Comp Clin Pathol (2012) 21:571–576
DOI 10.1007/s00580-010-1136-9

ORIGINAL ARTICLE

Distribution of collagen adhesin gene among various types of *Staphylococcus aureus* strains associated with bovine mammary gland

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Received: 20 June 2010 / Accepted: 3 November 2010 / Published online: 19 November 2010

Abstract A subset of 58 bovine mastitis-associated *Staphylococcus aureus* isolates with known *coa* types were investigated for the presence of collagen adhesin gene, *cna*. According to the enzyme restriction pattern of gene encoding coagulase, strains were divided into nine genotypes. All isolates were investigated by PCR for the presence of gene encoding Cna, which was considered as an important virulence factor associated with bacteria adhesion. Interestingly, 49 (84.5%) strains were found to be cna^+ with significant variations across the predominant and rare genotypes. According to the results of this study, it might be emphasized that *cna* is significantly more common in bovine mastitis-associated *S. aureus* isolates and distributed dependently between genotypes. The finding that collagen–adhesin is present in the majority of the bovine mastitis-associated *S. aureus* isolates encourages the development of new strategies to prevent mastitis, based on antagonist ligands able to interact with surface adhesin and block its specific binding with matrix collagen.

Keywords Collagen adhesin gene · *Staphylococcus aureus* · Bovine · Mammary gland

Introduction

Staphylococcus aureus can colonize and infect a variety of members of the animal kingdom, including mammals, reptiles, and birds (van Leeuwen et al. 2005). This organism is the most common etiologic agent of contagious bovine mastitis, with relevant losses in the dairy industry (Zecconi et al. 2003). Intramammary infections, caused by *S. aureus* and also by other bacterial species, are due to the entry of bacteria through the teat canal into the mammary gland. The induction of intramammary infection is thought to be due in part to its expression of a large number of secreted and cell surface-associated virulence factors enabling adherence, colonization, and invasion of the mammary cells of the bovine host by the *S. aureus* cells, evasion from the immune defense mechanism and survival in the host environment. The first step in the colonization of the mammary gland by *S. aureus* seems to be adhesion to epithelial cells, which prevent the bacteria from flowing out of the gland during milking (Brouillette et al. 2003) and thus, a risk factor for invasive disease (Foster and Hook 1998). Several studies have been demonstrated *in vitro* and *in vivo* that *S. aureus* is able to adhere to and penetrate inside bovine mammary epithelial cells (Gudding et al. 1984; Sutra and Poutrel 1994; Hebert et al. 2000; Hensen et al. 2000). However, the mechanism of *S. aureus* adhesion to mammary epithelial cells is not well known, but is likely to be multifactorial. It is now recognized that *S. aureus* express various types of wall-associated proteins promoting adherence to host cells and/or tissue components. *S. aureus* adhesins are grouped into a single family named microbial surface components recognizing adhesive matrix molecules (*MSCRAMMs*) (Foster and Hook 1998). These surface proteins play a major role in host–bacterium interaction. Two types of interactions could be involved: (1) non-specific physicochemical interactions, and (2) specific interactions between bacterial cell wall-associated receptors and host components (Sutra and Poutrel 1994). Among the surface proteins produced by *S. aureus* are a diverse group of adhesins capable of binding host proteins present in the

