Molecular investigation of bacterial communities on inner and outer surfaces of peripheral venous catheters

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Running title: Bacterial diversity on peripheral venous catheters.

Abstract

Peripheral venous catheters (PVCs) are some of the most widely used medical devices in hospitals worldwide. PVC-related infections increased morbidity and treatment costs. Inner surfaces of PVCs are rarely examined for population structure of bacteria as it is generally believed that bacteria at this niche are similar to those on the external surface of PVC. We primarily test this hypothesis and also study the effect of antibiotic treatment on bacterial communities from PVC surfaces. Inner and outer surfaces of PVCs from 15 patients were examined by 454 GS FLX Titanium 16S rRNA sequencing and the 'roll-plate' culture method. None of the PVCs were colonized according to the culture method and none of patients had a bacteraemia. From a total of 127,536 high quality sequence reads, 14 bacterial phyla and 268 diverse bacterial genera were detected. The number of Operational Taxonomic Units for each sample was in the range of 86-157 even though 60% of patients had received antibiotic treatment. *Stenotrophomonas maltophilia* was the predominant bacterial species in all examined PVC samples. There were noticeable but not statistically significant differences between the inner and outer surfaces of PVCs in terms of the distribution of the taxonomic groups. In addition, bacterial communities on PVCs from antibiotic treated patients were significantly different from untreated patients. In conclusion, surfaces of PVCs display complex bacterial communities although their significance is yet to be determined, these findings alter our perception of PVC-related infections.

Key words: Peripheral venous catheters; Catheter-related infections; Bacterial community; 16S rRNA gene sequences

1 **Introduction**

2 Peripheral venous catheters (PVCs) are vital for the administration of fluids and medications
3 and for multiple blood access. In the USA, it is estimated that 25 million PVCs are inserted
4 each year, accounting for 90% of all venous catheters [1]. PVC-associated infection is
5 considered to be implicated in 5% of cases of nosocomial bacteraemia which has a
6 prevalence of 670 per 100,000 patients with PVCs [2]. Although PVC-related infections may
7 not result in increased mortality, they contribute considerably to the burden of the health-care
8 system by increasing morbidity and treatment costs [1,3,4].

9 Intravenous catheter insertion provides a portal of entry for bacteria to cross from an unsterile
10 external environment to the normally sterile blood [5]. The precursor to bloodstream
11 infection (BSI) is usually device colonization; bacteria found adhering to the extra or intra
12 luminal surfaces of catheters are the principal source and cause of BSIs [6,7]. Post-
13 attachment, many microbial communities form biofilms [8,9]. Because of increased
14 resistance to antibiotics, the bacteria within biofilms are difficult to treat effectively [10].

15 Detection and identification of the bacteria found on intravascular catheters, including PVCs,
16 has relied on culture-dependent techniques such as the ‘roll-plate’ [11]. This method involves
17 culturing the external surface of the catheter tip by rolling a segment (2-3 cm) of the
18 intravascular catheter four times over the surface of a blood agar plate with sterile forceps.
19 However, the ‘roll-plate’ technique **fails** to detect bacteria on the inner surface of catheters
20 and does not identify fastidious bacterial species. Moreover, bacteria identified from the
21 positive cultures are rarely found to be associated with BSI [12-14]. As for the inner surfaces
22 of short-term catheters, they are generally not examined for colonization as it is presumed
23 that bacteria first colonize the outer surface before colonizing the inner surface, and that the
24 composition of bacterial population at these two surfaces are similar. However, the

availability of nutrition, temperature, oxygen tension, exposure to antibiotics and infusion fluids, and fluid dynamics are factors that could contribute to differences in the micro-environment at the inner and outer surfaces of PVCs. This may reflect in changes in bacterial composition at both PVC surfaces. The aim of this study **was** to provide data to test this hypothesis and to examine the effect of antibiotic treatment on microbial communities at PVC surfaces.

Materials and methods

Hospital setting and study population

After obtaining institutional ethics approval and patients' informed consent, the study was conducted from April 2008 to October 2009 at three teaching hospitals in Queensland, Australia. PVCs (Insyte™ Autoguard™ BD Medical, Franklin Lakes) were inserted by hospital nurses or doctors and cared for in accordance with usual hospital practice. Skin was decontaminated with 2% chlorhexidine gluconate in 70% ethanol before insertion of PVC. Transparent, semi-permeable dressings at the insertion site (Tegaderm™ 1624W, 3M, St Paul) were maintained by nurses according to hospital protocol.

When the PVC was no longer required, the nursing staff removed the PVC after treating the insertion site with 2% chlorhexidine in 70% ethanol. **Fifteen PVC samples were taken by qualified registered nurses with experience in preparation of specimens for culture.** The distal 2-3 cm of the tip was cut using sterile scissors and deposited in a sterile container. All PVC tips were handled under aseptic conditions and immediately transported to the laboratory for examination, where they were cultured by the semi-quantitative method [11].

