Prevalence of cna, fnbA and fnbB adhesin genes among Staphylococcus aureus isolates from orthopedic infections associated to different types of implant

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Abstract

Here are reported data on virulence determinants of Staphylococcus aureus from orthopedic surgical infections, emphasizing on the genes encoding fibronectin (fnbA, fnbB) and collagen (cna) adhesins. 191 S. aureus strains from orthopedic infections (53 from internal fixation devices, 29 external fixation devices, 15 knee arthroprostheses, 30 hip arthroprostheses, 45 surgical reconstruction and 19 non-associated to medical devices) were investigated for the presence of the genes of the collagen-binding protein Cna and of the two fibronectin-binding proteins, FnBA and FnBB. 87 (46%) strains were found to be cna+ without significant variations across the different surgical categories considered. Conversely, the fnbA and the fnbB genes were almost always present in all surgical categories. The finding that, among the investigated adhesins, fibronectin-adhesins are present in the majority of the implant associated S. aureus clinical isolates encourages the development of strategies to specifically block the interaction of bacteria with matrix fibronectin by antagonist ligands.

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1. Introduction

For Staphylococcus aureus, a very common pathogen involved in implant-associated infections, a series of adhesins have been recently identified that, during the early phases of cell adhesion, can potentially favor the active anchoring of bacteria to specific extracellular matrix proteins adsorbed on biomaterials surfaces. These adhesins, also named MSCRAMMs (the acronym for “microbial surface components recognizing adhesive matrix molecules”) [1], are protein components of the microbial surface that are able to interact with and bind to a variety of relevant mammalian extracellular proteins. Among adhesins, two fibronectin-binding proteins, (FnBA and FnBB), three fibrinogen-binding proteins (ClfA, ClfB and EfB), a collagen-binding protein (Cna), the elastin-binding protein (EbpS [2]) and the bone sialoprotein-binding protein (Bbp [3]) have been well characterized. Cna, FnBA and FnBB have been proved significantly to contribute to tissue
colonization in various pathological conditions such as eye keratitis [4–6], osteomyelitis and septic arthritis [7–9], and indwelling medical devices [10].

In *S. aureus*, different MSCRAMM proteins often coexist with other crucial surface components such as the polysaccharide intercellular adhesin (PIA) and the biofilm-associated protein (Bap) [11]. Adhesins expression can be temporally modulated as a function of the cell cycle and the phase of infection [2,12,13].

Diverse studies have shown that FnB, CNA and PIA are significantly more common in invasive isolates and that they contribute independently to virulence and their effect appears to be cumulative [14]. A key to interpret such cumulative contribution is that, although co-existent, they explicate their specific action in distinct phases of the infection process.

This molecular epidemiological study aimed at investigating the prevalence of selected adhesion mechanisms in a collection of 191 *S. aureus* strains isolated from orthopedic infections, 127 of which from infections clearly associated to prosthetic implant materials. PCR-based techniques were utilized to detect the gene encoding for the collagen-binding adhesin (*cna*) and the genes of the two fibronectin-binding proteins, respectively, *fnbA* and *fnbB*. In order to ascertain possible differences related to the typology of the implant, the isolates associated to prosthetic materials were further grouped in 4 different categories.

