Detection of viable but non-culturable staphylococci in biofilms from central venous catheters negative on standard microbiological assays

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Abstract

Viable bacteria were sought in 44 Maki-negative biofilms from central venous catheters (CVCs) using epifluorescence microscopy after live/dead staining. Thirty (77%) samples contained viable but non-culturable (VBNC) cells; the majority were positive on real-time PCR specific for *Staphylococcus epidermidis* (one also for *Staphylococcus aureus*). Viable cells were significantly ($p < 0.01$) associated with CVCs from febrile patients, three of whom showed *S. epidermidis*-positive blood cultures, suggesting that CVC-associated biofilms can be reservoirs for staphylococci in the VBNC state. The possible role of VBNC staphylococci in persistent infections related to medical devices requires further investigation.

Keywords: Biofilms, catheter-associated, infections, non-culturable bacteriastaphylococci

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Biofilm-related infections can persist for months, years, or even throughout life and have a well-known role in chronic disease pathogenesis [1–4]. They are among the major limitations to long-term use of indwelling medical devices, because of the well-established impaired susceptibility of biofilm-growing bacteria to current antibiotics [2,3,5,6]. Central venous catheters (CVCs) are indwelling medical devices at high risk of infection [1,3,4,7,8]. The hypothesis that bacterial clumps from mature biofilm may become septic emboli that disperse via the bloodstream is supported by *in vitro* catheter infection models and by microbiological and clinical data [4,7,9].

Biofilm-growing bacterial cells live in unfavourable microenvironmental conditions such as nutrient and oxygen depletion and pH and ionic strength variation, and may undergo transformation into slow-metabolism forms, including the Viable But Non-Culturable (VBNC) state, characterized by a number of molecular, structural and functional features [6,10–12].

The VBNC state is a transient dormancy condition aimed at stress adaptation and associated with significant cell dwarfing and failure to grow on culture media. Nonetheless VBNC cells are alive, preserve a degree of metabolic activity and can be 'resuscitated' to the culturable state by appropriate stimulation [11,12]. The role of CVC-associated biofilms as reservoirs for VBNC forms capable of regaining full culturability and virulence (after biofilm detachment) is therefore a major question, also considering their possible involvement in recurrent infections.

A total of 44 CVCs explanted from hospital patients from November 2007 to February 2008, selected from devices yielding no growth by the Maki procedure [13], were further investigated for VBNC forms in associated biofilms. The CVC implantation time ranged from 3 days to 3 months.

To detach any biofilm present on the catheter, the distal 5 cm of each CVC was subjected to three cycles of sonication and vortexing, followed by resuspension in PBS (5 mL). Aliquots of 200 μL were then inoculated in duplicate in 5 mL of tryptic soy broth and observed for 72 h to confirm lack of growth.

Overall, 39/44 biofilms yielded no microbial growth (Non-Culturable) and were investigated for viable bacterial cells by staining 1 mL of each sample with a live/dead viability assay using SYBR Green I 1× (Invitrogen, Eugene, OR, USA) and 40 mg/L propidium iodide (Sigma-Aldrich, St Louis, MO, USA) and examining it with an epifluorescence microscope at × 1000 magnification (Axioskop 2, Zeiss, Milan, Italy). Viable bacteria in amounts ranging from 2.5×10/mL to 3×10^4/mL were detected in 30/39 (77%) samples (Fig. 1), which were then analysed for bacterial DNA.

DNA was extracted from 1 mL aliquots using the FastDNA SPIN kit for soil (Q-Biogene, Irvine, CA, USA) after incubation with lysozyme (10 mg/mL) and lysostaphin

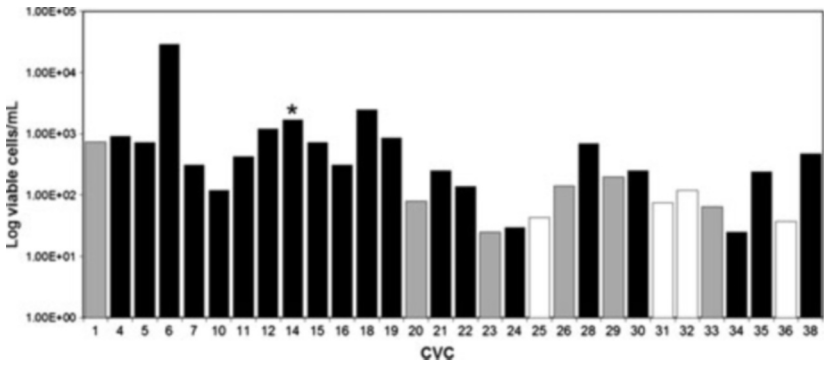

FIG. 1. Number of viable cells/mL counted in central venous catheter (CVC) -associated biofilms and presence of sequences specific to bacterial DNA, *Staphylococcus epidermidis* and *Staphylococcus aureus* in the 30 biofilms negative on culture tests (non-culturable; NC) and positive for viable cells. □ No detection of 16S rDNA; ▨ Detection of 16S rDNA; ■ Detection of 16S rDNA and species-specific sequences of *S. epidermidis* and *S. aureus* (*).

