Brief Original Article

The *ica*A gene in staphylococci from bovine mastitis

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Abstract

Introduction: *Staphylococcus aureus* and coagulase-negative staphylococci (CNS) are frequently isolated from cows with mastitis. A main virulence factor of CNS is the ability to adhere and form biofilms. The intercellular gene cluster adhesion (*ica*) operon is one factor involved in biofilm production although i*ca*-independent factors are also involved. Previous reports based on the results of *S. epidermidis* and *S. aureus* suggested that *ica* is highly conserved between species, but this detection decreases in other CNS biofilm producers. In this study we evaluated the presence of the *ica*A gene in strains of *Staphylococcus* spp. isolated from the milk of bovines with mastitis.

Methodology: Thirty-seven staphylococci strains were evaluated by detecting the *ica*A gene. A new set of PCR primers was designed by consensus region of eight staphylococci from GenBank. Species characterization was performed using the Kloos and Schleifer scheme.

Results: We identified the presence of the gene in *S. aureus* (n:4), *S. chromogenes* (n:4), and *S. sciuri* strains (n:2). We also, identified the presence of the gene in *S. xylosus* (n:5) for the first time. The *ica*A gene was not detected in *S. capitis* (n:1)*, S. epidermidis* (n:2)*, S. hominis* (n:2), *S. saccharolyticus* (n:1), *S. simulans* (n:4) and *S. saprophyticus* (n:3). The *ica*A gene was detected in 40.54% (15/37) of the CNS evaluated.

Conclusions: Our results confirm the presence of the *ica* operon in various species of CNS pointing to polysaccharide intercellular adhesin (PIA) as the most important component for the formation of biofilms.

Key words: *ica*A*;* coagulase-negative staphylococci; PCR; bovine mastitis

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Introduction

Coagulase-negative staphylococci (CNS) are emerging pathogens in bovine mastitis [1]. Although infections are usually subclinical or mild, they increase somatic cell counts in milk and decrease production [2]. Infections are opportunistic with the most commonly reported CNS being part of the normal muco-cutaneous microbiota. The ability of some CNS to form biofilms is thought to enable them to avoid the immune system and cause persistent intramammary infections [3]. This trait might be useful in differentiating pathogenic and contaminating strains [4,5].

Although multiple bacterial and external factors influence attachment and accumulation leading to biofilm formation [6], the production of a polysaccharide intercellular adhesin (PIA) or polymeric N-acetyl-glucosamine (PNAG) by intercellular gene cluster adhesion (*ica*) operon-encoded enzymes [7,8] is currently the best understood mechanism of biofilm formation in staphylococci *in vitro* and *in vivo* [9-14]. At present there is little information on the *ica* operon in CNS of veterinary importance other than *S. aureus* and *S. epidermidis*.

The *ica* gene has been evaluated by PCR with different primers [4,15-18]; however, it cannot always be detected in biofilm forming CNS species. In our study we evaluated consensus sequences of the *ica* gene from CNS to find primers that would be effective in detecting veterinary strains of CNS that form biofilms.

Methodology

Bacterial strains

A total of 37 staphylococci isolated from the milk of cows with both clinical and subclinical mastitis were used in this study. They were identified phenotypically by standard procedures [19,20] and API-Staph 20 Ident System (Biomerieux, Marcy l'Etoile, France). Confirmation of *S. xylosus* was by PCR [21]. As negative controls we used *S. carnosus* TM300 (kindly provided by F. Götz, Tübingen, Germany) and *S. epidermidis* ATCC12228 (GenBank

accession Nº004461), while S. *aureus* ATCC29740 was used as the positive control.

Microtitre-plate test

The ability of the strains to form biofilms was evaluated in a Microtitre-plate test (MPT) as previously described [22] with modifications [11,23].

DNA isolation, PCR amplification and sequencing

Chromosomal DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA), with lysostaphin (10 µg/ml) to lyse cells. In the first step in the detection of *ica*, forward primer pia1 5'-TCTCTTGCAGGAGCAATCAA (nucleotides 1337-1356 from *ica*A gene, accession number U43366), and reverse primer pia2 5'-TCAGGCACTAACATCCAGCA (nucleotides 1505-1524) were used [24]. In the second step we used primers we designed using clustal W software and consensus sequences for the *ica* operon of *S. aureus* (GenBank accession Nº: AF500262), *S. simulans* (AF500263), *S. capitis* (AF500269), *S. saprophyticus* (AF500270), *S. caprae* (AF246927), *S. sciuri* (AF500259), *S. cohnii* (AF500268) and *S. epidermidis* (U43366)] (Figure 1). These primers, fcv1 (5'-TGGAAACAAAGGGTTCGATGG nucleotides 1542-1562 from *ica* operon U43366) and fcv2 (5'-TAACCCAGTATAACGTTGGATACC, amplified a 355bp AD portion of the *ica* operon corresponding to 1873-1896. The PCR was performed using 150 ng of DNA, 120 pM of each respective primer, 2.5 mM of each dNTP, 1X reaction buffer (Invitrogen), Cl_2Mg 2 mM and 1U of Taq Platinum polymerase (Invitrogen, Sao Paulo, Brazil) to give a final reaction volume of 50 µl. The mixture was subjected to 35 cycles of amplification: at 94ºC for 30 seconds, 49ºC for 30 seconds and 72ºC for 1 minute, with a final extension at 72ºC for 7 minutes.

