Molecular analysis of bacterial diversity on central venous catheters recovered from cancer patients

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Abstract of the Thesis

Central venous catheters (CVC) are a known source of nosocomial blood stream infections. However, standard cultivation methods to identify causal pathogens of catheter-related blood stream infections (CRBSI) on catheters often fail. In this study, I compare the traditional cultivation method to the cultivation-independent PCR-DGGE method to examine bacterial colonization on central venous catheters retrieved from cancer patients.

In order to study the biofilm communities that colonize the catheters, an optimized sonication protocol was developed to remove biofilm bacteria from their substratum. I showed that a sonicating power of 12 Watts for 5 minutes could remove bacteria, such as *Pseudomonas putida* and *Staphylococcus epidermidis* cells, from a glass surface without killing the bacteria.

Twenty-four catheters were retrieved from cancer patients and used in this study. Five out of the 24 catheter samples (21%) showed growth in at least one of the culture media used. The isolates recovered from the catheters belonged to five bacterial species, including *Staphylococcus aureus*, *S. epidermidis*, *S. hominis*, a *Staphylococcus* sp., and *Streptococcus agalactica*.

The PCR-DGGE method showed that 100% of the catheters were colonized by bacteria. Furthermore, unlike the cultivation assay demonstrated that contaminated catheters were colonized by only one or two bacterial species, DGGE analysis showed that all the catheters in this study possessed a mixed-bacterial community of at least 4 bacterial species. By analyzing the sequences

of the DGGE bands, *Enterococcus faecalis* (100%), *E. faecium* (96%) and *Roseomonas* sp. (88%) were the most predominant species detected on the catheters. *Staphylococcus epidermidis*, *S. hominis*, *S. aureus*, *Corynebacterium* sp. and *Serratia* sp. were detected on 30-54% of the catheter samples. Other bacterial species, such as *E. coli*, *Aeroccous* sp., *Micrococcus* sp. and alpha proteobacterium were detected on less than 30% of the catheters. In addition, scanning electron microscopy (SEM) confirmed the DGGE findings in that biofilms were found on all the catheter samples. Biofilms were found in the lumens of the catheters but rarely associate with the outer surface.

The catheters were rinsed prior to the sonication process and the rinsing buffers were also analyzed by culturing and DGGE assays as described for the catheter samples. None of the buffer samples showed any positive growth in the growth media but all of them showed positive amplification by a pair of eubacterial universal primers and showed similar DGGE profiles as their respective catheter samples, showing that even gentle rinsing could remove enough biofilm cells for PCR-DGGE analysis.

In conclusion, the PCR-DGGE method is a superior method in identifying biofilm microbial communities on catheters. This method could detect and identify "viable but non-culturable" bacteria that were missed by the conventional cultivation methods. Furthermore, this study also revealed that some bacterial species (such as *Enterococcus*, *Roseomonas* spp. and *Serratia* spp.), that have yet been recognized as a major cause of catheter related infections, can potentially be important catheter-associated pathogens.

1. Literature Review

1.1 Problems associated with use of catheters

More than 150 million of intravenous catheters are used annually in North American hospitals (Crump and Collignon, 2000) and catheter-related blood stream infections (CRBSI) have been implicated as one of the major causes of nosocomial infections (Siegman-Igra *et al.*, 1997). Up to 250,000 documented cases of CRBSI occur annually in the USA (O'Grady *et al.*, 2002). Use of central venous catheters (CVC) is responsible for as many as 90% of total CRBSI developed in hospital (Bregenzer *et al.*, 1998). The annual cost associated with catheter-related infections in the United States in 1992 was estimated to exceed $4.5 billion (Polonio *et al.*, 2001). For each individual patient recovered from a nosocomial bloodstream infection in an intensive care unit (ICU), it can cost as much as $40,000 (Pittet *et al.*, 1994). Central venous catheter related blood stream infections cause considerable morbidity and mortality (Mermel *et al.*, 2000). Intra-vascular catheters related sepsis brings 80,000 deaths annually in USA alone (Pelletier et al. 2000). Also, Tcholakian and Raad (2001) indicated that frequency of infection was considerably greater in immunocompromised patients who require CVC for parenteral nutrition and chemotherapy treatment. Prolonged use of intravascular devices in patients who are older, sicker and immunosuppressed can lead to complications and increased morbidity and mortality rates (Goldmann and Pier, 1993; Siegman-Igra *et al.*, 1997)

1.2 Catheter contaminations

Central venous catheters (CVC), in general, are long, fine, sterile tubes that are introduced into a large blood vessel such as the jugular or subclavian vein for parenteral administration of fluids and/or medications (Long and Phipps et. al., 1985) or for measurement of central venous pressure (Carratalã *et al.*, 1999; Flowers *et al.*, 1989; Friel, 1981; Stamou *et al.*, 1999). In this study, I focus on the Hickman - Broviac catheters, which are specifically used for administration of drugs, such as chemotherapeutic agents, to cancer patients.

Catheters can be colonized by microorganisms, leading to catheter-related infections. Goldmann and Pier (1993) suggested that the vast majority of endemic infusion related infections were caused by catheter contamination. The consensus is that most of the infusion-related infections are initiated during insertion of catheter. Epidemiological data indicates that microorganisms at the insertion site are able to track into the wound made by the catheters and can rapidly colonize the catheters' intravascular segment. The bacteria are able to move down the external surface rapidly, infecting a larger area of the catheters.

The most common sources of CVC-related infections are bacteria from patients' skin, hands of medical staff, tools and chemicals (Murga et al. 2001). Since the most common source of microorganisms that colonize catheters is the patients' own cutaneous flora, the major type of bacteria that causes catheter-related bacteremia is coagulase-negative staphylococci, a common group of skin microflora (Goldmann and Pier, 1993). Snydman *et al.* (1990) found that with

each case of catheter contamination, at least one of the organisms cultured from the catheter was also present on the skin of the patient.

Health care personnel can also contribute to catheter infections by not washing their hands appropriately. This can lead to intrahospital bacterial dissemination (Maki *et* al., 1977). Use of 2% aqueous chlorhexidine for skin disinfection and proper hand washing can result in 2-fold decrease in CRBSI (Sandoe at. al, 2001). As it is a clinical standard to draw blood to ensure proper catheter placement, bacteria from the tip of a catheter can be disseminated by this procedure in catheter lumen. Blood proteins absorbed on the internal catheter surface serve as ideal adhesive material especially for Gram-negative bacteria (Murga *et al.* 2001). Critically ill patients frequently develop infections, such as pneumonia, pyelonephritis, urinary tract infections and septic vein thrombosis. Bacteria from these infections can be spread by circulatory system and cause CVC intra-luminal colonization (Raad *et al.*, 1995). Infusate contaminations during industrial production can also cause bloodstream infections but this is a rare source of bloodstream infections (Maki at. al., 1981).

1.3 The types of bacteria that colonize catheters

As mentioned earlier, one of the most frequently isolated bacteria from colonized catheters are coagulase-negative staphylococci (CoNS), such as *Staphylococcus epidermidis.* Other skin microflora such as *Staphylococcus aureus* have also been isolated (Stamou *et al.*, 1999; Raad, 2000; Goldmann and Pier, 1993). Center of Diseases Control of the USA (CDC 1999; Guidelines for the Prevention of Intravascular Catheter-Related Infections 2002) has reported

that coagulase-negative staphylococci cause 37% of all CRBSI. *S. aureus*, enterococci, *Candida* spp. and Gram-negative bacilli account for 12.6, 13.5, 8 and 14 % of reported hospital-acquired CRBSI, respectively. However, many more microorganisms have been linked to catheter-related bloodstream infections. These include *Enterococcus faecalis, Corynebacterium spp., Bacillus spp., Enterobacter aerogenes, Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella spp., Mycobacterium neoaurum, Serratia marcescens and Candida albicans*, (Atela *et al.*, 1997; Nahass and Weinstein, 1990; Raad, 1998; Raad, 2000; Stamou *et al.*, 1999).

1.4 CVC related infections in cancer patients

Solid tumors and hematological malignancies are frequently associated with neutropenia and weakened immune response. Septicemia is a frequent complication in immunocompromised patients undergoing chemotherapy using long-dwelling central venous catheters (Soufir, 1999). Such patients often do not demonstrate clinical symptoms of systemic inflammation. These make diagnostics of catheter related sepsis impossible and cause increasing mortality (Pelletier *et al.* 2000; Andremont *et al.*, 1988; DesJardin *et al.*, 1999). Catheters used in treatment of cancer patients usually remain in a patient's body for longer than 30 days (Andremont *et al.*, 1988). This further increases the potential of catheter infections of the cancer patients. Over 40% of the nosocomial infections observed in cancer patients are catheter-related blood stream infections (Armstrong *et al.*, 1986; Johnson *et al.*, 1986).

1.5 The formation of biofilm on catheters

It has been recognized that majority of the bacterial communities in the environment, as well as on medical devices, live in form of biofilms (Donlan, 2001). Donlan and Costerton (2002) described biofilms as sessile microbial communities that are characterized by cells irreversibly attached to substrate surfaces or to each other and are embedded in a matrix of extracellular polymeric substances (EPS). Biofilm bacteria have been shown to colonize the lumen of catheters and are usually immersed in a thick layer of EPS (Aschner *et al.*, 1987; Mamie *et al.*, 1984; Tenney *et al.*, 1986). Raad *et al.*, (1993) showed that biofilms can associate with either the outside or inner lumen of catheters. Due to the direct contact of catheters with the bloodstream, the catheter surfaces are usually coated with plasma proteins such as laminin, fibrinogen, fibronectin and albumin. These proteins can act as conditioning films for bacteria attachment. For instance, *S. aureus* adheres to proteins such as fibronectin, fibrinogen and laminin while *S. epidermidis* adheres to fibronectin only (Rand, 1998). Biofilm formation has been shown to occur as early as three days after catheterization (Anaissie *et al.*, 1995). Tenney *et al.*, (1986) demonstrated that Hickman catheters that were used to treat patients with chemotherapy drugs could be completely colonized by microbial biofilms on both the inner and outer surface of the catheters after three weeks. Short-term (less than 10 days) indwelling, non-cuffed catheters are usually colonized by skin microflora, such as coagulase-negative staphylococci (39% of the catheters used), *S. aureus* (26%), *Candida* species (11%) and Gram-negative bacilli (14%). In contrast, long-term (> than 10 days) CVC were

found being colonized mostly by coagulase-negative staphylococci (25%) and Gram-negative bacilli (45%) (Maki *et al.*, 2001; Maki *et al.*, 1998).