Culture method

This method is based on rolling a segment, usually the tip, of the removed PVCs back and forth on 5% sheep blood agar plates (Oxoid, Australia). The plates were incubated at 37 °C

under aerobic conditions for 2–4 days. Microorganisms were then isolated and identified according to standard microbiology protocols. The PVC tip was considered colonized if the plate grew ≥ 15 colony forming units (cfu).

Molecular techniques

In order to separate the bacterial communities of inner and outer PVC surfaces, 1cm at both ends of PVC tips were carefully heat-sealed. PVC tips were placed in 1.5 ml microcentrifuge tubes and gently sonicated to release the bacteria from the surfaces of the PVCs. Lysis buffer containing 20 mg/ml lysozyme, 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1.2 % Triton was added and incubated at 37 °C overnight. After that, PVC tips were taken out and bacterial DNA from outer surfaces of PVCs was collected. DNA extraction was continued using QIAamp DNA mini kit (Qiagen, Australia) according to the manufacturer's instructions.

In order to extract bacterial DNA from the inner surface, the external surface of the PVC section was washed and disinfected with ethanol, both ends of PVC tips were cut and 200 μ l of lysis buffer passed through. The PVC fragment was incubated at 37 °C overnight. Bacterial DNA from inner surfaces was collected and extracted using QIAamp DNA mini kit (QIAGEN, Australia) according to the manufacturer's instructions. A new PVC was suspended in lysis buffer and DNA was extracted as above for a negative control. 16S rRNA genes were amplified from purified genomic DNA using the primers F (5' AGA GTT TGA TCC TGG CTC AG 3') and R (5' GAG TTT GAT CCT GGC TCA G 3') which would cover variable regions (V1, V2 and V3). Thirty different self correction barcodes were designed and added to primers. For each 25 μ l reaction, conditions were as follows: 3 μ l of DNA template (concentration ranged from neat to 1:10³), 2.5 μ l of 10 $\times$ reaction buffer containing 20 mM MgCl₂, 2 μ l of 25 mM dNTPs, 1 μ l of each primer (10 μ M), 0.1 U of Taq DNA polymerase (Qiagen, Australia), 5 μ l of 5 $\times$ BSA and 10.4 μ l of sterile deionized water. Each

PCR run contained a negative control (sterile deionized water instead of template DNA) and a positive control (*E. coli* instead of template DNA). For each DNA sample, three replicate PCRs were performed.

PCR products were cleaned using Qiaquick PCR Purification kit (Qiagen, Australia). These were sequenced unidirectionally in the reverse direction by means of the Genome Sequencer FLX (GS-FLX) system (Roche, Australia) at 454 Life Sciences.

Informatics analysis

Pyrosequence reads were analysed using QIIME package (Quantitative Insights Into Microbial Ecology) as follows [15]. Briefly, bar coded 16S rRNA gene sequences were inputted into QIIME, then sequences were classified using the Ribosomal Database Project classifier, and sequences were aligned and chimera checked using Pynast and ChimeraSlayer. UniFrac distances were calculated, and data generated for summarizing the proportions of taxonomy and Principle Coordinates Analysis (PCoA) plots. All sequences were assigned to phylotypes (Operational Taxonomic Units, OTUs) at 97% sequence identity thresholds and 80% confidence was used in the QIIME-based wrapper of the RDP classifier program against the RDP core set. The Chao1 microbial richness estimate and overall community diversity (Shannon-Weaver index) were computed from the OTU data. Rarefaction curves were generated by plotting the number of observed genera versus the number of sequences sampled. A heatmap was generated on the basis of the relative abundance of OTUs using R version 2.15 (The R Project for Statistical Computing). PCoA was carried out based on theta-similarities of the relative abundance of the OTU in each sample. The top bacterial OTUs were selected after ranking their separability measured as the variance between classes using principal component analysis with respect to instrumental variables in R version 2.15 [16]. Therefore, bacterial OTUs which contribute to the significant difference in compared

bacterial groups were shown in box plots and two tailed *t*-Test was used to evaluate the difference between PVC groups. The Monte Carlo permutation test was used to test the statistical significance of the relationships bacterial communities on PVCs between patients with antibiotics and without antibiotics.