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extracellular matrix. The gene encoding *cna* was reported as an important virulence factor associated to bacterial adhesion (Montanaro et al. 1999; Nashev et al. 2004; Xu et al. 2004). The *cna* (collagen adhesin) gene (Fig. 1) encodes a 135-kDa protein with structural features common to surface proteins from other Gram-positive bacteria. An amino-terminal signal sequence is followed by a large non-repetitive region (55 kDa) which is referred to as ligand-binding A domain. Immediately following the A domain is a series of 187 amino acid repeated motifs present in one to four copies. On the basis of the number (one, two, three, or four) of B domains, the *S. aureus* collagen adhesion occurs in at least four forms. Finally, the repetitive region is followed by domains that anchor collagen binding protein (CBP) to the cell envelope. These domains included a 64-amino acid proline- and lysine-rich cell wall-associated region, a hydrophobic membrane-traversing domain, and finally, a short, positively charged carboxy-terminus located in the bacterial cytoplasm. The collagen-binding domain (CBD) of the collagen adhesin has been localized to a fragment containing amino acids 151–318 of the intact protein (within the non-repetitive region) (Patti et al. 1995). The crystal structure of the binding domain has been determined at 2 Å resolution (Symersky et al. 1997), and has a 'jelly-roll' topological pattern that is composed of two anti-parallel R-sheets and two short helices. A groove on one of the R-sheets exhibits the best complementarity to triple-helical collagen model probes and is the most likely collagen-binding region.

Cna, encoded by the *cna* gene, is the primary identified adhesion protein responsible for the ability of *S. aureus* to bind collagen substrates and collagenous tissues (Switalski et al. 1989; Patti et al. 1992; Gillaspy et al. 1998). This adhesin has been shown to significantly contribute to tissue colonization in various pathological conditions such as eye keratitis (Rhem et al. 2000; Jett and Gilmore 2002), osteomyelitis and septic arthritis (Hudson et al. 1999; Smeltzer and Gillaspy 2000; Elasri et al. 2002), and indwelling medical devices (Hudson et al. 1999; Arciola et al. 2005).

Investigating distributions and the virulent factors of *S. aureus* provides important information for establishing infection control strategies. Hence, we suggest that a study for adhesion molecules may help in clarifying the relevance of the different adhesion mechanisms in the pathogenesis of mammary-associated infections. The role of Cna in the virulence of *S. aureus* during intramammary infections (IMI) has not been definitively demonstrated. Molecular biology could provide the tools to clarify this point. We, therefore, investigated the prevalence of collagen adhesion gene in a collection of 58 *S. aureus* strains isolated from bovine mastitis, and analyzed for any correlation between the presence of *cna* gene and the different genotypes of *S. aureus*.

Materials and methods

Bacterial isolates and preparation of bacterial DNA

The 58 *S. aureus* strains used in this study were the same as those used in previous studies (Saei et al. 2009, 2010). Briefly, the tested isolates were obtained from clinical and subclinical bovine mastitis cases from nine dairy herds. All isolates were characterized by means of classic microbiological methods.

For chromosomal DNA purification, bacterial cells grown in 10 ml of nutrient broth (Merck, Germany) were collected by centrifugation (Sigma, Germany) at 13,000 rpm for 5 min and resuspended in 200 μl of TE buffer in a 1.5 microtube. Bacterial DNA was then extracted using Genomic DNA purification Kit (Fermentas, Germany) according to the manufacturer's protocol.

PCR mediated identification and genotype analysis

The determination of species specific parts of the genes encoding thermonuclease (*nuc*) was performed with oligonucleotide primers as described previously (Brakstad et al. 1992) and PCR conditions reported elsewhere (Saei 2010).

The results from previous study (Saei et al. 2009), in which amplification of the variable region of the *coa* gene and restriction enzyme analysis of the resultant *coa* amplicons were performed by a modification of the procedures described by Hookey et al. (1999), were used in the present study.

Detection of the *cna* gene

The amplification was carried out by a modification of the procedure described by Montanaro et al. (1999). The