According to Heilmann *et al.* (1996a), biofilm formation consists of 5 stages – attachment to a surface, proliferation, intercellular adhesion, extracellular slime production, and cells or even whole biofilm detachment. All these phases can be demonstrated on *S. epidermidis* biofilm development, one of the most frequently found inhabitants of human skin and CVC. Heilmann *et al.*, (1997) also discovered that a cell surface protein exhibiting vitronectin-binding activity was related to the primary attachment of *S. epidermidis* to a polystyrene surface. This protein is encoded by the chromosomal *atiE* gene (Heilmann *et al* , 1997). Other genes have been implicated in the second stage of biofilm formation, including three intercellular adhesion genes named *icaA, icaB* and *icaC*, which are found in an operon structure (Heilmann *et al.*, I996b). *Staphylococcus spp.* produce a group of cell wall-associated proteins regulating adhesion of the bacteria to surfaces and conducting protection against host immune system (Balaban *et al.*, 1998).

1.6 Biochemistry and physiology of Biofilm cells

Bacterial cells communicate with each other by quorum sensing (QS) mechanism during biofilm formation (Greenberg, 1997; Kleerebezum *et al*, 1977). Different bacterial species produce a number of different classes of QS signal molecules. *N*-acyl-L-homoserine lactone (AHL) and some of its derivates are shown to be the QS signals of Gram-negative bacterial species, such as *E. coli, Pseudomonas* and *Vibrio* spp. (Calfee *et al*, 2001; Mayville *et al.*, 1999). The QS

signals are constitutively produced by individual cells. However, the signal molecules will only be effective in transforming microcolonies of attached cells to mature biofilms when these signal molecules within the microcolonies reach their threshold concentrations. Once they reach their threshold concentrations, they turn on the biofilm genes that are responsible for the maturation of biofilms, producing a large amount of EPS that assemble the biofilm matrices.

Gram-positive bacteria communicate by other QS signal molecules such as peptide pheromone, cell-density dependent peptides and cascade of sensor proteins and histidine kinase, located in the cell membrane of the bacterium. Some of these peptides interact with membrane bound kinase sensors to transduce signals across the membrane. Cyclic thiolactone is another QS signal molecule used by *Staphylococcus aureus* for biofilm formation.

Biofilm cells are more resistant to pH and temperature changes, disinfectants and antibiotics. It has been shown that bacterial biofilms on medical catheters are 1000 times more resistant to antibiotics than their planktonic counterparts (Donlan, 2001, Costerton, 1995). This can be explained by changes in cell wall composition of and increase in EPS production by the biofilm cells (Rupp and Hamer, 1998). Due to the low immunogenic response elicited by the polysaccharide matrix of biofilms, the immune system may not be able to detect the presence of bacterial cells hidden in the biofilm matrices (Davies *et al.*, 1998; Fuqua *et al.*, 1994). Aerobic and anaerobic processes can also occur simultaneously at different sites of a biofilm (Davies *et al.*, 1998). When a biofilm

reaches its maturity, biofilm cells can be detached into the blood stream of a patient causing symptoms of bacteremia (Crump and Collignon, 2000).

1.7 Detection of catheter-related infections

Several methods are used to diagnose catheter-related infections. Some of these methods require removal of the catheters. They include:(1) cultivation of potential pathogens from the tip of the catheters (either qualitative or quantitative), (2) a rolling-tip plate method (a semi-quantitative cultivation assay by rolling the tip of a catheter sample on microbiological agar media), and (3) direct microscopic examination (staining with either Gram stain or acridine orange). Other detection methods do not require removal of the catheters. These include (1) Superficial cultures from sites of insertion, (2) endo-luminal brushing, (3) paired quantitative central :peripheral venous blood culturing, (4) unpaired quantitative culturing of blood samples obtained through CVC, and (5) direct microscopic examination of blood samples obtained through CVC.

Accurate diagnosis of catheter-related blood stream infection (CRBSI) requires quantitative blood smear counting, blood sample culturing and catheter tip smear counting and culturing. These methods are labor-intensive and costly (Raad, 2004). Presence of microbial contaminations on the external surface of central venous catheters can be detected by the semi-quantitative rolling tip culturing assay. A catheter is considered colonized when more than 15 CFUs are detected (Maki *et al.*, 1977; Buisson *et al.*, 1987; Collignon *et al.*, 1986). However, the rolling-tip plate method does not detect microorganisms that colonize the inner lumen of catheters because the inner lumen does not come into contact

with the nutrient agar. Therefore, this method may under estimate the incidence of CRBSI (Sherertz *et al.*, 1997; Crump and Collignon, 2000).

Sonication has been used to remove biofilm bacteria from catheter samples and found to be more sensitive than other methods to detect bacteria on contaminated catheters (DesJardin *et al.*, 1999). For catheters that have been in place for extended periods of time, the sonication method demonstrates 20% more sensitive than the roll-tip plate and catheter flashing methods (Sherertz *et al.*, 1997).

To detect CRBSI without removing the catheters, paired blood samples can be drawn from the central (via the central venous catheters) and peripheral veins of patients for quantitative culturing assays. Patients with CRBSI should show a high central :peripheral ratio of bacterial counts (Flynn *et al.*, 1987). A qualitative approach has also been proposed to detect CRBSI in cancer patients (DesJardin *et al.*, 1999; Raad *et al.*, 2004). Blood samples are taken from the peripheral and central veins of the patients. If the same bacteria are identified from both blood samples, the patients are considered positive for CRBSI.

"Endo-luminal brush method" is another method that allows CRBSI diagnosis without CVC removal (Kite *et al.*, 1997). When compared with the rolling-tip culturing method, the percentage of catheters tested positive were 82 and 66% for the endo-lumnal brushing and roll-tip plate methods, respectively (Kite *et al.*, 1997).

One of the most important limitations of direct cultivation from catheter tip samples is the demand for catheter removal. Despite the removal of catheters for

microbiological analysis will provide a more accurate diagnosis of CRBSI, catheter removal is not always preferred by clinicians (Press *et al.*, 1984) because only 25-45% of episodes of sepsis with long-term devices represent true CRBSI (Tacconelli *et al.*, 1997). Furthermore, some clinical studies show that CRBSI caused by coagulase-negative staphylococci can be successfully treated by antibiotic infusion through the infected catheters, with a success rate of 60-91% (Press *et al.*, 1984; Hartman *et al.*, 1987; Benezra *et al.*, 1988; Marr *et al.*, 1997; Messing 1990). Some studies also demonstrated that the quantitative blood culture method may allow diagnosis of CRBSI without the unnecessary removal of non-infected catheters (Flynn *et al.*, 1987; Maki *et al.*, 1977; Yagupsky and Menegus, 1989; Sherertz *et al.*, 1997). However, leaving an infected catheter in place increases the risk of recurrent of bacteremia up to 20% (Raad *et al.*, 1992; Elishoov *et al.*, 1998; Press 1984).

Nevertheless, a common consensus of whether a catheter should be removed for CRBSI analysis has yet been reached. Siegman-Igra *et al.* (1997) performed a meta-analysis of different diagnostic methods to evaluate their effectiveness of detecting CRBSI. These methods include (1) qualitative culture of the tip, (2) semiquantitative culture ("rolling-tip plating"), (3) quantitative culture of the tip, (4) direct microscopic examination (Gram staining and acridine orange staining), (5) paired quantitative blood cultures (central :peripheral vein blood samples) and (6) unpaired quantitative blood samples obtained through the CVC. The most accurate method was the quantitative culture of the catheter tip

samples (90% accuracy) and the unpaired quantitative blood culturing had the lowest accuracy of 78%.

1.8 Analysis of CVC colonization by PCR-DGGE

Fast identification of causative microorganisms on catheters is important for CRBSI treatment and prognosis. Most of bacterial cells on an infected catheter are hidden inside of a thick layer of extracellular polymeric substances (EPS) and are not accessible for the host's immune system. This also makes routine blood culturing ineffective and serological diagnosis of CRBSI uncertain (Crump and Collignon, 2000). In a mature biofilm, bacterial cells undergo differential genotypic and phenotypic expressions according to their topographic position in the matrix. Coch *et al.*, (2002) showed that bacterial communities found on catheters exhibited viable but non-culturable (VBNC) behavior. Furthermore, bacterial cells taken from biofilms fail to grow on most standard microbiological growth media and this becomes a major limitation of most clinical culturing methods (Marshall, 2000).

Studies have shown that both bacterial DNA and enzyme activity are present on medical devices even when no bacteria are recovered by plating (Costerton *et al.*, 1999). The presence of messenger RNAs on these devices also proves metabolic activity of biofilm cells (Vandecasteele *et al.*, 2002). Molecular detection of microbial DNA by polymerase chain reaction (PCR) is sensitive and quantitative and has been successfully used to establish bacterial presence in different clinical samples. Amplification of 16S rDNA of bacteria makes possible to avoid frequently inconclusive cultivating methods. PCR is

extremely sensitive and can detect as small amount of bacteria as 10 cells per one µl of blood sample (Schabereiter-Gurtner *et al.*, 2001; Walter *et al.*, 2000). In case of CRBSI, bacterial presence on catheter lumen can be detected by PCR (Dobbins *et al.*, 2003). PCR amplification can detect even one bacterial cell in a blood sample drawn via a catheter (Warwick *et al.*, 2004).

Biofilm on a long-dwelling catheter can be composed of mixed microbial species. According to Donlan (2001), samples provided for blood culturing taken from long dwelling catheters are always colonized by Gram-positive and Gram-negative mixed cultures. The denaturing gradient gel electrophoresis (DGGE) can be used to detect the composition of bacterial communities without prior information about the possible identity of the microbes in these communities.

DGGE has been used to identify bacterial communities in various environments, such as soil, feces and food (Leung *et al.*, 2003; Satokari *et al.*, 2001). PCR amplification of DNA extracts from samples with a pair of universal eubacterial 16S rDNA primers produces 16S rDNA fragments of all bacteria found in the samples. The amplified 16S rDNA fragments from various bacterial species can be separated by electrophoresis in a denaturant (urea and formamide) gradient gel. DNA fragments are separated based on their GC:AT nucleotide ratio, length and arrangement of nucleotide base sequence and melting temperature. All these factors influence speed and distance that the DNA fragments travel in the gel. The DGGE profile of a sample represents the specific bacterial composition of the sample. The DNA bands in the gel can be excised,

amplified, sequenced and identified by comparing with known bacterial DNA sequences in the GenBank database.

