Biofilms in chronic bacterial prostatitis (NIH-II) and in prostatic calcifications

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Abstract

The prevalence of inflammatory conditions of the prostate gland is increasing. In Italy, there is a high incidence of prostatitis (13.3%), also accompanied by prostatic calcifications. Cat NIH-II chronic bacterial prostatitis (CBPs) are the most frequent. Their aetiology theoretically involves the whole range of bacterial species that are able to form biofilms and infect prostate cells. The aim of our study was to isolate potential biofilm-producing bacteria from CBP patients, to evaluate their ability to produce in vitro biofilms, and to characterize intraprostatic bacteria and prostatic calcifications using scanning electron microscopy. The 150 clinical bacterial strains isolated from chronic prostatitis NIH-II patients were: 50 Enterococcus faecalis; 50 Staphylococcus spp.; 30 Escherichia coli; 20 gram-negative bacteria, Staphylococci and Enterococci strains were strong or medium producers: 63–30%, 75–15%, 46–36%, and 58–14%, respectively. Prostatic calcifications consisted of bacteria-like forms similar to the species isolated from biological materials and calcifications of patients. Our study proves, for the first time, that bacterial strains able to produce biofilms consistently are present in CBP. Additionally, prostatic calcifications are biofilm-related.

Introduction

Human prostate pathologies are one of the clinical problems with the greatest impact in the third millennium, both as prostate cancer, causing morbidity and death (Haas et al., 2008), and as inflammatory conditions of the gland. The latter inflammatory pathologies are strongly increasing in the males between 20 and 40 years old, with important impacts in terms of social, health-related and individual costs (Turner et al., 2004). Additionally, they have a very high impact on fertility and patient quality of life (Rizzo et al., 2003). In Italy, according to two recent studies (Rizzo, 2005; Bartoletti et al., 2007), chronic prostatitis (CP) is an emerging problem in young males of fertile age: the mean age is usually very low and the condition is confirmed at about 40 years. The main chronic characteristics of prostatitis are the early start of symptoms, the persistence of symptoms for years, the relatively young age of the patients, the possible impact of sexually transmitted disease (STD) infections acquired during early sexual intercourse, and the serious long-term sequelae such as infertility. A recent multicentre, cohort, observational Italian study on 750 male patients showed a high prevalence of prostatitis (13.3%), probably due to the use of systematic diagnostic criteria (Meares–Stamey test and urethral swab) on all patients (Bartoletti et al., 2007). Cat NIH-II chronic bacterial prostatitis (CBP) is the most frequently detected condition. The aetiology theoretically involves the whole range of bacterial species (Mazzoli, 2007): Escherichia coli, other Enterobacteriaceae, Enterococcus, Staphylococcus and Pseudomonas spp. All these microorganisms are able to form biofilms (Mazzoli, 2009), as well as E. coli, Staphylococcus, Enterococcus, and Ureaplasma spp. (García-Castillo et al., 2008), and infect prostate cells. Bacteria living in a biofilm usually have significantly different properties compared with free-floating bacteria (planktonic) of the same species, as the dense and protected environment of the film allows them to
cooperate and interact in various ways. One benefit of this environment is the increased resistance to antibiotics, as the dense extracellular matrix and the outer layer of cells protect the interior of the community. Inside the in vivo biofilms, the bacteria are prominently persistent. A small fraction of cells within biofilms are dormant and almost immune to the effects of antibiotics because of their very low level of metabolic activity. In addition, these dormant persistent bacteria are able to induce recurrences, as has been proved in chronic cystitis (Opal, 2007; Wellens et al., 2008) and proposed in CBP (Nickel & McLean, 1998; Soto et al., 2007; Mazzoli, 2009), and proved for E. coli acute prostatitis (Kanamaru et al., 2006). It has been suggested that if bacteria persist from acute or, more likely, clinically subacute prostate inflammation, they can form small, sporadic bacterial microcolonies or biofilms within the ductal system adherent to the epithelium (Nickel & McLean, 1998). There is a sort of quiescent period, a sort of 'hibernation', when the environment becomes adverse to and difficult for bacterial existence. The presence of focal sites of bacterial persistence can be postulated by areas of inflammation with subsequent lymphocyte invasion and infiltration of plasma cells and macrophages; this has been proved in both nonbacterial (Liu et al., 2008; Kim et al., 2009) and bacterial prostatitis (Matsumoto et al., 1992). It appears that the persistence of bacteria in the prostate gland in these focal biofilms leads to persistent immunologic stimulation and subsequent chronic inflammation. It is suspected that the creation of a chemically and immunologically distinct microenvironment may induce microorganisms to crystallize, calcify and form calculi (Nickel & McLean, 1998). In fact, another characteristic of biofilm-forming bacteria is the property to mineralize and calcify; this happened to the first bacteria colonizing the ‘orbe terraqueous’ in stromatolites (Kazmierczak & Kempe, 2006) and in Phanerozoic oceans (Arp et al., 2001), and nowadays occurs in hot springs biofilms (Allen et al., 2000). Extraterrestrial calcified biofilms were suspected by a NASA project ‘Life on Mars’, in Mars meteorites (Thomas-Keptra et al., 1998) from the Antarctic, in samples from Mars (Parro et al., 2008), and, by analogy, from terrestrial sites (Westall et al., 2004; Parro et al., 2008). They are also a modern and common phenomenon in urinary catheter-associated infections (Nickel et al., 1985; Morris et al., 1999; Drinka, 2006) and in dental roots in vitro (Kishen et al., 2006; Estrela et al., 2009) and in vivo (Costerton et al., 1986, 1987; Costerton, 1999; Schaudinn et al., 2009). Enterococcus faecalis or coccoid bacteria are involved. A bacterial-induced apatite precipitation on mature biofilm was also observed. This ability of E. faecalis to form such calcified biofilms on root canal dentin may contribute to their persistence after endodontic treatment. Enterococcus faecalis is also a well-represented cause of bacterial prostatitis, and has been confirmed for my cases (Mazzoli, 2007).