A 355bp product obtained from the *S. sciuri* strain with the fcv primers was ligated into pGEM-T (Promega, Madison, WI, USA) and cloned in competent *E. coli* JM109. The plasmid was sequenced on a Genetic Analyzer 3130 xl (Applied Biosystems, Foster City, CA, USA) and compared with sequences in GenBank.

Results

Of the 37 staphylococci investigated, 29 (78%) were identified as CNS [*S. capitis* (n:1), *S. chromogenes* (n:7), *S. epidermidis* (n:2), *S. hominis* (n:2), *S. saccharolyticus* (n:1), *S. saprophyticus* (n:3), *S. sciuri* (n:3), *S. simulans* (n:4) and *S. xylosus* (n:6)] and 8 (22%) as coagulase positive *S. aureus*. Biofilm production was observed in 50% of the CNS strains, but only four strains, *S. sciuri* (#50a), *S. xylosus* (#263), *S. aureus* (#1942a) and *S. hominis* (#2056), produced a strong biofilm in the MPT.

Using pia1/2, only two *S. aureus* and two *S. chromogenes* out of the 37 (11%) staphylococci isolates were positive. Notably, the four staphylococci that produced the strongest biofilms in the MPT were all negative. More positive results were obtained with the fcv1 and fcv2 primers we designed (15/37; 40.54%), although *S. hominis* (#2056), a strong biofilm-producer, was not amplified. In total, the *ica*A gene was found with the fcv1 and fcv2 primers in *S. aureus* (4/8), *S. chromogenes* (4/7), *S. sciuri* (2/3) and *S. xylosus* (5/6) but not in *S. capitis* (0/1*), S. epidermidis* (0/2), *S. hominis* (0/2), *S. saccharolyticus* (0/1), *S. saprophyticus* (0/3) and *S. simulans* (0/4). Some (#2015b, #2102) of the *S. chromogenes* strains were positive with both primers while some (#1942b, #111a) were only positive with fcv1/2.

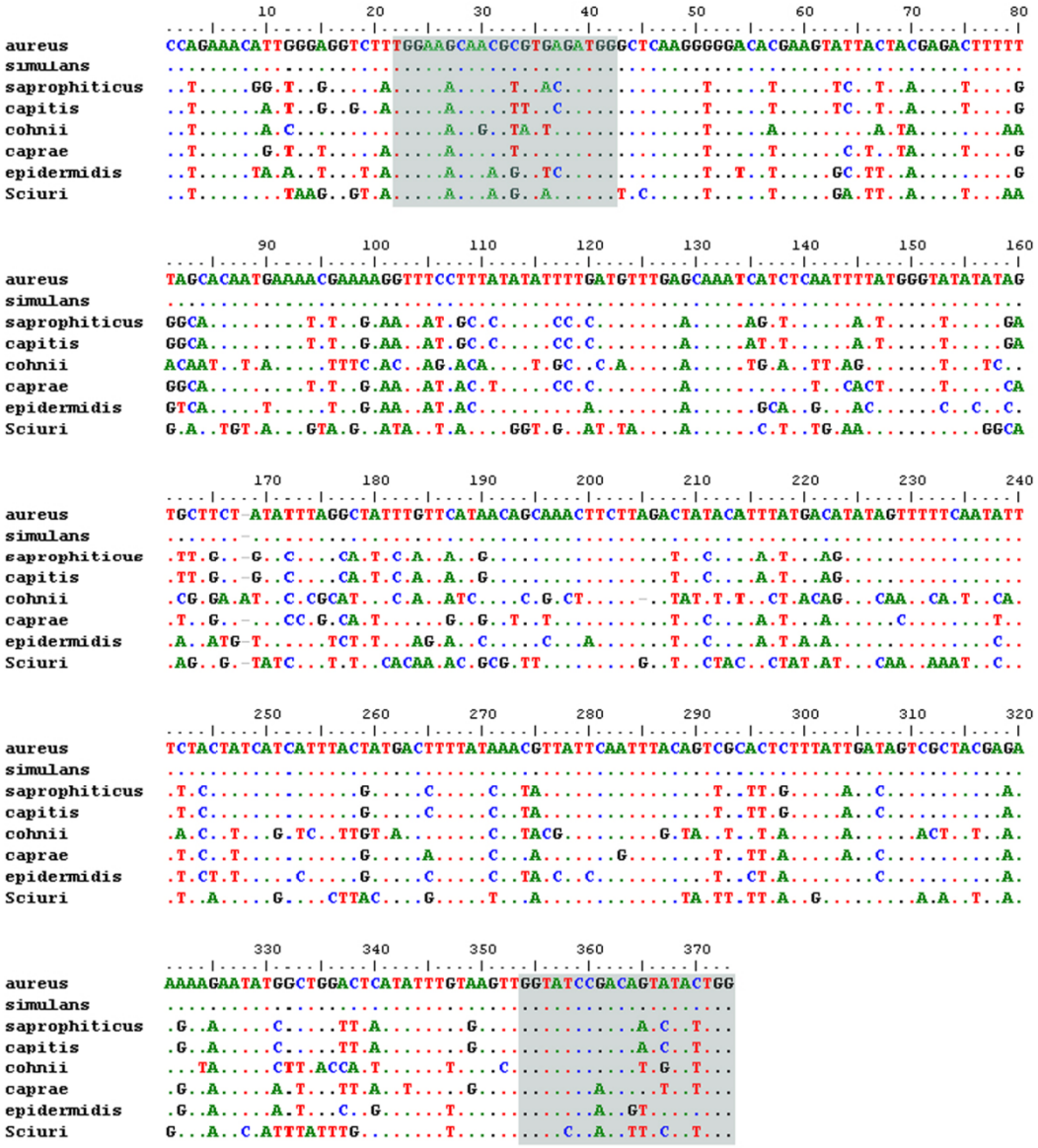
Considering all positive and negative biofilm-former strains, the pia1/2 showed 17% sensitivity, 100% specificity and 56% efficiency. On the other hand, fcv1/2 showed 61% sensitivity, 94% specificity and 76% efficiency.