DGGE has been widely used in environmental microbiology for evaluation of microbial community in natural environments (Davies *et al.*, 2004). However, it is rarely used in medical diagnosis and has yet to be used for detection of CRBSI. Therefore, the aim of this study is to compare the traditional cultivation method to the cultivation independent PCR-DGGE method to examine bacterial colonization on central venous catheters recovered from cancer patients.

1.9 Objectives of the Thesis

Specific objectives of this study are:

(i) to develop an optimal protocol to remove biofilm bacteria from a substratum;

(ii) to isolate and identify bacteria on central venous catheters (CVC) collected from cancer patients by cultivation using various microbiological media;

(iii) to determine the diversity and identity of the bacterial community on CVC recovered from cancer patients by the PCR-DGGE method; and

(iv) to examine the presence of biofilm on the CVC by scanning electron microscopy.

2. Materials and methods

2.1 Biofilm removal protocol

A protocol to remove bacterial biofilms from surfaces was developed for this study. A Sonic Dismembrator Model 100 sonicator (Fisher Scientific) was used to remove biofilm bacteria from the catheter samples. Knowing that high energy of sonication can destroy bacterial cells, the tolerance level of bacterial cells to various intensity of sonication was evaluated. Taking into account that bacterial community on a catheter can be represented by both Gram-positive and Gram-negative bacteria, *Staphylococcus epidermidis* (Gram-positive) and *Pseudomonas putida* (Gram-negative) were used as model strains to optimize the sonication procedure.

A *S. epidermidis* and a *P. putida* strain were grown in Tryptic Soy Broth (TSB; Becton Dickinson and Company, Sparks, MD, USA) to an early stationary phase at 37° and 30°C, respectively, harvested, washed twice and suspended in sterile phosphate-buffered saline (PBS; 0.14M NaCl, 2.68 mM KCl, 10.10 mM Na_2HPO_4 and 1.76 mM KH_2PO_4, pH 7.4) at OD_{600} 1.0. One ml of bacterial suspension of each strain was exposed to different intensity of sonication ranging from 0 to 16 Watts for 5 minutes. Cell density of the sonicated and non-sonicated samples were determined by plate counting on Tryptic Soy Agar (TSA; Difco Detroit, MI, USA) incubated at 37°C for *S. epidermidis and* 30°C for *P. putida* for 48 hours. Viability of the cell suspensions was examined at different sonicating powers and an optimal setting was chosen for biofilm removal (Figure 1).

Statistical analyses, such as the ANOVA and standard derivations, were performed by the SPSS statistical software package ver 9.0.

Sonication was applied to *P. putida* biofilm grown on glass surface in order to demonstrate the ability of the sonication method to remove biofilm. Two ml of overnight *P. putida* culture were added to 18 ml of TSB containing 2 pieces of sterile pre-cleaned cover slips and triplicate samples were used for this experiment. The samples were incubated at 30°C for 24 hours with shaking at 50 rpm. After 24 hours, the growth medium was removed from all beakers and the cover slips were rinsed 3X with 20 ml sterile PBS. One glass cover slip from each beaker was submerged in 20 ml sterile PBS and was sonicated at 12 Watts for 5 minutes. The second glass cover slip was used as an un-sonicated control. The glass cover slips were stained with acridine orange and examined under an epifluorescent microscope for biofilm cells.

2.2 Catheters

Catheters collected from 24 cancer patients, who were under chemotherapeutic treatments at the Northwestern Ontario Cancer Care Centre between 2003 and 2004, were used for this study. Blood stream infection (BSI) was suspected in 13 patients based on clinical symptoms. Peripheral blood culture assays were performed on blood samples obtained from these patients by the Thunder Bay Regional Health Centre Clinical laboratory and two samples produced growth. For seven patients, peripheral blood culture assays were repeated twice but the results were negative. Inflammation of an insertion site and dermal tunnel was observed in three patients but in each case culturing of

the insertion site probe was negative. For the remaining 11 patients, symptoms of BSI were not observed. The catheters were removed from the patients between 20 and 180 days depending on the length of the treatments and potential BSI (Table 1).

2.3 Catheter processing

Catheter tips (about 5 cm in length) were received in sterile plastic bottles. For each catheter sample, three 1-cm pieces were cut from the tip of the catheter sample. One piece of the catheter was used for cultivation assay, one for the DGGE analysis and the last piece was for SEM imaging. The catheter pieces were separately placed in sterile 1.5 ml Eppendorf tubes and gently washed by pipetting 1 ml of sterile PBS solution from both ends of the catheter pieces to wash away blood residues that adhered on the catheter samples. Each of the washed catheter pieces was split vertically into 4 equal pieces and placed in a sterile 1.5 ml Eppendorf tube for further processing, by the cultivation assay, DGGE analysis or SEM imaging. The wash solution from each catheter sample was also saved for the corresponding analysis.

2.4 Culturing assay

To remove biofilm cells from the catheters, 500 μl of sterile phosphate-buffered saline (PBS; 0.14M NaCl, 2.68 mM KCl. 10.10 mM Na_2HPO_4 and 1.76 mM KH_2PO_4, pH 7.4) were used to immerse the catheter samples and the samples were sonicated at an output power of 12 Watts for 5 minutes. The sonicated liquid samples were divided into six equal portions and were cultivated

on TSA, MacConkey Agar and Sheep Blood Agar, both aerobically and anerobically at 37°C for 72 hours. When colonies were found, they were picked and streaked onto the same, but fresh medium. The isolates were Gram stained and identified by 16S rDNA sequencing.

The PBS solution samples that were used to wash the catheters prior to the sonication process were also analyzed by culturing assay as described for the catheter samples.

2.5 DNA extraction and 16S rDNA sequencing from isolates

Bacterial isolates were grown aerobically in 50 ml of TSB at 37° C to late log phase (OD at 600 nm 1.0). One ml of this broth suspension was placed in a 1.5 ml tube and centrifuged at room temperature at speed 14,000 x g for 5 minutes. The cell pellet was washed with 1.0 ml of sterile deionized water 3 times. Finally, the pellet was resuspended in 1.0 ml of the sterile deionized water and the tube was placed in a 100°C water bath for 10 minutes to kill cells and release their DNA. The cap of the tube was pierced to allow the release of steam pressure. The tube was centrifuged for 5 minutes at 14,000 x g and the supernatant was transferred to a sterile 1.5 ml tube and one µl of the crude DNA extract was used for 16S rDNA amplification.

A pair of universal eubacterial primers, 341-Forward (5'-CTACGGGAGGCAGCAG) and 534-Reverse primers (5'-ATTACCGCGGCTGCTGG), were used to amplify the V3 region of the 16S rDNA of the catheter DNA extracts, producing an amplicon of 194 bp. Amplification was performed under the following conditions:denaturation at 95°C for 1 min,

annealing at 55°C for 1 min, and extending at 72°C for 1 min. A total of 30 cycles were performed and then followed by a final extention step at 72°C for 10 min. The PCR was performed in 200 µl PCR tubes using HYBAID PCR Sprint Thermocycler (Midwest Scientific, MO, USA). One µl of DNA template was used for each 50 µl reaction product mixture containing 0.5 mM of each primer, 0.2 mM of each dNTP (MBI Fermentas, Burlington, ON), 1.0 U of *Taq* polymerase (MBI Fermentas, Burlington, ON), 5 µl of 10x PCR buffer without $MgCl_2$ to 1x final concentration, 3 µl of 25mM $MgCl_2$ to 1.5 mM final concentration. The PCR product was examined by electrophoresis in a 1% agarose gel. DNA was stained with ethidium bromide (100 µg/ml) and visualized under UV light.

DNA product concentration was measured in each sample by comparison with a known concentration of DNA standard marker. Concentration of the amplicon was adjusted to about 150 – 200 ng per µl. The sample was sent to Mobix Lab (MacMaster University, Ontario) for DNA sequencing. The amplicon DNA sequences were identified by comparing to known bacterial DNA sequences in the GenBank database at the NCBI Blastn website (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi).

2.6 DNA extraction from catheter samples

Five hundred µl of the Instagene matrix solution (Bio-Rad Laboratories Inc, Hercules, CA) were used to immerse each catheter sample for sonication at 12 Watts for 5 min. After sonication the catheter samples were incubated at 56°C for 30 minutes and vortexed at high speed for 10 seconds. The samples were then placed in a 100°C water bath for 8 minutes. Again, each sample was vortexed at

high speed for 10 seconds and centrifuged at 14,000 x g for 5 minutes. The supernatant was transferred to another sterile 1.5 ml tube and the volume was estimated. Using the GeneClean (Q-Bio Gene, Carlsbad, CA) Kit reagents, DNA from the crude DNA extract was purified by binding to a silica matrix. The DNA-silica matrix was washed with buffered ethanol and the purified DNA was eluted from the silica by 25 µl of sterile deionized water as recommended by the manufacturer. The purified DNA extracts were stored at -20°C for the PCR-DGGE assay.

2.7 PCR-DGGE

PCR primers targeting the V3 region of 16S rDNA gene of eubacteria at the nucleotide positions 341 (Universal Primer Forward 341-f-GC) and 534 (Universal Primer Reverse 534-r) were used to amplify the 16S rDNA fragments of the bacterial population in the catheter DNA extracts. The sequences of the forward and reverse primers were:Universal 341-Forward-GC, GC clamp underlined, (5'-

CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGC CTA CGG GAG GCA GCA G) and 534-Reverse primers (5'- ATT ACC GCG GCT GCT GG). The PCR assay was performed in a 25 µl PCR mixture containing 0.5 unit of Taq DNA polymerase (MBI Fermentas, Burlington, ON), 0.5 µl of each 25 µM primer, 2.5 µl 10X buffer without $MgCl_2$ and 3 µl of 25 mM $MgCl_2$, 2.5 µl of dNTP mix (0.2 mM for each kind of nucleotide), deionized water 12.5 µl and one µl of catheter DNA extract. The PCR reaction mixtures were prepared aseptically and processed in a Hybaid PCR Sprint Thermal cycler (Midwest Scientific, MO, USA).