Results and Discussion

Sample characteristics

The 15 patients of whom 60% were females had a median age of 58 years. All PVCs were short-term and duration of PVC left in situ varied from 2-4 days. Approximately 80% of PVCs were in the forearm and 60% of patients were receiving systemic antibiotic therapy during the observation period. **No local signs of inflammation were noticed.** No patients were suspected or diagnosed with PVC-related BSI. According to the ‘roll-plate’ culture results, none of PVCs were colonized as no bacteria were grown on Columbia blood agar plates. In this study bacterial DNA was extracted from all PVC samples although they were “non-colonized” as defined by the ‘roll-plate’ method. These DNA samples were amplified and used for constructing pyrosequencing libraries. No bacterial DNA was detected and amplified from negative control PVCs. These control results indicate that the contribution to the bacterial community from manufacture of PVCs, DNA extraction procedures and PCR reagents is negligible.

Features of the bacterial community on PVC

Sequence lengths outside bounds of 150 and 600, ambiguous bases exceeding limits, missing or mean quality score below a minimum of 25, maximum homopolymer run exceeding a limit of 6, and mismatches in primers exceeding limits of 1 were discarded. Of the 16S rRNA gene sequences generated, 127,536 high-quality sequence reads from 30 samples (15 outer- and inner- surfaces of PVCs) passed the set threshold, and were used for further analysis.

Chimera checks showed that all sequences were unlikely to be chimeric. The average number of sequences per sample was 4251 (range 1413-10957) and average base-pair in length was 496 (range 150-599).

Overall, microbial communities on PVCs had a high diversity and complex community structure. 16S rRNA gene sequences were assigned to fourteen different bacterial phyla: Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Firmicutes, Fusobacteria, Gemmatimonadetes, Planctomycetes, Proteobacteria, TM7, Thermotogae and unclassified bacteria (Fig. 1a). The predominant phylum was Proteobacteria, including Alphaproteobacteria (4% of 16S rRNA reads), Betaproteobacteria (8.4%) and Gammaproteobacteria (68%). Four phyla (Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria) represented, on average 98% of individual bacterial communities on PVC surfaces. In all, 140 OTUs were from unclassified bacteria. All examined sequences were assigned to 1026 OTUs (predominant OTUs were shown in Fig. 2). A high number of OTUs was observed for each sample, ranging from 86 to 157. Sample richness was between 119 and 268, and the overall diversity (Shannon-Weaver index) was in the range of 2.24 and 3.64.

Bacterial diversity was very complex when examined using pyrosequencing analysis.

The limitations of culture methods to determine bacterial communities from environmental and clinical samples have been reported previously [17]. Several possible explanations for the discordant results from the two methods included: 1) antibiotic usage in patients which are common for acute hospital patients [18-21]; 2) sonication as part of the protocol in molecular methods may help detachment of bacteria in biofilms; 3) some bacteria are more easily cultured than others; 4) DNA based molecular methods might detect DNA from dead bacteria. Overall, the molecular methods provide much greater sensitivity than culture methods for this work.

Comparisons between bacterial communities on inner and outer surfaces of PVCs

Difference in Shannon-Weaver diversity indices between bacterial communities on inner and outer surfaces of PVCs was not significant, with $p=0.365$ ($H_{\text{Inner surface}}=3.05\pm0.35$, $H_{\text{Outer surface}}=2.95\pm0.22$). Similarly, results from PCoA suggested that bacterial communities on inner and outer surfaces of PVCs had no statistically significant difference, as they did not separate. There were noticeable differences between the inner and outer surfaces of PVCs in terms of the distribution of the taxonomic groups. For example, PVC inner surfaces had higher abundance of Firmicutes, Gemmatimonadetes, Planctomycetes and TM7 than the outer surfaces (Fig. 1b). More Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria and Proteobacteria were detected on the outer surfaces of PVCs than on the inner surfaces. Chloroflexi and Deinococcus-Thermus were only present on the inner surfaces, and conversely, Fusobacteria and Thermotogae were only present on the outer surfaces of PVCs. It is unknown whether those low abundance bacteria play a significant role in PVC-related infections or not and future research **is** required to verify this. Although these results indicate variations in bacterial diversity, they are at very low abundance. No significant difference was **found** between the two groups.

Effect of antibiotic treatment on bacterial communities of PVCs

There were 12 PVC samples (inner and outer surfaces of PVCs) from six patients without antibiotic treatment. We compared bacterial communities on these to the 18 PVC samples from nine patients treated with intravenous antibiotics to those from untreated patients. Results from PCoA (Fig. 3), Shannon-Weaver diversity index ($H_{\text{Antibiotic}}=2.85\pm0.30$, $H_{\text{Noantibiotic}}=3.13\pm0.21$; $p=0.005$) and Monte Carlo permutation test (Fig. 4) showed significantly different bacterial communities on PVCs from patients that were untreated and treated with antibiotics in the two weeks prior to PVC collection. The analysis found that