(100 mg/L; both from Sigma-Aldrich) for 1 h at 37°C and used in real-time PCR assays targeting bacterial 16S rDNA [14] and genes specific to *Staphylococcus epidermidis* [15] and *Staphylococcus aureus* [16], the species growing most frequently as CVC-associated biofilm [4,8,17,18].

The PCR assays targeting 16S rDNA were performed as described previously using primer pair P891F and P1033R [14]. Those targeting *S. epidermidis* and *S. aureus* were developed for this study using primer pairs Se705-1/Se705-2 [15] and Sa442-1/Sa442-2 [16] and the following cycling conditions: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 54°C (*S. aureus*) or 56°C (*S. epidermidis*) for 20 s and 72°C for 20 s. The ramping to generate melt curves was 0.5°C/10 s. Reactions were run in a total volume of 25 μL containing 5 μL DNA, 0.8 μM of each primer and Supermix iQ SYBR Green 1×, using the iQ5 iCycler (both from Bio-Rad Laboratories, Hercules, CA, USA). Samples were analysed in triplicate together with crude extracts of a culture of *S. epidermidis* ATCC 35984 or *S. aureus* ATCC 25923. Twenty-six of the 30 biofilms harbouring viable cells were positive on real-time PCR assays targeting bacterial 16S rDNA; 19 (73%) were also positive on *S. epidermidis*-specific PCR and one (NC14) contained both *S. epidermidis*-specific and *S. aureus*-specific sequences (Fig. 1).

Blood culture results were also examined where available (17/30 patients), *S. epidermidis* was recovered from three patients, accounting for 18% of all CVCs carrying VBNCs and for 27% (3/11) of those also positive for *S. epidermidis* species-specific PCR.

To relate these data to actual clinical outcomes, findings were compared with patient data, which were available for 26 patients from intensive-care units and haematology (the wards where most of the CVCs had been collected). Patient temperature, CVC implantation time and antibiotic therapy administered were analysed. A significant ($p < 0.01$; chi-square test) correlation was found between high temperature (≥38°C) and presence of viable cells, which were detected in 81% of CVCs from febrile patients and 30% of CVCs from non-febrile patients. Moreover, 62% of VNBC cell-containing CVCs from febrile patients were positive for *S. epidermidis* (Fig. 2), highlighting the proneness of this species to colonize indwelling medical devices and its ability to persist in a dormant state.

Biofilm involvement in chronic infections is well documented [1,2,4,5,8,9]. Our findings demonstrate that CVC-associated biofilms testing negative on routine microbiological assays can nonetheless carry viable bacteria. The significant association between VBNC forms and fever documented here suggests a role for them in clinical symptoms, supposedly after resuscitation and growth of a small number of non-culturable cells, and stresses the value of molecular diagnostic tests in routine

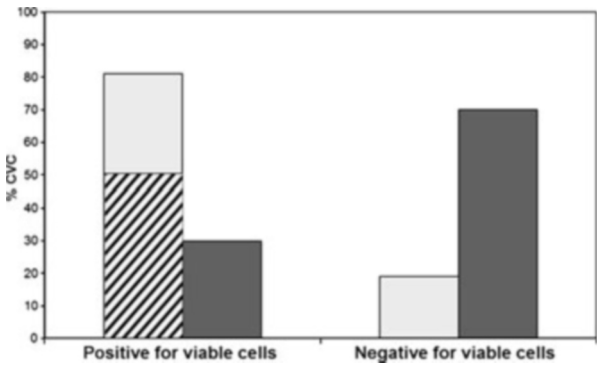

FIG. 2. Percentage of central venous catheters (CVCs) from febrile (□) and non-febrile (■) patients found positive/negative for the presence of viable cells. The proportion of CVCs containing *Staphylococcus epidermidis* species-specific gene is indicated as ▨.

microbiological investigations, at least when high-risk patients are involved.

To our knowledge this is the first evidence that *S. epidermidis* and *S. aureus* can enter the VBNC state. If positively documented, their ability to persist in this state could help to explain the well-known prevalence of both species in recurrent CVC-related infections [4,8,17,18]. Detection of *S. epidermidis* in 62% of CVCs demonstrated to contain non-culturable bacteria (removed from febrile patients, 27% of whom also had a positive blood culture) supports this hypothesis.

Further research into the relationship among VBNC forms found in CVC-associated biofilms, their actual metabolic activity and recurrent infection, as well as careful recording of concurrent clinical data, are required to gain insights into the possible role of VBNC bacterial cells in indwelling medical device-related persistent infections.

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Transparency Declaration

The authors declare no conflicts of interest.

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