Amplification was performed under the following conditions:denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extending at 72°C for 1 min. A total of 30 cycles were performed and then followed by a final extention step at 72°C for 10 min. The PCR amplification product was examined by electrophoresis in a 1% agarose gel. DNA was stained with ethidium bromide (100 µg/ml) and visualized under UV light. DNA concentrations were estimated with a Chemigenius BioImaging system and a GeneTools ver. 3.00.22 software program (Synoptic Ltd., SynGen division, Beacon House, Cambridge, England)

A 40% Acrylamide/Bis solution, 37.5:1 (DCode Universal mutation Detection System, Bio-Rad Laboratories Inc, Hercules, CA) was cast with an increase of denaturant gradient from 25 to 65 % (top to bottom). One hundred percent of denaturant was defined as 7M urea plus 40% formamide. The DGGE gel was equilibrated in a DGGE unit containing 7 L of 1X TAE running buffer (Bio-Rad) maintained at 60°C. The PCR products of the catheter samples (containing the 341F GC clamp) were mixed with an equal volume of 2x loading dye (1.5 ml of bromphenol blue-xylene cyanol mixture and 3.5 ml of 100% glycerol) and were loaded to the DGGE gel. The gel was processed at 150 Volts and a constant temperature of 60°C was kept during whole DGGE procedure. *Staphylococcus epidermidis*, *Enterococcus faecalis* and *Escherichia coli* 16S rDNA amplicons were used as positive controls and sterile double-deionized water was used as a negative control. The DGGE process was terminated after 8 hours and the gel was stained for 15 minutes in 150 ml of 1X TAE buffer contained 15 µl of 10,000x SYBR Green I (Sigma-Aldrich, St. Louis, MO). The

image of the gel was captured by a Chemigenius BioImaging documenting system and a GeneTools ver. 3.00.22 software was used to detect and quantify DNA bands with concentration higher than 5 ng. The computer selected DNA bands were also confirmed by visual examination. The DGGE profiles of the samples are presented by binary codes in Table 2.

2.8 DNA extraction from DGGE gel

Selected bands were excised and rinsed in 1 ml double distilled water (ddH_2O). The excised gel was homogenized in 50 µl of sterile ddH_20 and allowed to equilibrate overnight at 4°C. The gel homogenate was spinned at 14,000 x g for one minute. One µl of the homogenate was amplified with 341-Forward and 534-Reverse primers as described earlier, except the forward primer lacked GC clamp. The PCR product was purified by 1% agarose electrophoresis. The DNA from agarose gel was extracted by centrifugation through a glass wool stuffed 0.5 ml microcentrifugal tube which had a tiny whole punched in its bottom. The DNA collected was purified with phenol-chlorophorm extraction and ethanol precipitation. To confirm that PCR product from an excised band was identical to the original band, the band homogenate was also amplified with the DGGE primers and confirmed by DGGE.

2.9 Sequencing and identification of DGGE bands

The PCR amplified products of selected bands were cloned into the pGEM ® -T Easy cloning vector (Promega corporation, Madison, WI) using a modified protocol. In brief, one µl of the PCR product (about 20 ng/µl) were mixed with 5 µl

of the 2x Rapid Ligation buffer (60mM Tris-HCl, pH 7.8, 20mM MgCl2, 20mM DTT, 2mM ATP 10% PEG), one µl of T4 DNA ligase, and 3 mcL of sterile ddH_2O. After overnight ligation at 4°C, the ligate product was transformed into an *E. coli* JM109 cells prepared by the Fermentas Bacterial Transformation Kit (MBI Fermentas, Burlington, ON). One and a half ml of an overnight *E. coli* culture grown at 37°C in LB broth, shaken at 200 rpm, was spun down for 1 minute at 14,000 x g at room temperature. The cell pellet was washed once with double distilled water, centrifuged at 14,000 x g, resuspended in 300 µl of TransformAid T- solution and pelleted at 14,000 x g after incubating on ice for 5 minutes. The cells were resuspended in 120 µl of TransformAid T- solution and incubated on ice for 5 minutes. One µl of the ligation mixture was mixed with 50 µl of cell suspension and incubated on ice for 5 minutes. The mixture was immersed in 42°C for 90 seconds and placed immediately back on ice for 5 minutes. The transformation mixture was mixed with 1 ml of TSB pre-warmed at 37°C and placed in a 37°C incubator for 1 hour. Three hundred µl of the suspension were plated on selective agar media for bacterial selection. The antibiotic selective agar was prepared based on the Promega protocol with Ampicillin (100 µg/ml), X-Gal (20 µg/ml) and IPTG (200 µg/ml). White colonies were isolated for further analysis.

To confirm the insertion of the 16S rDNA fragment, crude DNA extracts were extracted from the selected clones by the boiling method as in Section 2.5. The crude DNA extracts from the white transformants were amplified with the T7 (5'- TAATACGACTCACTATAGGG) and SP6 (5'-

GCTATTTAGGTGACACTATAG) primers. The PCR products were visualized on an agarose gel as described earlier. Clones that gave the expected 200 bp PCR fragment were chosen for DNA sequencing analysis.

The Promega Wizard Plus Mini-Plasmid Purification Kit (Promega corporation, Madison, WI) was used to extract the pGEM Easy-16S rDNA plasmids from the selected clones. In brief, aliquots of 10 ml of LB broth with 100 µl/ml of Ampicillin were inoculated with the selected clones and incubated overnight at 37°C in a shaker incubator. Cells were centrifuged for 5 minutes at 14,000 x g, suspended in 250 µl of suspension solution and 250 µl of cell lysis solution, incubated at room temperature for 5 minutes and neutralized with 350 µl of Neutralization solution. After centrifugation of the tube at 14,000 x g for 10 minutes, 850 µl of the supernatant were centrifuged through the Spin Column and double-washed with 750 µl of the Column Wash Solution. Plasmid DNA was dissolved and removed from the Spin Column by centrifugation with 100 µl of nuclease-free water. Sequencing of the 16S rDNA insert in the plasmid was performed Mobix DNA laboratory (MacMaster University, Ontario). Sequences of the 16S DNA insert were compared and identified with GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi).

Some DGGE bands were identified by direct sequencing from the PCR products obtained the excised DNA bands. To confirm that PCR product from an excised band was identical to the original band, the band homogenate was also amplified with the DGGE primers and confirmed by DGGE before DNA sequencing.

2.10 Electron Scanning Microscopy

A Scanning Electron Microscope/Energy Dispersive Spectrometer (SEM/EDXA, JEOL-USA Inc., Peabody, MA) was used for examining of external and internal surfaces of catheters. Catheter samples were prepared by desiccation in a biosafety hood at room temperature. Time for desiccation was selected by comparison of images of control samples desiccated for 6 hours, 12 hours, 18 hours, 24 hours, 36 hours and 48 hours. Most effective resolution was observed in samples desiccated for 24 hours. Catheter samples were cut into 5-mm pieces and prepared for microscopy by desiccation for 24 hours, gold-sputtered and observed under the SEM.

3. Results

3.1 Optimization of a sonication procedure to remove biofilm cells

Comparing the viability of the planktonic *Pseudomonas putida* and *Staphylococcus epidermidis* cells before and after sonication revealed that both bacterial species maintained their viability with an output power of ≤ 14 Watts for 5 minutes (Figure 1). Using an ANOVA test, survival ability of both bacterial strains was not significantly different when exposed to sonication power between 5 to 14 Watts for 5 minutes. However, at an output power of 16 Watts for 5 minutes, the cell densities of *P. putida* and *S. epidermidis* declined significantly ($p < 0.01$) at about 1.7-2.6 log and 2.0-2.8 log CFU/ml, respectively.

Based on the above testing, a setting of 12 Watts for 5 minutes was used to remove 72-h old biofilm *P. putida* cells attached on glass cover-slips. Laser scanning confocal microscopy showed that all the biofilm cells were essentially removed from the cover-slip after sonication at this energy level (Figure 2c).

3.2. Detection of bacterial colonization on catheters by culturing

Samples collected from the sonicated catheters were cultured on TSA (or in TSB), MacConkey Agar and Blood Agar aerobically and anerobically. Five out of 24 catheter samples (21%) showed positive growth in at least one of the culture media used (Table 3). Bacterial isolates from each catheter were screened with the ERIC-PCR (data not shown). Isolates that showed distinct ERIC-PCR DNA fingerprints were chosen for further identification by 16S rDNA sequencing. Among the five culture-positive catheters, all of them contained

specific member(s) of the *Staphylococcus* genus, including *S. aureus*, *S. epidermidis*, *S. hominis* and *Staphylococcus* sp., and one of the catheters possessed a *Streptococcus agalactica* strain (Table 4). However, 100% of the samples showed positive amplification with eubacterial universal primers in PCR analysis, indicating presence of bacteria (or bacterial DNA) on all the catheters (Table 3).

Buffer samples that were used to rinse blood residue from the catheters were also analyzed by the culturing methods, but none of the buffer samples showed any positive growth in the growth media. Again, all of the buffer samples showed positive amplification by eubacterial universal primers (data not shown).

3.3 DGGE analysis of catheter bacterial community

PCR amplification of the catheter samples by a pair of universal 16S rDNA primers (amplifying the V3 variable region of the 16S rDNA of eubacteria) produced amplicons at about 200 bp for all 24 catheters (Table 3). The DGGE profiles of the amplicons showed that every single catheter in this study contained bacterial DNA that belonged to a multitude of bacterial species (Figure 3). Despite the fact that the catheters were collected by more than one oncologist over a one-year span, the DGGE profiles were relatively similar and dominated by seven major DNA bands (C1, C2, C3, C7, C8, C22, C23) that were found in ≥ 40% of the 24 catheters. While other DGGE bands, such as, C11, C12, C13, C16 and C21, appeared only in limited number of the samples.

The major DGGE bands were excised, amplified and re-examined with the DGGE assay to confirm their purity prior to sequencing analysis. In order to

recover enough DNA from the DGGE bands for sequencing, individual DGGE bands were either cloned into a cloning plasmid or directly amplified from the excised bands. Both, cloning and direct sequencing from amplified DGGE band were performed on bands C1, C2, C8, C11, C21 and C22. Sequencing data from these bands showed that both approaches produced quality DNA sufficient for sequencing analysis (data not shown). Based on this finding, the rest of the DGGE bands were identified by direct sequencing.

All the DGGE bands chosen for DNA sequencing analysis showed high % of similarity (98 – 100%) to known DNA sequences deposited in the GenBank database (Table 5). Bands C1 and C2 were identified as *Enterococcus faecium* and *Enterococcus faecalis*, respectively, and they ware present in all catheter samples. Other dominant species included *Roseomonas* sp. (band C3), *Corynebacterium* sp. (band C7), *Serratia* sp. (band C8), *Staphylococcus epidermidis* (band C22) and *Staphylococcus hominis* (band C23). Bacterial species such as *Aerococcus* sp. (band C11), *Micrococcus* sp. (band C12), unculturable proteobacterium (band C13), *Staphylococcus aureus* (band C16) and *Escherichia coli* (band C21) were found in less than 40% of the catheter samples (Figure 5).