PVCs from patients without antibiotic use were associated with a significantly higher abundance of *Thermomonas fusca*, *Flavobacteriaceae*, *Stenotrophomonas maltophilia*, *Brevibacterium sp.*, *Chryseobacterium sp.*, *Streptococcus suis* and *Leucobacter sp.* *Stenotrophomonas* were the predominant bacteria on all examined PVCs (Fig. 5). However, *Stenotrophomonas* abundance in patients with antibiotic treatment was dramatically decreased compared with patients **who were not treated with antibiotics** and it was shown in Fig. 5. PVCs from patients with antibiotic treatment had a significantly higher abundance of *Phyllobacterium sp.*, *Propionibacterium acnes*, *Acidovorax sp.* and *Escherichia coli* (Fig. 5). Results from this study show that antibiotic therapy reduces bacterial diversity and alters the structure of bacterial communities-reducing some bacteria while selecting for others. More attention should be paid on bacterial communities of PVCs from patients with antibiotic treatment since they have great potential to resist antibiotics currently used and might be difficult to manage in the future infections. Larger sample numbers generally have greater statistical powers. In practice, however, considering the time consuming and money spending, it could be extremely difficult to include a huge number of samples in a study. In this study, 30 samples were reported to indicate the statistical trends that larger clinical trials might exhibit.

Bacteria identified in PVCs

Bacterial species such as *Staphylococcus spp.*, *Streptococcus spp.*, and *Pseudomonas spp.* are well known as the major causes of colonization and related BSI [22,23], and they were also present on **examined PVCs** in this study. Nikkari *et al.* examined bacteria in venous blood from four healthy individuals [24]. They examined 192 clones and found *Pseudomonas fluorescens* (77%), *Acidovorax defluvii* (17%), *Propionibacterium acnes* (2%), *Staphylococcus epidermidis* (2%), *Stenotrophomonas maltophilia* (1%) and *Riemerella anatipestifer* (1%) [24]. The origins of these bacteria may be skin and/or blood [24]. The

1 above study, despite some limitations, suggests the presence of bacterial communities in the
2 blood of healthy people. All bacterial species found in blood microbiota were also identified
3 in our study. These species are presumably cleared efficiently by the healthy reticulo-
4 endothelial system, but may be given the opportunity to colonize and persist on vascular
5 catheters when they are present. Specific conditions that then may lead to the establishment
6 of infection are yet to be determined.

7 The pyrosequencing library was dominated by *Stenotrophomonas maltophilia*, **an**
8 **opportunistic pathogen** that can cause serious infection and bacteraemia in critically ill
9 patients. The treatment of these can be difficult because *Stenotrophomonas maltophilia* is
10 intrinsically resistant to many commonly used antimicrobial agents including most
11 cephalosporins, penicillins and quinolones [25-27]. *Stenotrophomonas maltophilla* **has** been
12 previously reported as the predominant species on arterial catheters [28].

13 It was noted that PVC samples with an increase in the proportion of *Staphylococcus*
14 (Firmicutes) showed a decrease of *Stenotrophomonas* (Gammaproteobacteria) at the same
15 time. A similar shift of bacterial composition was also seen in *Propionibacterium*
16 (Actinobacteria) and *Stenotrophomonas* (Gammaproteobacteria). These findings raise the
17 possibility that low level colonization of PVCs is the norm and changes in bacterial
18 composition favouring the expansion of population of pathogens or opportunity pathogens
19 such as *Staphylococcus spp.* might trigger the process of clinically significant bacterial
20 colonization and BSI. Further investigation should be undertaken to determine whether these
21 potential pathogens represent non-pathogenic variants or simply do not cause infection
22 despite being present on PVC samples.

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6 **Conflict of Interest**

7 All authors declare no conflicts of interest relevant to this article.

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Figure legends

Fig. 1. Microbial phyla detected in (a) PVC samples, and (b) outer surfaces and inner surfaces of PVCs. A represent inner surface of PVCs and B represents outer surfaces of PVCs. Each column represents one sample.

Fig. 2. Heatmap show the relative abundance of bacterial genera across PVC samples of 15 patients. A represents inner surface of PVCs and B represents outer surfaces of PVCs. Each column represents one sample.

Fig. 3. The PCoA plot of bacterial communities on individual PVC sample as determined using UniFrac. AN represents PVC samples from patients with antibiotic treatment. NOAN represents PVC samples from patients without antibiotic treatment.

Fig. 4. Monte Carlo permutation analysis of bacterial communities on PVCs from patients with and without antibiotic treatment. Red ball represents with antibiotic treatment and green ball represents no antibiotic treatment.

Fig. 5. OTUs are significantly different on PVCs from patients with and without antibiotic treatment ($P < 0.05$). AN represents with antibiotic treatment and NOAN represents no antibiotic treatment.

Fig. 1a

Fig. 1b

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.