAR | 5'- AAGATATAGCGATAAGTGC-3' | | |
| icaD | ICADF | 5'-AAACGTAAGAGAGGTGG-3' | 381bp | Vasudevan et al. (2003) |
| | ICADR | 5'-GGCAATATGATCAAGATAC-3' | | |
| Vancomycin Resitance | VanA 1 | 5'- ATGAATAGAATAAAAGTTGC-3' | 1032 bp | Saha et al. (2008) |
| | VanA 2 | 5'-TCACCCCTTAACGCTAATA-3' | | |

Table 2. The result of Slime production for each isolates

| No | CRA | icaA gene | icaD gene | No | CRA | icaA gene | icaD gene |
|----|-----|-----------|-----------|----|-----|-----------|-----------|
| 1 | + | - | - | 19 | + | + | + |
| 2 | - | - | - | 20 | + | + | + |
| 3 | + | - | - | 21 | + | - | - |
| 4 | + | - | - | 22 | - | - | - |
| 5 | - | - | - | 23 | + | + | - |
| 6 | + | + | + | 24 | + | + | - |
| 7 | - | - | - | 25 | - | - | - |
| 8 | + | + | + | 26 | + | + | - |
| 9 | + | - | - | 27 | + | - | - |
| 10 | - | - | - | 28 | + | + | - |
| 11 | - | - | - | 29 | - | - | - |
| 12 | + | - | - | 30 | + | - | - |
| 13 | + | + | + | 31 | + | + | - |
| 14 | + | - | - | 32 | + | + | - |
| 15 | + | - | + | 33 | + | + | - |
| 16 | - | - | - | 34 | + | + | - |
| 17 | - | - | - | 35 | + | + | - |
| 18 | + | + | - | | | | |

-20 °C until further use. PCR was carried out for the virulence associated genes using primers as shown in Table 1. PCR analyses; primer pairs used for the PCR analyses in this study were also showed in Table 1.

Biofilm-associated genes of *Staphylococcus* strains (icaA, icaD) were detected by PCR. The PCR conditions were as follows: initial denaturation step at 95°C for 8 min, followed by 30 cycles of 95 °C for 30 s, annealing

temperature of each primer pair for 30 s and 72 °C for 30 s, and completed with a 10 min final extension at 72 °C. The sizes of PCR products were analysed by electrophoresis on 1.5% (wt/vol) agarose gels stained with ethidium bromide (0.5 µg/mL), and visualized under the ultraviolet illuminator gel documentation system.

Results

In the PCR analyses of isolates, VanA and antiseptic resistance genes (qacAB and qac C) were not detected in any of the isolates. The results of slime production for each isolate are shown in Table 2.

In the analyses for detection of slime production in CRA, 71.42% (25/35) of isolates gave a positive result, while in the PCR analyses (icaA and icaD genes), a positive result was obtained in 42.85% (15/35) and 17.14% (6/35), respectively.

While 16 strains (45.71%) were detected as biofilm positive by both CRA and PCR assays, 10 strains (28.57%) were unable to produce biofilm by both methods. Some discordance was also recorded between the CRA and PCR essays: 9 strains (25.71%) were biofilm positive by CRA but biofilm negative by PCR essays. None of the strains were positive for the production of biofilm

by PCR essays and negative by CRA. The results suggested that CRA had a higher detection rate for the biofilm-producing capacity of *Staphylococci* strains than PCR essays. Specific bands for icaA and icaD genes are shown in Figure 1.

Biofilm-associated genes in staphylococcal strains

The distribution of different biofilm associated genes (BAGs) in 35 *Staphylococci* strains is showed in Table 4. The most prevalent gene was icaA (42.85%), followed by icaD (17.14%). However, the VanA, qacAB or qac genes were not amplified in any strains.

Discussion

To combat the host immune response and to remain stable in the hostile environment, biofilm formation is an important defensive mechanism of the pathogenic *Staphylococci*. This study indicated that a considerable proportion of staphylococcal strains have the capability to form biofilm.

The present results of slime production by staphylococci strains were slightly lower than in China (87.6% biofilm formation; Li et al., 2011). This suggests that the slime producing strains of *staphylococcus* from bovine mastitis are highly prevalent in Algeria. In USA, Fox et al. (2005) reported that 41.4% of bovine *S. aureus* strains formed biofilm. They reported a high proportion of biofilm positive strains in dairy farms of developed regions. Using the CRA method, Kenar et al. (2012) found that 55.2% of coagulase-negative strains of *Staphylococcus* produce biofilm. In this study, two important assays, CRA and PCR were used to detect biofilm formation in *Staphylococcus*. The proportion of biofilm-producing strains tested by CRA (71.42%) was obviously higher than that by PCR assay (42.85%).