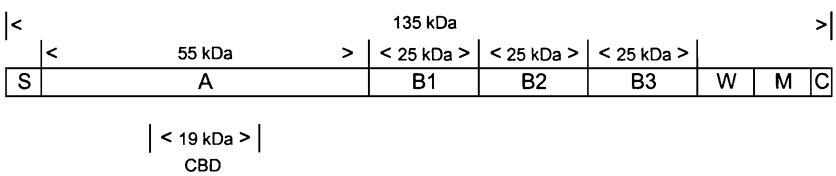

Fig. 1 Model of the collagen MSCRAMM gene *(cna)* from *S. aureus* FDA strain 574 (Symersky et al. 1997). *S*, signal peptide-encoding region; *A*, non-repetitive domain; *Bl*, *B2*, *B3*, repeated regions; *W*, cell wall domain; *M*, membrane-spanning domain; *C*, carboxy-terminal domain. The 19-kDa CBD (collagen binding domain) in the *A* region is also indicated

primers to detect the presence of the *cna* gene were as follows: 5′-AAAGCGTTGCCTAGTGGAGA (forward primer) and 5′-AGTGCCTTCCCAAACCTTTT (reverse primer), including a region of 192 bp (corresponding to nucleotides 1,291–1,482). The thermal cycler programs were: 94°C for 5 min, 1 cycle; 94°C for 45 s, 55°C for 45 s, 72°C for 45 s, 40 cycles; 72°C for 10 min, 1 cycle. PCR products (8 μl) were analyzed on 1.2% (*wt*/*v*) agarose gel stained with ethidium bromide (0.5 μg/μl), and visualized under ultraviolet transillumination and photographed using gel doc apparatus. The *S. aureus* ATCC 25923 was used as a positive control for *cna* gene detection.

Results

According to culture and biochemical properties, all 58 isolates used in the present study could be identified as *S. aureus*. The identification of the isolates was also confirmed by PCR amplification of thermonuclease gene, *nuc*. The amplicons of this gene showed a uniform size of approximately 270 bp (data not shown).

Results from *coa* gene PCR-RFLP analysis of the studied isolates have been described previously in details (Saei et al. 2009). Briefly, amplification of the variable region of the *coa* gene from these isolates produced five different PCR products ranging in size from approximately 490 to 850 bp. To obtain RFLP patterns of the PCR products, they were subjected to digestion with restriction endonuclease *Hae*III. Nine *coa* gene RFLP patterns, numbered I–IX, were observed, with 23 isolates (39.66%) assigned to RFLP pattern I and 14 isolates (24.14%) assigned to RFLP pattern III. The genetic analysis of *S. aureus* isolates showed that all of them were positive for *coa* gene as expected, and thus, this gene was further analyzed by enzyme restriction pattern using *Hae*III.

The PCR technique allowed the identification of cna^+ strains by the appearance of an amplified DNA fragment of 192 bp, as described earlier (Montanaro et al. 1998, 1999) (Fig. 2). The amplicon indicating the presence of *cna* gene was detected in 84.5% of all the 58 isolates. As a result, majority of the bovine mastitis-associated *S. aureus* isolates had gene encoding collagen adhesin.

The different prevalence of *cna* among the nine categories of *coa* types is reported in Table 1. As shown, the *cna* gene was detected in both predominant and rare *coa* types but mostly in predominant types than rare types. On the other hand, different genotypes exhibit differences in prevalence of the *cna* gene. As shown, all of the 37 predominant types were positive for *cna* gene, but nine out of the 21 rare genotypes were negative for the mentioned gene.