In 2004, Geramoutlos et al. (2004) found a clinical correlation of prostatic lithiasis with chronic pelvic pain syndromes (CPPS) in young adults. They examined any possible correlation with CP/CPPS and found that type B calculi (large calculi) were more often associated with symptoms and CP/CPPS (P = 0.007). These large prostatic calculi seemed to be related to underlying inflammation.

According to our studies, prostatic calcifications are a common finding in prostatitis. In a recent study (Bartoletti et al., 2007) intraprostatic calcifications were found in 59% of the patients and only 1% of the controls (P < 0.001), confirming their specific relation to prostate inflammation.

Additionally, in prostatitis there are difficulties in interpreting microbiological findings owing to the presence of contaminating, indigenous microbiota, organisms derived after passage through distally contaminated urethra [i.e. voided urine, urethral swabs, expressed prostatic secretion (EPS)], the presence of inhibitory substances in prostatic secretions and total ejaculate (TE), history of multiple courses of antibiotics in patients, presence of difficult-to-culture cell wall-deficient/defective bacteria, nonculturable bacteria, interaction between multiple microorganisms, interaction with the host, infections and inflammatory responses, and finally environmental stresses in tissues (Mazzoli, 2007). Many of these characteristics can be related to and confirm biofilm production inside the prostate.

The aims of the present study were to isolate potential biofilm-producing bacteria (Staphylococcus spp., Enterococcus spp., E. coli, Enterobacteriaceae, Pseudomonas aeruginosa) from urinary samples, TE, prostate/seminal vesicle secretions, and intraprostatic calcifications of CP NIH-II patients to evaluate their ability to produce in vitro biofilms. Further, we characterized intraprostatic bacteria and prostatic calcifications using electron microscopy to determine whether they might be biofilm-related.

**Materials and methods**

Bacterial strains were isolated from patients aged > 18 years with symptoms of CBP NIH-II, present for at least 6 months. The CBP NIH-II patient population was linked to the STDs Center in Florence from the whole of Italy during the year 2008. From these well-characterized patients with CBP (NIH-II) a population of bacterial strains causing the infection was isolated.