Buffer samples used to rinse blood residue from the catheters were also analyzed by the PCR-DGGE assay. Their DGGE profiles were similar to their catheter counterpart samples (Figure 4). However, the intensity of the DGGE profiles from the buffer samples was significantly weaker than that of the catheter samples, indicating a lower bacterial DNA density in the wash buffers. DGGE

bands of the wash samples were identified (Table 6). Similar to the catheter samples, dominant species (≥ 40%) were *Enterococcus faecalis*, *Enterococcus faecium, Roseomonas* sp., *Corynebacterium* sp., *Staphylococcus hominis*, *Serratia* sp. and *Staphylococcus aureus*. Other species including *Staphylococcus epidermidis*, *E. coli, Aerococcus* sp. and *Streptococcus agalactiae* were identified in the wash buffer samples (Figure 5).

3.4 Electron Scanning Microscopy

Biofilms were found in the lumens of all catheter samples, but rarely associate to the outer surface of the catheters. Various types of biofilm structure were observed on the surface of the catheter lumens (Figure 6), some with single cell-layer covered with either a thin or thick layer of extracellular polymeric substances (Figures 6A and 6B), some with multiple cell-layers (Figure 6C), and some with thick biofilm that had few bacterial cells visible on the matrix surface (Figure 6D). Some biofilms also contained both cocci and rod-shaped cells of various sizes (Figure 6E), indicating a mixture-culture contamination. In one of the catheter samples, bacterial cells in the biofilm were connected with thin tubular structures (Figure 6F). It is not clear if these tubular structures were conjugation pili or artifacts of extracellular polymeric substances.

Tables

Table 1. Clinical information about source of catheters

NN	Cancer Of	Reason for removal	BSI	Anti Bact-erial lock	Dermal Tunnel Inflammation Symptoms	Blood culture Repeated	Urine culture
BM	ND	E/T	Neg	yes	Neg	WNP	WNP
PM	ND	E/T	Neg	yes	Neg	WNP	WNP
HI	Lympho ma	BSI	Pos	yes	Neg	Four times/NG	WNP
JS	ND	E/T	Neg	yes	Neg	WNP	WNP
KD	Esophag us	BSI	Pos	yes	Pos/NG	Two times/NG	MF
PE	Breast	E/T	Neg	yes	Neg	WNP	WNP
SH	ND	E/T	Neg	yes	Neg	WNP	WNP
FT	Rectum	BSI	Pos	yes	Pos/NG	Two times/NG	NG
AR	Melano ma	E/T	Neg	yes	Neg	WNP	*Growth* [1] *E. coli*
DJ	ND	E/T	Neg	yes	Neg	WNP	WNP
GD	Myelom a	BSI	Pos	yes	Neg	Four times/NG	Growth [2] C.albicans
MR	Breast	BSI	Pos	yes	Pos/NG	Two times/NG	WNP
KK	Leukemi a	BSI	Pos	yes	Neg	Six times/NG	WNP
QN	ND	E/T	Neg	yes	Neg	WNP	WNP
WA	Breast	E/T	Neg	yes	Neg	WNP	WNP
JA	Breast	BSI	Pos	yes	Neg	Two times/NG	WNP
SA	Lympho ma	BSI	Pos	yes	Neg	Six times/NG	Twice NG
SN	Colon	BSI	Pos	yes	Neg	Three times S. maltophilia[3]	NG
DT	Colon	BSI	Pos	yes	Neg	Three times P. agglomerans[4]	NG
NN	Esophag us	BSI	Pos	yes	Neg	Two times/NG	WNP
KP	Rectum	BSI	Pos	yes	Neg	Two times/NG	MF
PL	Colon	BSI	Pos	yes	Neg	Two times/NG	WNP
KKw	ND	E/T	Neg	yes	Neg	WNP	WNP
BE	ND	E/T	Neg	yes	Neg	WNP	WNP

Abbreviations:

Samples (First column) were labeled by first letters of patients names, ***ND*** – No data, ***BSI*** – Blood Stream Infection, ***E/T*** – End of therapy, ***A/B lock*** – instillation of the catheter with antibiotic, ***Pos*** – positive presence of symptoms, ***Neg*** – Negative absence of symptoms, ***WNP*** – Was not performed, ***NG*** – No growth, "MF –"Mixed flora" – means no other data.

Growth detected:

[1] - *Escherichia. coli,* [2] - Clostridia albicans, [3] - Stenotrophomonas maltophilia, [4] - Pantoea agglomerans

Table 2. Bacteria detected in all samples by DGGE and culture method (part 1)

Bacterial species	BM			PM			HI			JS			KD			PE			SH			FT		
	So	Wa	Cu	So	Wa	Cu	So	Wa	Cu	So	Wa	Cu	So	Wa	Cu	So	Wa	Cu	So	Wa	Cu	So	Wa	Cu
Aerococcus spp.C11B11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Corynebacterium minutissimumC7B7	-	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-
Enterococcus faecalis C2B2	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	-	-	+	+	-
Enterococcus faeciumC1B1	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-
Escherichia coliC21B21	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Micrococcus luteusC12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Roseomonas genomospeciesC3B3	-	+	-	+	+	-	-	+	-	+	+	-	+	+	-	+	+	-	-	-	-	+	+	-
Serratia spp.C8B8	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-
Staphylococcus aureusC16B16	-	+	-	+	+	-	-	+	+	-	+	-	-	+	-	-	+	-	-	+	-	+	+	-
Staphylococcus epidermidisC22B22	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	+	+	-	-	-
Staphylococcus hominisC23	+	*	-	-	-	-	+	*	+	-	-	-	+	*	-	+	*	-	+	*	-	-	*	-
Streptococcus agalactiaeB24	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
Uncultured alpha proteobacteriumC13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Abbreviations:
Samples (top row) were labeled by first letters of patients' names, So - materials removed by sonication, Wo - materials removed by pipetting wash, Cu - culture esssay.

Table 2. Bacteria detected in all samples by DGGE and culture method (part 2)

Bacterial species	AR			DJ			GD			MR			KK			QN			WA			JA		
	So	Wa	Cu	So	Wa	Cu	So	Wa	Cu	So	Wa	Cu	So	Wa	Cu	So	Wa	Cu	So	Wa	Cu	So	Wa	Cu
Aerococcus spp.C11B11	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-
Corynebacterium minutissimumC7B7	-	-	-	+	+	-	+	-	-	+	-	-	+	-	-	+	+	-	-	+	-	-	-	-
Enterococcus faecalis C2B2	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	-	-	+	+	-	+	+	-
Enterococcus faeciumC1B1	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-
Escherichia coliC21B21	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Micrococcus luteusC12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Roseomonas genomospeciesC3B3	+	+	-	+	-	-	+	-	-	+	+	-	+	+	-	+	-	-	+	-	-	+	+	-
Serratia spp.C8B8	-	-	-	+	+	-	+	+	-	+	-	-	+	-	-	-	+	-	-	+	-	-	-	-
Staphylococcus aureusC16B16	-	-	-	-	+	-	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus epidermidisC22B22	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	+	-	+	-	-	-	+	-	-
Staphylococcus hominisC23	+	*	-	-	*	-	+	*	-	+	-	-	+	-	-	-	-	-	+	-	-	+	-	-
Streptococcus agalactiaeB24	-	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
Uncultured alpha proteobacteriumC13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-

Abbreviations:

Samples (top row) were labeled by first letters of patients' names, So - materials removed by sonication, Wo - materials removed by pipetting wash, Cu - culture esssay.

Table 2. Bacteria detected in all samples by DGGE and culture method (part 3)

Bacterial species	SA			SN			DT			NN			KP			PL			KKw			BE		
	So	Wa	Cu	So	Wa	Cu	So	Wa	Cu	So	Wa	Cu	So	Wa	Cu	So	Wa	Cu	So	Wa	Cu	So	Wa	Cu
Aerococcus spp.C11B11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Corynebacterium minutissimumC7B7	+	-	-	+	-	-	+	+	-	+	+	-	+	+	-	+	+	-	-	+	-	-	-	-
Enterococcus faecalis C2B2	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-
Enterococcus faeciumC1B1	+	-	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-
Escherichia coliC21B21	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-
Micrococcus luteusC12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Roseomonas genomospeciesC3B3	+	-	-	+	+	-	+	+	-	+	+	-	+	+	-	+	-	-	+	-	-	+	-	-
Serratia spp.C8B8	+	-	-	+	-	-	+	-	-	+	+	-	+	+	-	+	+	-	-	+	-	-	-	-
Staphylococcus aureusC16B16	-	-	-	+	-	-	+	-	+	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-
Staphylococcus epidermidisC22B22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	+	-	-
Staphylococcus hominisC23	-	-	-	-	*	-	-	*	-	-	-	-	-	*	+	-	-	-	-	-	-	-	-	-
Streptococcus agalactiaeB24	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Uncultured alpha proteobacteriumC13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Abbreviations:
Samples (top row) were labeled by first letters of patients' names, So - materials removed by sonication, Wo - materials removed by pipetting wash, Cu - culture esssay.

Table 3. Bacterial growth from materials removed by sonication.

Cat[1]	Aerobic[2]			Anerobic[3]			PCR[4]
	TSB/TSA[5]	MacConkey[6]	Blood[7]	TSB/TSA	MacConkey	Blood	
BM[8]	-	-	-	-	-	-	+
PM	-	-	-	-	-	-	+
HI	-	+	-	-	-	-	+
JS	-	-	-	-	-	-	+
KD	-	-	-	-	-	-	+
PE	-	-	-	-	-	-	+
SH	+	-	-	-	-	-	+
FT	-	-	-	-	-	-	+
AR	-	-	-	-	-	-	+
DJ	-	-	-	-	-	-	+
GD	-	-	-	-	-	-	+
MR	-	-	-	-	-	-	+
KK	-	-	-	-	-	-	+
QN	+	-	-	-	-	-	+
WA	-	-	-	-	-	-	+
JA	-	-	-	-	-	-	+
SA	-	-	-	-	-	-	+
SN	-	-	-	-	-	-	+
DT	+	-	-	+	-	-	+
NN	-	-	-	-	-	-	+
KP	+	-	-	-	-	-	+
PL	-	-	-	-	-	-	+
KKw	-	-	-	-	-	-	+
BE	-	-	-	-	-	-	+

Footnotes:

[1] – Cat abbreviation of "catheter"

[2,3] – Aerobic, Anerobic – description of growing condition

[4] – PCR – polymerase chain reaction was performed with 16S Universal forward and reverse primers producing 200 bp size amplicons. "+" means DNA product amplification was detected by horizontal gel electrophoresis