### 2. Materials and methods

#### 2.1. Bacterial isolates

The 191 staphylococcal strains used in this study were clinical isolates derived from orthopedic infections sequentially observed in patients of the Rizzoli Orthopedic Institute over a 20 months period. Clinical infections were identified based on criteria and definitions previously described in Arciola et al. [15], opportunely adapted to surgical and prosthesis associated infections: stage 1, colonization at the surgical wound or implant surface; stage 2, localized infection, revealed by local inflammatory signs at biopsy; stage 3, systemic infection, documented by bacteraemia and stage 4, sepsis. Strains were isolated from tissue biopsies. Depending on the type of orthopedic implant infected, 127 of these strains were classified into 4 different categories: internal fixation devices, external fixation devices, hip arthroprostheses, and knee arthroprostheses. The category of internal fixation devices included a variety of prosthetic implants such as plates, pins, screws and so on. Conversely, 19 strains were isolated from infections that were not associated to medical devices such as those post-trauma. The remaining 45 isolates classified in the category of reconstructive surgery included infections developed following interventions of reconstructive surgery in oncology, pelvis surgery, tendon and ligaments reconstructions. In these 45 cases, the device could not be correctly classified in one of the 4 categories, the complete patient history was not promptly available, the infection could potentially have started from a suture used in a surgical procedure, but there was no sound proved association with biomaterials. All isolates were characterized by means of classic microbiological methods. In particular, the staphylococcal species was identified by Api-Staph test (Biomérieux, France), an identification kit, and coagulase test. Bacterial strains coming from the same surgical unit, or even when coming from different units but isolated within the same week, were subjected to automated EcoRI ribotyping by RiboPrinter® (DuPont) in order to check if they were originating from monoclonal outbreaks. A clonal relationship among these strains was excluded, because they, compared with the 303 ribotypes of the Dupont Release (2003 updating), appeared as individual strains and therefore all the collected isolates were included in the study. For the storage, a single colony of each isolate was seeded in 8 ml of trypticase soy broth (TSB) and incubated for 24 h at 37 °C. The broth culture, supplemented with 15% glycerol, was finally fractionated in 1-ml aliquots and stored at −80 °C.

#### 2.2. Detection of *cna* sequence

The sequence of *cna* gene (Accession No. M81736) was checked by the GenBank Sequence Database of the National Centre for Biotechnology Information. A couple of primers specific for *cna* gene were picked by the program Primer3 as previously described [16,17]. The respective sequences were: 5′-AAAGCGTTGCCATGTTGAGAGA (forward primer) and 5′-AGTGCCCTTCCAAAACCTTT (reverse primer), including a region of 192 bp (corresponding to nucleotides 1291–1482). Both primers were synthesized by M-Medical Genenco (Firenze, Italy). PCRs were performed in a DNA thermal cycler model Gene Amp PCR System 9600, Perkin Elmer. PCR conditions were those described in [16]. Electrophoretic patterns in agarose gels were analyzed by a “Scanner Agfa Ars II” image analyzer equipped with the software GelPro Analyzer 3.0.

#### 2.3. Binding of bacteria to immobilized collagen

The phenotypic ability of all *S. aureus* strains to bind to collagen type II and IV (all from Sigma) was assessed following an adaptation of the procedure already described by Gatermann and Meyer [18], as mentioned in [16]. Briefly collagen (50 μg/ml in PBS) was dispensed in 96-wells microtiter plates (5 μg/well) for 16 h at 4 °C. Wells were then washed with PBS. Fresh cultures of bacteria in TBS were pelleted, washed with PBS and
adjusted to 3.0 \( A_{600} \) in PBS. Bacterial suspensions (0.1 ml) were added to each collagen-coated well. After 2 h at 37 °C, bacterial suspensions were aspirated and the wells washed with PBS. The plates were dried and read at 404 nm. Binding was considered positive when an \( OD_{405} > 0.2 \) was recorded.

2.4. Detection of the fnbA and fnbB sequence

The sequences of the fnbA gene (STAFNBP, Accession No. J04151) and of the fnbB gene (SAFNBP, Accession Nos. X62992, S76179) were checked by the GenBank Sequence Database of the National Centre for Biotechnology Information. The primers to detect the presence of the fnbA gene were as follows: 5'-GATACAAACCCAGGTTGTGG (forward primer) and 5'-TGTGCTTGACCATGCTCTTC (reverse primer), including a region of 191-bp (nucleotides 2011–2201). Those for the fnbB gene were: 5'-TGTGCTTGACCATGCTCTTC (forward primer) and 5'-AGTTGATGTGCGCTGTATG (reverse primer), including a region of 201-bp (nucleotides 562–762). PCR was performed as described elsewhere [19].