A total of 150 bacterial strains were evaluated: 50 E. faecalis; 50 Staphylococcus spp. (three Staphylococcus aureus, 23 Staphylococcus haemolyticus, 10 Staphylococcus epidermidis, five Staphylococcus warneri, nine Staphylococcus capitis, Staphylococcus hominis, Staphylococcus lugdunensis, Staphylococcus simulans); 30 E. coli; 20 gram-negative miscellaneous (eight Klebsiella oxytoca, three Citrobacter spp., four Enterobacter spp., two Pseudomonas spp., three Proteus spp.).
Bacterial strains were isolated from urine (U) and EPS, TE, postmassage urine (PMU) obtained from adults with clinical and microbiological diagnoses of CP NIH-II. Biological materials from the lower and upper genital tract were obtained from adults with diagnoses of CBP (NIH Cat: II) of varying severity (moderate to severe) according to symptoms. Bacteria isolated from these samples were cultivated aerobically in Columbia blood agar (BioMerieux, Italy) and in a 10% CO2 atmosphere in Columbia CNA agar (BioMerieux; BD, Italy). They were identified and characterized biochemically using the species identification cards of the Vitek II semi-automated System for Microbiology-BioMerieux; in addition each of the 150 strains under study was tested for antibiotic chemosensitivity on 16 different antibiotics cards of the Vitek II semi-automated System for Microbiology (BioMerieux). After this characterization all the strains were tested for biofilm production. Quantitative assay of biofilm was used to classify isolates as nonproducers, weak producers, moderate producers or strong producers.

Quantitative assessment of slime production was performed using crystal violet binding assay method according to the classic Christensen microwell assay (Christensen et al., 1985). Adherence measurements, read with an ELISA microreader apparatus (ETI-System S800, DIASORIN, Italy) at 630 nm with the photometer switched to the single-wavelength mode, were performed in triplicate and averaged. According to the ODs and cut-off value, bacteria were classified as nonbiofilm producer (nonadherent) or weak, moderate or strong biofilm producer (adherent). I modified the classic Christensen method by introducing a new category of ‘medium’ biofilm producers, to better differentiate the strong producer strains.

Prostatic calculi
Prostatic calculi were collected in a sterile manner during transurethral resection of prostate (TURP) from five patients with CBP in the previous population who were affected by dysuria characterized by sterile uriculature and caused by benign prostatic hyperplasia. Patients signed a written consent form for calculi studies. For each patient a minimum of at least two calculi were sampled: one for the culture and one for scanning electron microscopy (SEM). The ones undergoing SEM were stored aseptically in sterile saline and stocked at ~80 °C until analysis. Prostatic calculi for culture were immediately cultivated in brain–heart infusion broth and grown subsequently on Columbia blood agar (BioMerieux).

SEM
Electron microscopy was performed using the new Nikon Neoscope Benchtop SEM. The NeoScope operates in both low and high vacuum modes and has three settings for accelerating voltage suitable for a variety of applications, all of which can be programmed in special prestored recipe files. Magnifications of ×10 to 20,000 without lens change were performed.

All biofilm samples were prefixed in 5% glutaraldehyde directly in the microplate wells, kept out of the well, allowed to dry and prepared by gold sputtering by JFC-1300 metallizer device. Prostatic calculi were allowed to dry at room temperature for 1 h before being prepared directly by gold sputtering and observed by SEM.

Results

Wild-type biofilm production
Only 6% of E. coli strains were nonbiofilm producers. The majority of the strains were strong or medium producers, 63% and 30%, respectively. The thick tridimensional biofilm and slime production is demonstrated by SEM in Figs 1 and 2. Probable initial crystallization bodies are visible in the pictures.

The gram-negative miscellanea were strong or medium producers, 75% and 15%, respectively. Klebsiella spp. were the most productive biofilm strain of the gram-negative bacteria. The biofilm images (Fig. 3) from SEM demonstrate the tridimensional orientation of the single bacterial cells inside the biofilm.

Among gram-positive bacteria, Staphylococci were strong producers in 46% and medium producers in 36% of the cases. Figure 4 shows a thick biofilm due to a S. epidermidis strain. Enterococcus faecalis strains, one of the most frequently isolated bacterial strains in CP in my case studies,
were strong biofilm producers in 58% of the cases and medium producers in 14%. Figure 5 shows a high-resolution image of *E. faecalis* biofilm.

Globally, 85% of the strains were strong or medium producers. Total results for biofilm production are showed in Fig. 6.

**Prostatic calcifications**

Prostatic calcifications from the five patients showed the presence in culture of *E. faecalis* strains in calcifications from two patients, *S. epidermidis* was found in one patient, and *E. coli* in another two patients. SEM showed the presence of coccoid bacteria calcifications in both patients with *E. faecalis* isolated by biological samples and calcifications. Figs 7 and 8 show these coccoid forms from calcifications, which are better defined at higher magnification (Fig. 8), with perfectly rounded bacterial forms. These coccoid forms are quite similar to *E. faecalis* forms found in the literature from dental surface biofilms. *Staphylococcus epidermidis* calcification showed a similar surface to that of the *S. epidermidis* biofilms formed *in vitro* in Figs 9 and 10. *Escherichia coli*-like forms were shown over the surface of the calcification (Fig. 11).