The cloned amplicon obtained from *S. sciuri* (#50a) [BankIt1494026 Seq1 JQ244772] had 96% similarity with the published sequence of *S. sciuri* (accession Nº AF5000259). Notably, this strain was negative for amplification with primers pia1/2. There was partial homology (69% and 82%) between the *ica*A gene sequence we obtained and that of other species of CNS and *S. aureus* in GenBank.

Discussion

The ability of *S. aureus* and CNS to cause mastitis is well recognized, but there is little information on the *ica* profiles of isolates from cases of bovine mastitis. Our results confirm that *ica* is present in *S. aureus, S. chromogenes* and *S. sciuri*. Furthermore, we have shown it also occurs in *S. xylosus*, where it has not been previously reported. Consistent with a previous report [25] we found that *S. sciuri* strains had different slime-producing profiles in MPT tests; one was strongly positive, one weakly positive, and one negative. Only the fcv1/2 primers detected the two biofilm positive *S. sciuri* strains. The pia1/2 failed to recognize the *ica* in the *S. sciuri* strains, possibly due to its having a lower homology with *S. epidermidis* and *S. aureus,* as shown in Figure 1. Previously, gene diversity has been demonstrated between bovine and

Figure 1. Staphylococci multiple alignment



Multiple alignment (GenBank accession numbers: *S. aureus* AF500262; *S. simulans* AF500263; *S. saprophyticus* AF500264; *S. capitis* AF500269; *S. caprae* AF246926; *S. cohnii* AF500260; *S. sciuri* AF500259; *S. epidermidis* U43366) showing sites where the primers were designed (grey blocks).

human strains of *S. aureus* [26] and this might also be possible for the *ica* operon which has been considered to be highly conserved [11]. Our finding that *S. sciuri* (#V290) was *ica* positive with fcv primers but was not a biofilm producer might be explained by its not having a complete gene, or because its regulon was not able to produce a biofilm. The low sensitivity of both PCR methods could be explained by biofilm production being a result of genes other than *ica*. The *S. hominis* (#2056) strain showed the same biofilm-positive/*ica*-negative profile as previously reported for *S. epidermidis* from humans [27], representing a newly emergent subpopulation of CNS strains showing heterogeneity in the mechanisms of biofilm development. Surprisingly, the #2056 showed strong biofilm formation and it is thus reasonable to consider it has a major variation in the *ica* operon or it has a different locus which plays an important role in biofilm development. Recent studies have shown a PIA-independent mechanism mediating biofilm formation in clinical isolates of *S. epidermidis* and *S. aureus* [14,28] and a biofilm-associated protein (Bap)

being involved in strains isolated from chronic mastitis cases [29] and *S. aureus* [30]. Similarly, Simojoki *et al.* [3] found that primers used for detecting *bap* and other biofilm-associated genes do not identify all CNS capable of biofilm production. Single pairs of primers do not appear to be diagnostic for biofilm production but could be useful in epidemiological studies.

Conclusion

The fcv primers we designed to detect a region of the *ica* operon were effective in a wide range of CNS and may facilitate the genotypic study of a wider variety of strains of veterinary interest. The fact that they were effective in some strains, but the *pia* 1 and 2 were not, shows there are variations in the *ica* operon, which has been regarded as a highly conserved region between species. Furthermore, our results confirm the presence of the *ica* operon in various species of CNS, pointing to PIA as the most important component for the formation of biofilms. Our results, however, do not exclude the presence of additional factors in biofilm production which could be *ica*-independent, such as *bap*.

Finally, we would note that biofilm formation in staphylococci is a complex process that could include different mechanisms and gene regulation. It plays an important role in colonization and evasion of phagocytosis, which could explain the persistence and chronicity of infections. Further studies should be conducted to identify factors related to strong and weak biofilm producer staphylococci.

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