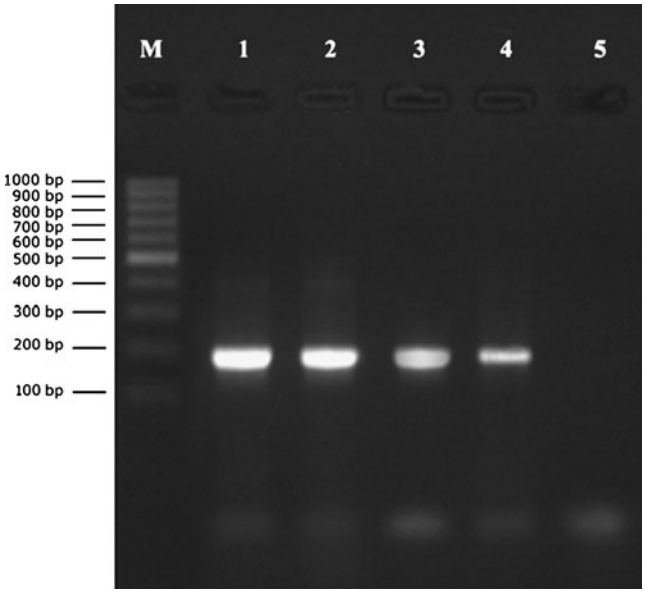

Fig. 2 Agarose gel electrophoresis of polymerase chain reaction (PCR) amplification of *cna* gene. *Lane M*, 100 bp DNA molecular size marker; *lane 1*, Positive control (*S. aureus* ATCC 25923); lanes 2–4, PCR amplicons obtained with DNA amplification of *S. aureus*; *lane 5*, negative control (reaction mixture without DNA)

Discussion

In the present study, the frequency of the isolates classified in the different restriction pattern clusters confirmed that there is a prevalent clone of *S. aureus* isolated from bovine mastitis. This is in agreement with the finding of other researchers (Gilot et al. 2002; Schlegelova et al. 2003).

It is now well established that to initiate infection at a particular site, bacteria must adhere to host cells or to layers covering these cells. There is mounting evidence to suggest that proteins that belong to a family defined as MSCRAMMs are involved in the adhesion process. These include proteins covalently bound to bacterial cell wall

Table 1 Presence of *cna* gene among the nine categories of *coa* types

Type	No. of isolates	Presence of the *cna* gene	
		cna-positive	*cna*-negative
I	23	23	–
II	3	2	1
III	14	14	–
IV	2	2	–
V	4	2	2
VI	2	1	1
VII	2	–	2
VIII	7	5	2
IX	1	–	1

peptidoglycan (Foster and Hook 1998), such as collagen-binding protein (Cna) (Hudson et al. 1999; Smeltzer and Gillaspy 2000). The role played by the Cna adhesin as a virulence determinant in the pathogenesis of septic arthritis is well documented (Patti et al. 1994). The presence of collagen adhesin (Cna) is also correlated with pulmonary manifestations (Gonzalez et al. 2005). Similarly, inactivation of the collagen adhesin gene, *cna*, has been correlated to reduce virulence in animal models of endocarditis (Patti et al. 1994; Hienz et al. 1996). In this study, the presence of gene encoding for the CBP in the majority of the 58 isolates of *S. aureus*, suggests that adherence to epithelial cells may play an important role in the pathogenesis of *S. aureus* mastitis and the presence of *cna* gene and expression of collagen adhesin may be very relevant, almost essential, trait for the virulence action in bovine mammary tissue. Receptors for collagen have been demonstrated on strains isolated from bovine IMI (Mamo et al. 1988, 1992). However, our finding is controversial when compared with earlier observations where most strains of *S. aureus* do not encode *cna* (Smeltzer et al. 1997). Results from previous studies have shown that the frequency of isolates harboring *cna* gene both in human (Montanaro et al. 1999; Nashev et al. 2004) and in animal isolates (van Leeuwen et al. 2005; Zecconi et al. 2005; Reinoso et al. 2008) was lower. In a study carried out by Reinoso et al. (2008), the *cna* gene of 15 isolates from bovine subclinical mastitis yielded no amplicon. Further expanded studies using large number of isolates from clinical and subclinical mastitis is necessary to investigate the extent of *cna* presence in mastitis-associated *S. aureus* strains.