[5] – TSB/TSA – Tryptic-Soy Broth, Tryptic-Soy Agar

[6] – MacConkey agar

[7] – Agar with sheep blood

[8] – Hospital's coding system for removed catheters

[+] – bacterial colonies growth

Table 4. Origin of grown bacteria established by 16S rDNA sequencing

Cat[1]	Identity[2]	Growth conditions	Homology %[3]
KP	*Staphylococcus hominis*	Aerobic	100
DT	*Staphylococcus. sp..*	Aerobic/Anaerobic	100
QN	*Staphylococcus epidermidis*	Aerobic	100
SH	*Streptococcus agalactica*	Aerobic	100
SH	*Staphylococcus epidermidis*	Aerobic	100
HI	*Staphylococcus aureus*	Aerobic	100
HI	*Staphylococcus hominis*	Aerobic	100

Footnotes:
[1] – "Cat" – abbreviation of "catheter"
[2] – origin of bacterial DNA according to GenBank
[3] – percentage of similarity of nucleotide sequence in analyzed DNA and GenBank analog

Table 5. Bacterial DNA band origin defined by sequencing (removed by sonication)

Bands designation	Phylogenic affiliation[1]	Homology %[2]
C1	*Enterococcus faecium*	98
C1/2	*Enterococcus faecium*	98
C1/3	*Enterococcus faecium*	98
C2	*Enterococcus faecalis*	99
C2/2	*Enterococcus faecalis*	99
C2/3	*Enterococcus faecalis*	99
C2/4	*Enterococcus faecalis*	99
C2/5	*Enterococcus faecalis*	100
C3	*Roseomonas genomospecies*	99
C3/2	*Roseomonas genomospecies*	99
C7	*Corynebacterium minutissimum*	100
C7/2	*Corynebacterium minutissimum*	99
C8	*Serratia spp.*	100
C8/2	*Serratia spp.*	100
C11	*Aerococcus spp.*	98
C12	*Micrococcus luteus*	100
C13	*Uncultured alpha proteobacterium*	100
C16	*Staphylococcus aureus*	100
C16/2	*Staphylococcus aureus*	100
C21	*Escherichia coli*	100
C21/2	*Escherichia coli*	100
C21/3	*Escherichia coli*	100
C22	*Staphylococcus epidermidis*	100
C22/2	*Staphylococcus epidermidis*	100
C22/3	*Staphylococcus epidermidis*	100
C22/4	*Staphylococcus epidermidis*	100
C22/5	*Staphylococcus epidermidis*	100
C23	*Staphylococcus hominis*	99
C23/2	*Staphylococcus hominis*	99

Footnotes:

[1] – origin of bacterial DNA according to GenBank

[2] – percentage of similarity of nucleotide sequence in analyzed DNA and GenBank analog

Table 6. Bacterial DNA band origin defined by sequencing (removed by pipetting wash)

Bands designation	Phylogenic affiliation[1]	Homology %[2]
B1	*Enterococcus faecium*	99
B1/2	*Enterococcus faecium*	99
B2	*Enterococcus faecalis*	100
B2/2	*Enterococcus faecalis*	100
B2/3	*Enterococcus faecalis*	99
B3	*Roseomonas genomospecies*	99
B7	*Corynebacterium minutissimum*	100
B7/2	*Corynebacterium minutissimum*	100
B8	*Serratia spp.*	100
B11	*Aerococcus spp.*	98
B16	*Staphylococcus aureus*	98
B21	*Escherichia coli*	100
B21/2	*Escherichia coli*	100
B22	*Staphylococcus epidermidis*	100
B22/3	*Staphylococcus epidermidis*	100
B24	*Streptococcus agalactiae*	100
B24/2	*Streptococcus agalactiae*	100
B24/3	*Streptococcus agalactiae*	100

Footnotes:
[1] – origin of bacterial DNA according to GenBank
[2] – percentage of similarity of nucleotide sequence in analyzed DNA and GenBank analog

Figures

Figure 1. Comparison of CFU count under different levels of sonication power.

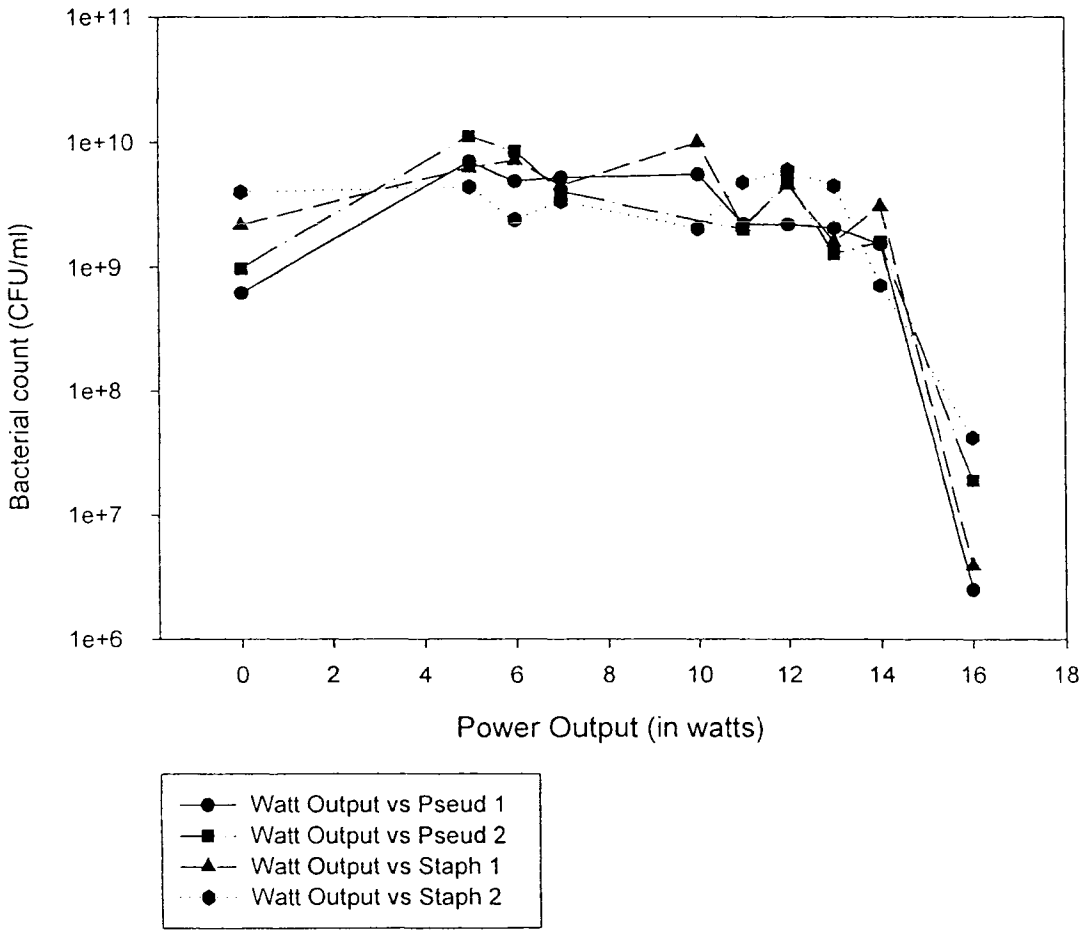


Figure 2a: Epi-fluorescent microscope picture of *P. putida* pure culture cells

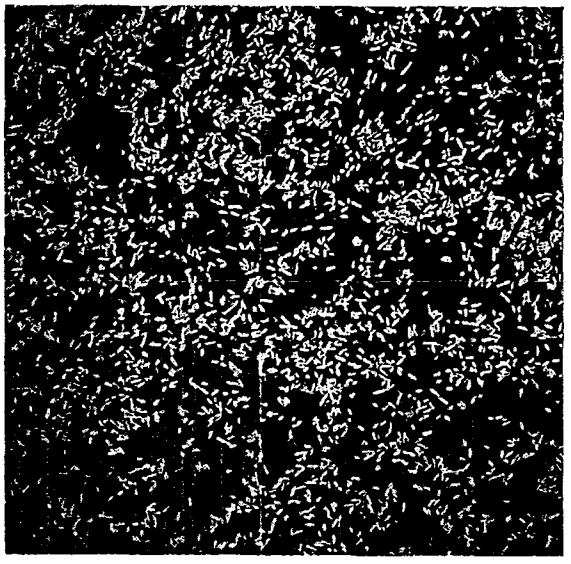


Figure 2b:Confocal microscope picture of *P. putida* biofilm crumbs grown for 72 hours on glass slides.

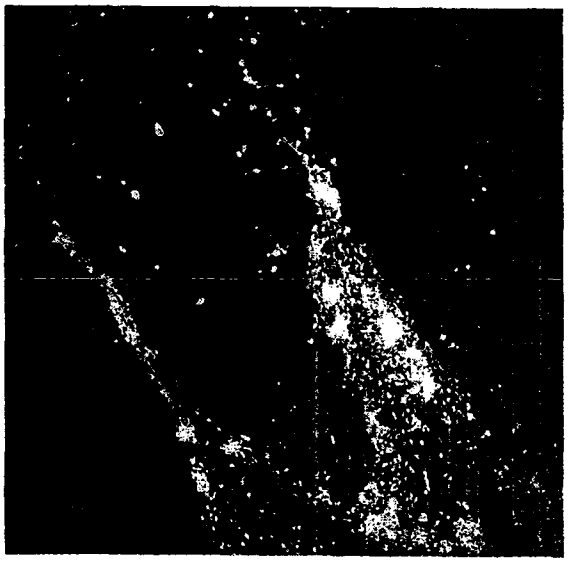
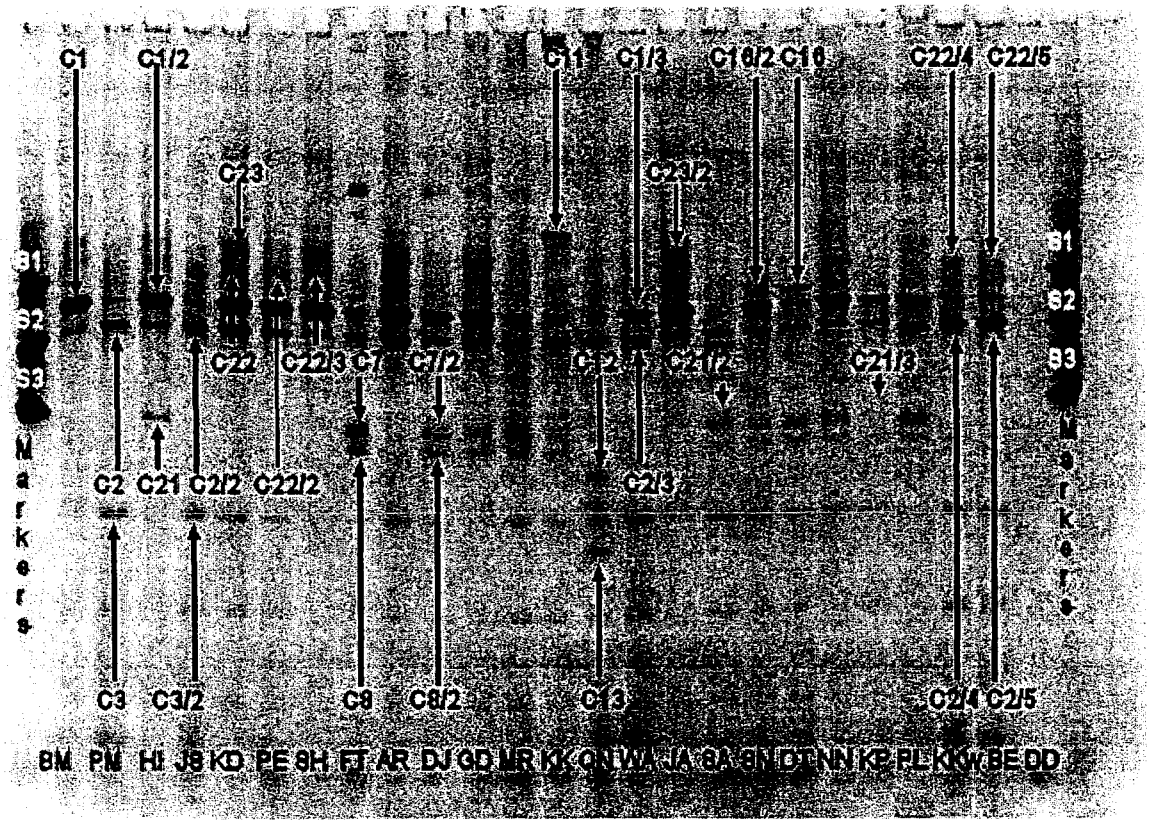