2.5. Statistics

All data were analyzed by \( \chi^2 \) test, comparing the prevalence of cna+ strains within the different categories of infection sources, using the Statview 5.01 (SAS Institute, NC, USA) statistical software package. A \( P \)-value of less than 0.05 was considered significant. For additional information, 95% confidence intervals (95% CI) were also calculated.

3. Results

3.1. Detection of cna gene by PCR

The PCR technique adopted allows to identify cna+ strains by the appearance of an amplified DNA fragment of 192 bp, as earlier described in Montanaro et al. (1998 and 1999) [17,16]. The amplicon indicating the presence of cna gene was detected in 46% of all the 191 clinical isolates (95% CI, 38.5–52.6%). Non-adherent \( S. aureus \) strains as classified per collagen-binding test did never give the 192-bp band. Table 1 shows the frequency of cna+ isolates within the entire strain collection. The different prevalence of cna among the 4 categories of medical devices are reported in Table 2. The cna gene was detected, respectively, in 36% of strains derived from internal fixation devices (95% CI, 22.8–48.9%), 40% of those from knee arthroprostheses (95% CI, 14.3–65.7%), 43% of those from hip arthroprostheses (95% CI, 25.3–61.4%), and 52% of those from external fixation devices (95% CI, 33.2–70.2%). Among the isolates obtained from infections, which were not associated to medical devices, the percentage of cna+ strains was 47% (95% CI, 24.3–70.4%). Finally, the category of reconstructive surgery, which included 45 strains isolated from different types of orthopedic surgical interventions, exhibited a slightly higher prevalence of the cna gene: 56% (95% CI, 40.9–70.2%).

3.2. Phenotypic expression of collagen binding activity

All cna+ strains were found to be positive also to in vitro collagen binding activity, while the cna-negative strains were found negative to the in vitro binding assay. These results suggest that, when present, the adhesin encoded by the cna gene is tendentially always expressed.

3.3. Detection of the fnbA and the fnbB genes

The presence of the adhesin genes was revealed, respectively, by a 191 bp band for the fnbA gene and a
201 bp band for fnbB, as expected from the respective gene sequence [18]. According to previous works, these adhesins were found to be very frequent and widely distributed. The vast majority of all isolates analyzed was found to possess the genes for both the homolog fibronectin-binding proteins FnbA and FnbB, as shown in Table 1. This was a common finding across all the 6 different defined categories of infections (Fig. 1) and, within the entire collection, only 2 strains of S. aureus, both isolated from hip arthroprosthesis-associated infections, were found, respectively, to be fnbA- and fnbB-.

3.4. Statistics

The broad and consistently high prevalence of the fnbA and the fnbB genes observed did not require statistical evaluation. Vice versa, different frequencies of cna+ strains were observed in the 6 categories of infections. However, none of these variations were found to be statistically significant (Table 2).

4. Discussion

In this study, the genes encoding for both the fibronectin-binding proteins appeared to be present in a vast majority of all 191 isolates of S. aureus (98.4% of the strains were fnbA+, 99.5% fnbB+, and 99.5% either fnbA+ or fnbB+), this independently on the category of the prosthetic implants involved. Thus, in agreement with other more general reports on isolates of S. aureus from nosocomial infections [20], these two adhesins appear to be very relevant, almost essential, traits for the virulence action in human hosts.