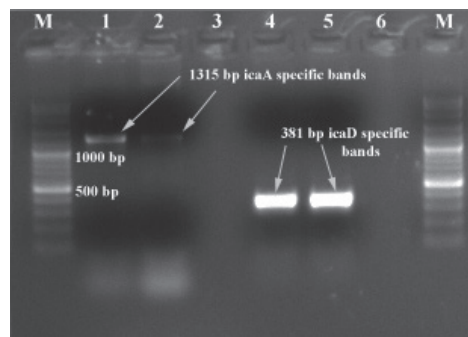


Figure 1. Detection of specific bands for icaA and icaD genes

Another study in the US demonstrated that 91.4% of *S. aureus* strains isolated from bovine mastitis were biofilm-positive using the CRA method, while 68.6% isolates were positive by quantitative assay (Vasudevan et al., 2003). Other reports are contrary to our findings, detecting a lower rate of CRA method for slime formation than using quantitative methods (De-Castro Melo et al., 2013).

The high prevalence of slime-producing staphylococcal isolates by the CRA and PCR assays confirmed that this kind of virulent characteristic is widely distributed among herds in Algeria.

Each method has its own advantages. The CRA method is slightly easier to perform, less time consuming, more sensitive and specific (Kwon et al., 2008), and it has been recommended by many authors (Jain and Agarwal, 2009; Kouidhi et al., 2010). Quantitative methods, such as PCR also has high specificity, sensitivity, and positive predictive value, but is time-consuming and complicated to operate (Mathur et al., 2006). Both of these assays could be chosen as applicable tools for detecting slime production.

Since phenotypic characteristics may arise from different genetic determinants, assessment of biofilm formation at the genetic level is important. There was a good correlation between phenotypic biofilm production and the existence of various genes. In this study, at least one gene in biofilm producing phenotypes of *Staphylococcus* strains was found.

In this study, the *icaA* and *icaD* genes were widely distributed among Staphylococcal strains, which is in accordance with other reports (Vasudevan et al., 2003; Ote et al., 2011).

In this report, despite the resistance to vancomycin inducible *in vitro*, the *vanA*, *qacAB* and *qac* genes were not amplified in any strains. This finding is contrary to the study of Biswajit et al. (2008). The genes *qacAB* and *qac* are likely absent

since antiseptics are not used in udder health on Algerian dairy farms.

Conclusions

Slime formation in staphylococcal strains isolated from clinical and subclinical bovine mastitis in Algeria was reported here for the first time, The majority of strains produced slime, and two associated genes, *icaA* and *icaD*, were detected, though the genes *VanA*, *qacAB* and *qac* were absent. CRA detection rate was higher than PCR assays for the slime producing capacity of *staphylococcus* strains. The study suggests the presence of the genes *icaA* and *icaD* in slime formation.

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Istraživanje prisutnosti proizvodnje sluzi, VanA gena i gena za rezistenciju na antiseptike u stafilocoka izoliranih iz goveđeg mastitisa u Alžiru

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Sojevi stafilocoka često su povezani s kliničkim i supkliničkim intramamarnim infekcijama goveda. Faktori virulencije stafilocoka nisu dobro istraženi u Alžiru. Cilj ove studije bio je odrediti učestalost proizvodnje sluzi, VanA gena i gena za rezistenciju na antiseptike u sojevima stafilocoka izoliranih iz goveđeg mastitisa u Alžiru. Istraživanja su obuhvatila 35 soja stafilocoka dobivenih iz upalnog sekreta mliječnih žlijezda krava s mastitisom. Proizvodnja sluzi ustvrđena je metodom lančane reakcije polimeraze (PCR) istraživanjem *icaA* i *icaD* gena, odnosno metodom s Kongo crvenim agarom (CRA). Prisutnost *qacAB* i *qac C* gena za rezistenciju na antiseptike i VanA gena za rezistenciju u tim izolatima ispitana je pomoću PCR. Rezultati ove studije otkrili su da je od 35

izolata stafilocoka, 42,85 % (15/35) i 17,4 % (6/35) sadržavalo gen za proizvodnju sluzi putem analize *icaA* i *icaD* gena, odnosno 71,42 % (25/35) CRA metodom. Međutim, VanA i geni za rezistenciju na antiseptike (*qacAB* i *qac C*) nisu detektirani niti u jednom izolatu. Stoga se iz podataka može zaključiti da je većina sojeva stafilocoka bila sposobna proizvoditi sluz, a detekcija CRA metodom je bila više od PCR metode za kapacitet proizvodnje biofilma sojeva stafilocoka. Stoga, prisutnost *ica* u sojevima stafilocoka potvrđuje njegovu ulogu kao faktora virulentnosti u patogenezi goveđeg mastitisa.

Ključne riječi: *krava, mastitis, geni za rezistenciju, proizvodnja sluzi, stafilocoki, faktor virulencije, Alžir*