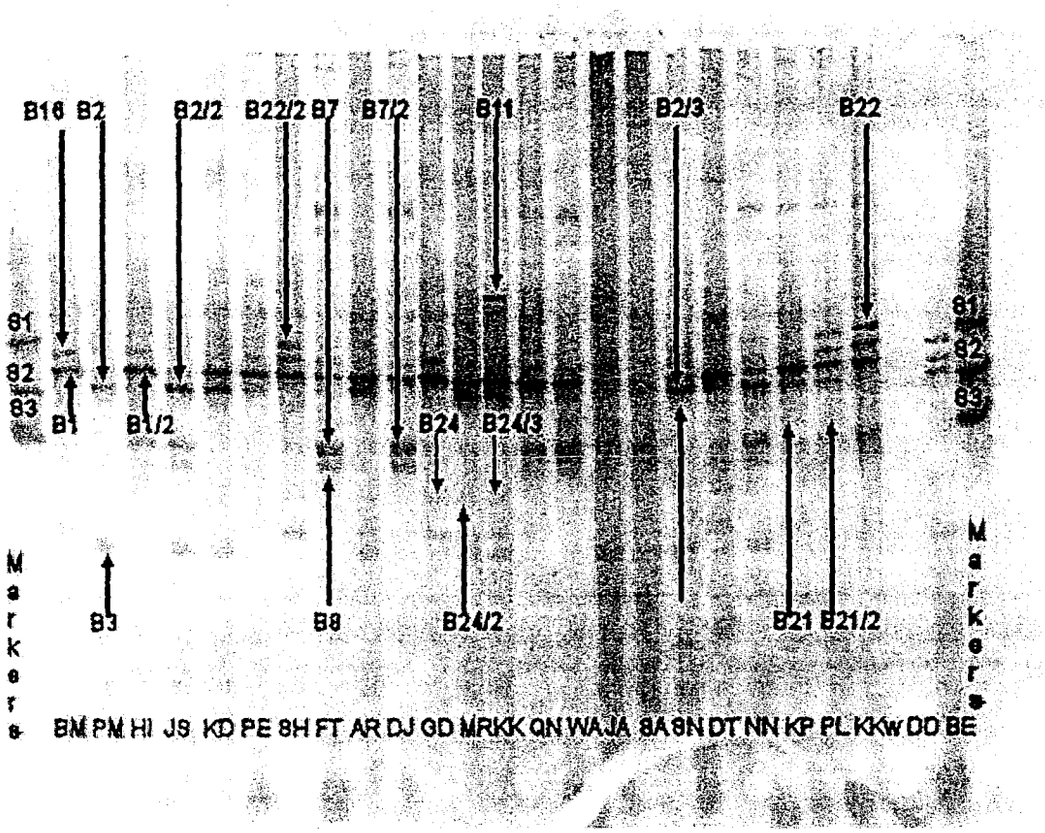
Figure 2c: Confocal microscope picture of a glass slide after removal of *P. putida* biofilm by sonication.

Figure 3. DNA bands in sonication removed materials



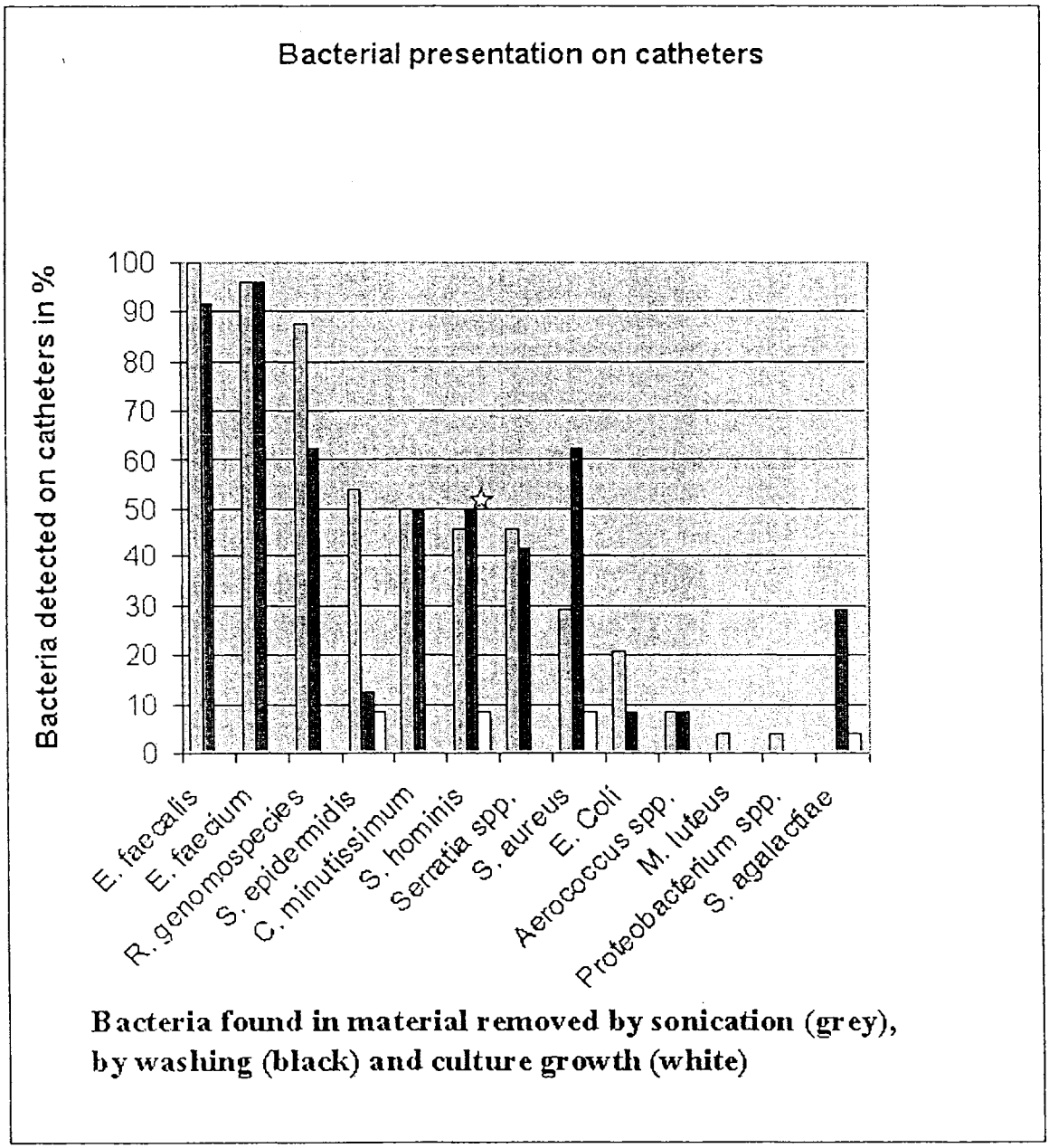
– S1 (*S. epidermidis*), S2 (*E. faecalis*), S3 (*E. coli*):labels bands of known bacterial strains DNA used as markers.
– Bands labeled with capital C correspond to DNA bands labeling shown in Table 5 of biofilm samples removed from catheters by sonication.
– Double capital letters label sample source by patient and also correspond to labeling shown in Table 5.

Figure 4. DNA bands in washing by pipetting removed materials



– S1 (*S. epidermidis*), S2 (*E. faecalis*), S3 (*E. coli*):labels bands of known bacterial strains DNA used as markers.
– Bands labeled with capital B correspond to DNA bands labeling shown in Table 6 of blood samples removed from catheters by pipetting wash.
– Double capital letters label sample source by patient and also correspond to labeling shown in Table 6.

Figure 5. Bacterial DNA detected in material removed by sonication, pipetting wash and culture.



Footnotes:

☆ – Amount of bands marked with star in blood samples (black bar, *S. hominis*) was counted visually based on bands' position in DGGE gel.

Figure 6. Bacterial biofilm on catheters

Figure 6A. Single layer biofilm.

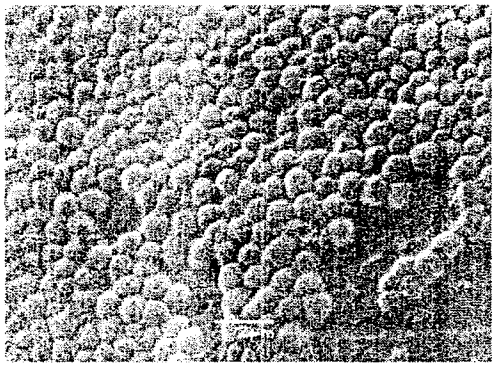


Figure 6B. Biofilm cells covered under EPS layer.