It seems from our images that the presence of bacteria can be detected by SEM in prostatic calcifications or calculi in...
two different ways, first, bacteria may seem to be an essential constituent of the calcifications (Enterococcus- and Staphylococcus-like forms) with a sort of repetitive globular unit forming the calcification itself (Fig. 12). The globular unit could be the calcified microcolony, formed by single bacterial cells. Secondly, bacteria may seem to colonize the surface of the previously formed calcifications (E. coli-like forms).

**Discussion**

Our study establishes, for the first time, the presence of bacterial strains able to produce biofilms consistently in patients affected by CBP NIH-II. Globally, both gram-positive bacteria and gram-negative bacteria were good producers of biofilm, confirming the high aggressivity of these strains, biofilm production being accompanied by evidence of other virulence factors (haemolysin production) and patterns of high antibiotic resistance (data not shown). Staphylococcus haemolyticus strains were the best biofilm producer, confirming their importance in prostatitis not only epidemiologically (Mazzoli, 2007) but also pathogenetically. The presence of bacteria-like forms in prostatic calcifications, confirmed by positive culture of the same microorganisms morphologically present in calcifications,

![Fig. 6](image6.png)

**Legend:** NP, non-producer; WP, weak producer; MP, medium producer; SP, strong producer.

**Fig. 6.** Biofilm wild-base production (%) by bacterial strains isolated from chronic bacterial prostatitis.

![Fig. 7](image7.png)

**Fig. 7.** SEM picture (×70) of a prostatic calcification from which Enterococcus faecalis was isolated; surface appears broken and in the distal part a grapes-like preserved area is evident.

![Fig. 8](image8.png)

**Fig. 8.** SEM picture (×20,000) of a prostatic calcification from which Enterococcus faecalis was isolated; evidence of rounded bacterial cells calcified and filling the ravines of the grapes-like calcification part.

![Fig. 9](image9.png)

**Fig. 9.** SEM picture (×900) of a prostatic calcification from which Staphylococcus epidermidis was isolated; the surface appears full of rounded calcified microcolonies-like forms.
opens up a new field of study in prostatitis and defines the essence of calcifications as nests of bacteria and bacterial persistence, with consequential explanation of the chronic inflammation processes as due to bacteria in these patients. A recent paper (Sfanosa et al., 2009) proves that acute inflammatory proteins constitute the organic matrix of prostatic corpora amylacea and calculi in men with prostate cancer and presents a definitive analysis of the protein composition of prostatic corpora amylacea and calculi. That paper suggests that acute inflammation has a role in calculi biogenesis: in fact, proteins identified in calcifications, including calprotectin, myeloperoxidase and defensins, are contained in neutrophil granules. Immunohistochemistry suggested the source of lactoferrin to be prostate-infiltrating neutrophils as well as inflamed prostate epithelium, and suggested prostate-infiltrating neutrophils as a major source of protein for calprotectin, because this protein was absent from other prostate compartments. These data confirm the inflammatory genesis and components of prostatic calcifications: inflammation, which resembled the ‘primum movens’ of the calcifications, may now also be the starting point for an additional cascade, amplifying and perpetuating the inflammation itself and tissue damage. Inflammatory cytokines, especially interleukin-8, were additionally proved to be present in men with prostatitis as a surrogate marker of prostatitis (Khadra et al., 2006; Penna et al., 2007; Lotti et al., 2009), in general as well as in well-specified Chlamydia trachomatis (Mazzoli et al., 2007) prostatitis.

This phenomenon of inflammation can explain the persistence of symptoms in CP patients, the high resistance to antibiotic treatment in vivo and the necessity for long-term antibiotic treatment regimens. The presence of a biofilm producer strain can additionally determine a therapeutic decision to administer those antibiotics able to act intracellularly to destroy persistent bacterial nests inside these prostatic biofilms.

The reports described in our study present a new front in the field of prostatitis and their classification. They additionally open up a new perspective on the role of calcifications in the persistence of prostatitis and speculation about prostate cancer induction and genesis by chronic bacterial inflammation.
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References