In the current study, distribution of the *cna* gene among different categories of the *coa* genotypes indicate that certain subtypes of *S. aureus* may have special properties increasing their potential for adherence to and colonization of bovine udder tissue in comparison with other subtypes less adapted to bovine udder. This result is in agreement with the findings of other researchers, who reported that the adherence capacity of *S. aureus* isolated from bovine mastitis to mammary cells to be strain dependent (Frost et al. 1977; Opdebeeck et al. 1988; Iturralde et al. 1993; Kuzma et al. 2006). Furthermore, a study by Otsuka et al. (2006) demonstrated that the pandemic type (ST30) and continent-specific type (ST1, ST8, ST59 or ST80) of community-acquired methicillin-resistant *S. aureus* (CA-MRSA), each had a unique adhesin gene. These findings are in consistent with the results from Poland, where the prevalence of *cna* positive strains was significantly higher in *spa*≤7 type than in *spa*>7 type (Kuzma et al. 2006). Results from study by Hensen et al. (2000) also indicate that strain differences of adherence and invasion exist for *S. aureus*. The study by van Leeuwen et al. (2005) showed that the *cna* gene was not equally distributed among the different lineages of *S. aureus*. These results may explain differences in the ability of the strains to spread. However, distribution of other adhesins such as fibronectin (FnbA and FnbB), fibrinogen (ClfA, ClfB and Efb) and elastin (EbpS) binding proteins must be investigated between different subtypes of *S. aureus* isolates. Overall, we suggest that different types of *S. aureus* seemed to use different adhesion mechanisms, some mediated by collagen binding, others independent of collagen binding. This issue may influence the target cell specificities of the major subtypes in comparison with rare types. This idea is supported by the observation that the presence of (combinations of) virulence factors plays an important role in host or even tissue specificity in *S. aureus* infections (van Leeuwen et al. 2005).

It would also be interesting, if a vaccine containing purified surface proteins involved in *S. aureus* adhesion (e.g., CBP) belonging to predominant types could be used to immunize cows. An advantage of vaccines directed at surface proteins is that they will block adherence of bacteria as well as promoting phagocytosis of the non-adherent bacteria. However, results presented by Mamo et al. (1994a), mice vaccinated with the glutathione S-transferase-CBP fusion protein were not protected against challenge infection with *S. aureus*. A possible explanation for this finding might be the fusion protein used in that study contained only the A domain responsible for collagen binding; it is still possible that CBP presented in another way could induce protection. Other speculations could be that the synthesis of the proteins present on the bacterial surface is influenced by growth conditions, such as the composition of the growth medium (Ellwood and Tempest 1972). On the other hand, it must be noted that *S. aureus* adheres to several different host proteins, so it might be necessary to include epitopes from several different binding proteins to be fully effective. A preliminary study has shown that vaccination of cows with a fusion protein containing the fibronectin-binding domain of Fnbp lead to significant protection against experimental *S. aureus* IMI (Nelson et al. 1991). Immunizations with fusion proteins encompassing the fibronectin binding D-domains of a FnBP from *S. aureus* have been shown to induce the production of antibodies with adherence blocking activity (Luk et al. 1989; Ciborowski et al. 1992). It was also reported that immunization with these fusion proteins induced protection against *S. aureus* rat endocarditis (Schennings et al. 1993) and mouse mastitis (Mamo et al. 1994b).

In conclusion, although *S. aureus* can bind to a verity of host proteins, isolates associated with bovine mastitis seem to have an enhanced propensity to bind to collagen and predominantly express receptors for this matrix protein. Hence, it can be supposed that CBP encoded by *cna* gene could mediate *S. aureus* adhesion, especially predominant types, to epithelial cells and to micro-lesions of the mammary epithelium where basal lamina and inflammatory conjunctive

tissue, which are rich in collagen, are exposed. Identification and characterization of the *S. aureus* adhesins that contribute to collagen binding is important in light of the fact that *S. aureus* strains that cause mammary gland infections almost invariably bind collagen. This correlation suggests that therapeutic strategies aimed at the inhibition of collagen binding might be useful for prevention and treatment of mammary gland infections. However, the development of such strategies will require a clear understanding of the bacterial factors that contribute to collagen binding by performing both *in vitro* and *in vivo* models of adherence and infection of mammary cells.

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