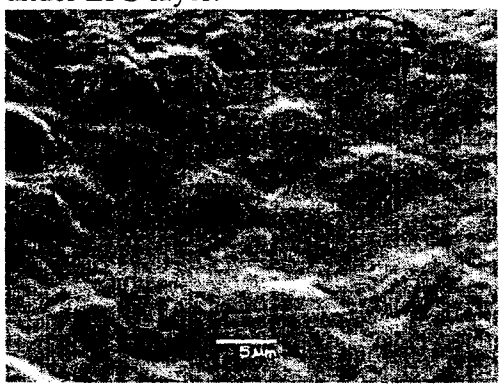


Figure 6C. Multilayer biofilm structure.

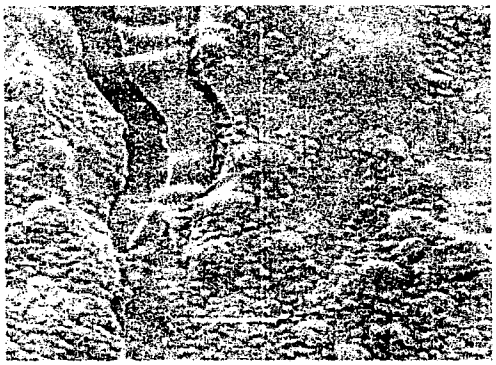


Figure 6D. Thick biofilm matrix without visible cells.

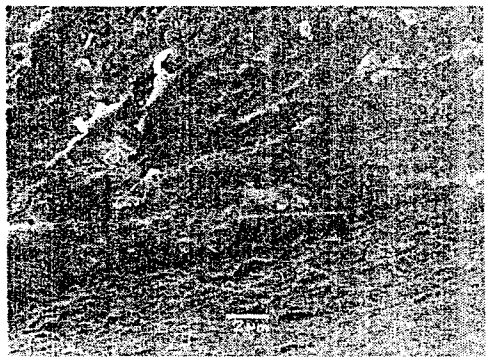


Figure 6E. Biofilm containing cells with various shapes and sizes.

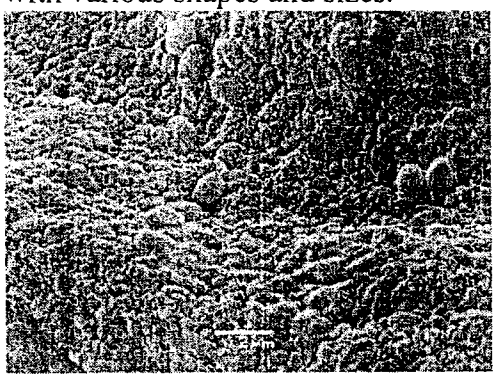
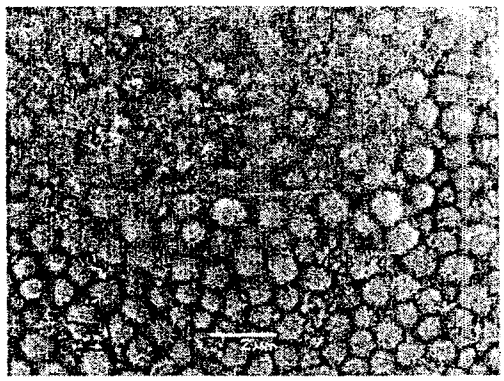


Figure 6E. Biofilm cells with intercellular tubular structures.



4. Discussion

Cultivation of microbial isolates from catheters has been the standard protocol to examine catheter related bacterial infections (Siegman-Igra *et al.*, 1997). However, it is common to have negative microbiological test results on catheters from patients who show symptoms of catheter-associated bacterial infections (Costerton *et al.*, 2003). Recently, PCR detection methods have been used to circumvent the short-coming of the conventional cultivation methods to diagnose microbial infections in clinical settings (Dobbins *et al.*, 2003). However, these methods target only the major bacterial pathogens that are commonly implicated for catheter-associated bacterial infections. The shortcoming of these methods is that they can miss potential pathogens that are uncommon or unanticipated in catheter related infections. To our knowledge, this study is the first attempt at examining bacterial community compositions on catheters that were used in chemotherapy for cancer patients, using the PCR-DGGE method.

Despite 100% of the catheters having tested positive for bacterial colonization by the PCR assay, only 5 catheters (21%) contained bacteria that could be recovered by cultivation methods. Our observations agree with others, in that only a small percentage of the suspected catheter-related bacterial infection patients produce positive microbiological cultures from their catheters (Collignon *et al.*, 1986; Maki *et al.*, 1977; Pinilla *et al.*, 1983). For instance, Nahass and Weinstein (1990) showed that only 20 (45%) of 44 catheters recovered septic patients were colonized by microorganisms. Because none of these studies have tested their samples with non-cultivation methods such as the

PCR or SEM methods, the assumptions were that these infections were either not catheter related, or were from some unknown sources. It is well known that bacteria produce copious amounts of extracellular polymeric substances to adhere on the medical devices as biofilms (Franson *et al.*, 1984). Besides being highly refractory to biocide treatments and resistant to environmental stresses, biofilm cells are usually at a resting phase (Lewis, 2001) and a portion of the biofilm cells also loses their ability to replicate in standard growth media despite their ability to respire (Yu and McFeters, 1994). In this study, I showed that cultivation methods are not effective in detecting some biofilm bacteria that colonize catheters.

Six out of the seven bacterial isolates recovered from the catheters were identified as members of the *Staphylococcus* genus. It is similar to the findings of Butt *et al.* (2004) that bacteria isolated from long-dwelling catheters used in cancer treatments had a high incidence of colonization by coagulate-negative staphylococci (73%). With a weakened immune system of patients undergoing chemotherapy treatments, it is not surprising to see high incidence of infection caused by staphylococci, which are part of the normal microflora that inhabit human skin. *Streptococcus agalactiae* is one of the most common causes of nosocomial infections that can also be related to catheter infections (Strampfer *et al.*, 1987).

Unlike most cultivation studies showing that contaminated catheters are colonized by one, or simple mixture, of microbial species (Maki *et al.*, 2001; Maki *et al.*, 1998), DGGE analysis showed that all the catheters in this study contained

a mixed culture of at least 4 bacterial species. This could be due to the fact that these catheters stayed in the patients for as long as 6 months.

Enterococcus faecalis (100%) and *E. faecium* (96%) were the most predominant species detected on the catheters. This disagrees with the many reports that *Staphylococcus* species are the most common bacterial agents causing catheter-related blood infections (Kloos and Bannerman, 1994; Frebourg *et al.*, 2000; Arciola *et al.*, 2001). Both *Enterococcus* species are gastro-intestinal bacteria thought to be innocuous to humans. Recently, they emerged as the major causes of sepsis in intensive care units and of immunocompromised patients in hospitals and nursing homes. Because enterococci can easily transmit from person-to-person through contaminated gloves or clothing, enterococci may be a more common catheter-related infection agent than I thought they were.

Roseomonas (88%) was the next dominant group of bacteria found on the catheters. It is a slimy, waterborne, Gram-negative coccobacillus and has recently been isolated from catheter and blood samples of cancer patients (Lewis et al., 1997). It has been considered primary or secondary pathogen causing high fever on infected patients. However, reported cases of *Roseomonas* infection have been relatively limited (Indra *et al.*, 2004). Our study shows that *Roseomonas* could be an important causal agent of catheter-related infections.

Other common catheter-related infection bacteria, such as *S. epidermidis*, *S. hominis*, *S. aureus*, *Corynebacterium* sp. and *Serratia* sp. were also found on the catheters of this study, ranging between 30-54%. However, other studies showed that 100% of catheters were colonized with *S. epidermidis* within 3

weeks of installation (Tenney *et al.*, 1986). Similar to other studies (Donlan *et al.*, 2001, Costerton *et al.* 1995), *E. coli*, *Aeroccous* sp., *Micrococcus* sp. and alpha proteobacterium were found on the catheters in low percentages.

Blood residues adhering on the surface the catheters were removed by rinsing. Neither culture nor DGGE assays from the wash samples did not match with the microbiological tests from blood samples obtained from the patients (Table 1). For instance, several bacterial isolates, such as *Pantoea agglomerans* and *Stenotrophomonas maltophilia*, were identified in the blood samples of some patients but none of these isolates were isolated from or identified in the DGGE profiles of the wash samples. However, the DGGE profiles of the wash samples and their corresponding catheters were almost identical. This indicates that the microbial communities obtained from the catheters were genuinely biofilm bacteria and not contaminations from the blood system. Since a small portion of the biofilm cells can be rinsed away from the catheters easily, it raises the possibility of collecting these loosely attached biofilm bacteria by drawing blood samples from suspected contaminated catheters. This may allow PCR-DGGE test on catheter biofilm samples without removal of catheters from patients.

The bacterial communities found on the catheters by the DGGE were significantly more diverse than the cultivation methods. Twelve major bacterial species (i.e. counting those that have been sequenced and identified) were detected by DGGE but only four bacterial species, including three *Staphylococcus* spp. and one *Streptococcus* sp., were isolated from the same set of catheter samples. The DGGE analysis of the catheter and wash samples

revealed all the bacterial cultures recovered from the catheters. However, some major species, such as *Enterococcus faecalis*, *E. faecium, Roseomonas* sp., *Corynebacterium* sp. and *Serratia* sp., that were detected by the DGGE were not recovered by cultivation methods. It is not known whether these bacterial species from the catheters are not adjusted to the growth media and conditions used in this study, or they are more susceptible to become "viable but non-culturable".

Scanning electron microscopy confirms the DGGE findings that biofilms were found on all catheter samples. Absence of biofilm on the external surface of a catheter can be explained by the fact that bacteria on the outside surface of the catheter are exposed and prone to host cellular and humoral immune systems. While inside the catheter lumen, there is limited possibility for the immune system to destroy the biofilm. Furthermore, blood proteins, water and other nutrients can diffuse into the lumen of a catheter providing essential conditions for bacterial growth. Colonization inside the lumen of a catheter can occur rapidly and result in a coagulation cascade inside the catheter (Maki, 1994).

This study shows that the PCR-DGGE method is a superior method in identifying microbial communities on catheters and has revealed some potentially important catheter-associated pathogens (*Enterococcus*, *Roseomonas* spp. and *Serratia* spp.). It also shows that only 13 out of the 24 cancer patients who carried the biofilm-associated catheters exhibited symptoms of infection. However, further investigations are required to determine if catheter-associated infections are controlled by (1) detachment of biofilm bacteria, (2) composition of

the biofilm bacteria, (3) expression of pathogenic genes, and/or (4) strength of the patient's immune system.

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