In a limited collection of 29 clinical isolates from orthopedic implant-associated infections, Peacock et al. [21] observed that these strains exhibited phenotypic characteristics of significantly greater adherence to immobilized fibronectin than isolates from other pathological conditions such as endocarditis or septic arthritis/osteomyelitis did. They described a prevalence of strains positive for both fnb genes ranging from 85.3% for endocarditis down to 63.6% for nasal carriage. The strains found positive to the fnbA and the fnbB genes were, respectively, 99.4% and 77.9% in their entire collection, and 96.6% and 79.3%, among the orthopedic implant associated infections. More recently, Rice et al. [22] reported a prevalence of strains positive for both genes in 91% of methicillin-resistant (n = 44) and 78% of methicillin-susceptible (n = 18) isolates.

The greatest differences with respect to the findings of Peacock et al. concerned the fnbB gene, in which case we generally observed a slightly higher prevalence within the entire collection of 191 isolates as well as among the 126 strains from orthopedic implants. Probably, this is partly to be ascribed to the different technique of gene detection utilized (rapid screening by PCR gene amplification of specific size-checked amplicons vs Southern blot analysis) and possibly by the different region of the locus analyzed by the couple of primers.
The role played by the Cna adhesin as a virulence determinant in the pathogenesis of septic arthritis is well documented. An experimental study based on an animal model quantified that cna+ strains can cause signs of arthritis in over 70% of injected mice, while less that 27% of the animals were affected when cna− strains were used [8]. Other recent results underline the important contribution of Cna in the pathogenesis of bone infection through haematogenous spread [9], acting almost like a homing receptor. However, several clinical studies indicate this adhesin is not essential in the pathogenesis of human musculoskeletal infection and little is still known on how the expression of this adhesin exerts its influence during the different phases of the pathogenesis of infection and if cna has a role in determining the severity or the prognosis of the disease [23].

Our characterization of 191 isolates shows that the cna gene is present in 46% of the strains. Thomas et al. [23] analyzed 159 strains from diverse geographic regions, respectively, 102 from United Kingdom and 57 from New Zealand. They observed a different prevalence of cna gene, respectively, of 67% and 44% in the two regions. Interestingly, in another study always conducted in United Kingdom on 155 clinical isolates, Peacock et al. [14] described a cna prevalence of 52%.

Previous studies conducted in North America reported a prevalence of 43% [24], while, in Sweden, Ryding et al. [25] reported a prevalence for cna gene of 57%, in isolates from patients with endocarditis or bacteraemia with bone or joint infection, and of 56%, in isolates from patients without signs of endocarditis or bone or joint infection. These variations have been suggested to depend either on the specific geographic region or, alternatively, on methodological differences.

The prevalence of cna slightly varied among different types of prosthetic device, but such variations were not found to be statistically significant. The indications of other authors [25] that, generally, the prevalence of such adhesin is consistent regardless of the clinical source, would support the hypothesis that type of implant and tissue location therefore do not significantly influence the frequency of cna gene.

When collagen-binding activity was phenotypically investigated, cna+ strains were all found adhesive to collagen coated substrata. Confirming other previous reports, the cna gene therefore appears to be frequently expressed in cna+ S. aureus strains.

Overall, this work confirms the large diffusion of both fnbA and fnbB genes in a conspicuous collection of S. aureus isolates from orthopedic infections. The frequency of the fnb genes remained high in strains from all 4 defined categories of implants. Thus, fibronectin-binding activity appears to play an essential role in the mechanisms of staphylococcal adhesion, which is a prerequisite for surface colonization. This encourages the development of new strategies to prevent implant infections, based on molecules such as heparin, able to interact with fibronectin and block its specific binding by staphylococcal adhesins [26,27]. Conversely, the cna gene appeared to be not strictly necessary for the development of orthopedic infections, appearing only in approximately half of all strains considered. Moreover, S. aureus strains isolated from healthy nasal carriers harboring cna gene were found to be about 48% [28].

This percentage is very near to that observed in the present study, in particular to that observed for external fixation device and reconstructive surgery categories. Our observations indicate that, under these circumstances, the conditions locally originated by orthopedic implants do not significantly alter the composition of staphylococcal populations as far as the prevalence of this gene is concerned.

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References


