Molecular investigation of bacterial communities on intravascular catheters: No longer just Staphylococcus

Molecular investigation of bacterial communities on intravascular catheters: not just *Staphylococcus* anymore

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Running title: Bacterial communities on intravascular catheters.

Abstract

Intravascular catheter-related bloodstream infections (IVC-BSIs) are associated with significant morbidity and mortality. Culture-independent molecular approaches can reveal and capture the composition of complex microbial communities, and are now being used to reveal "new" pathogens as well as the polymicrobial nature of some infections. Patients with concurrently sited arterial and central venous catheters whom had clinically suspected IVC-BSIs, were examined by high-through put sequencing of microbial 16S rRNA. An average of 100 operational taxonomic units (OTUs, phylotypes) was observed on each IVC, indicating that IVCs were colonized by complex and diverse bacterial communities. *Ralstonia* (53% of 16S rRNA sequences), *Escherichia* group (16%), *Propionibacterium* (5%), *Staphylococcus* (5%), and *Streptococcus* (2%) were the most abundant genera. There was no statistically significant difference in the bacterial communities examined from arterial and central venous catheters; from those with and without systemic antibiotic treatment; or from conventionally colonised and uncolonised IVCs. The genome of the predominant bacteria, *R. pickettii* AU12-08, was found to encode resistance to antimicrobial drugs of different classes. In addition, many encoded gene products are involved in quorum sensing and biofilm formation that would further contribute to increased antimicrobial drug resistance. Our results highlight the complex diversity of microbial ecosystems on vascular devices. High-throughput sequencing of 16S rRNA offers an insight into the pathogenesis of IVC-related infections, and opens up scope to improve diagnosis and patient management.

Keywords: intravascular catheter; catheter related bloodstream infections; biofilm; antibiotic resistance; high-throughput sequencing.

Introduction

Intravascular catheters (IVCs), including central venous catheters (CVCs) and arterial catheters (ACs), are the most frequently used invasive medical devices in hospitals [1]. However, IVCs are associated with life threatening bloodstream infections (IVC-BSIs) which have high associated rates of morbidity, mortality and additional personal and medical costs [2,3].

Bacteria cause IVC-related infections when they gain access to the surface of IVCs and establish biofilm, allowing sustained surface colonization and ultimately, dissemination into the bloodstream leading to IVC-BSI. Early detection and adequate treatment of causative pathogens is critical for a favourable outcome, yet the majority of patients with a suspected catheter-related infection yield negative diagnostic investigations, necessitating empiric, rather than optimal, antimicrobial therapy [4].

Unfortunately, less than 1% of bacteria in nature can be recovered using culture-based methods [5]. Our previous studies have shown that many fastidious bacteria on medical devices, potentially responsible for sepsis, are not diagnosed using current standard culture examination as used in hospital laboratories [6]. Thus the most frequently isolated bacteria might not be the dominant bacteria on colonised IVCs, or, indeed be responsible for many patients' clinical conditions. The next generation sequencing now provides a key approach by which to examine these complex microbial communities in a high-throughput manner. Importantly, these approaches can reveal, and capture, the genetic potential present in complex microbial communities without having to isolate and culture the microorganisms. The cultivation-independent molecular approaches are now being used to reveal "new" pathogens, as well as the polymicrobial nature of some infections [7,8].

Current strategies for control of IVC-BSI have focused more heavily on CVCs than ACs [9-11]. However, the rate of IVC-BSIs arising from ACs has recently been demonstrated to be

comparable to that of CVCs [12-14], thereby rendering ACs an important device to study. Little is known as regards to the microbial colonisation of CVCs and ACs sited concurrently in the same patient as is a common situation in the intensive care unit (ICU) patient where most patients need both IVCs. Despite the differing circulations and use of the two device types, it is possible that bacteria which colonize one device may also colonize other concurrently sited IVCs, in similar fashion, since the skin is the common portal of entry. Because CVCs have been considered a higher infective risk than ACs, for patients with suspected IVC-BSI, clinicians may remove the CVC but not the AC. Even if both catheters are removed, it may be that the CVC is only sent for investigation, despite the AC contributing equally to the infection, and being important for diagnosis and treatment. The main aim of this study was to test this hypothesis and evaluate the bacterial profiles on the surfaces of both ACs and CVCs removed concurrently from patients suspected of IVC-BSIs.

Materials and Methods

Hospital setting and study population

The study was carried out in the ICU of the Royal Brisbane and Women's Hospital (RBWH), Queensland, Australia. This is a multi-disciplinary adult ICU that treats all conditions except cardiac surgical and solid organ transplant. Fifteen adult patients (18 years of age or older) with concurrently sited ACs and CVCs and with clinically suspected IVC-BSI were recruited for the study (Table 1). Ethical approval for the study was granted by the RBWH Human Ethics Board and Griffith University Human Research Ethics Committee. Written informed consent was provided by participants or their representatives.

IVCs were inserted after skin decontamination with chlorhexidine in alcohol, by experienced ICU medical staff using a Seldinger approach according to accepted guidelines for the prevention of IVC-BSI [11]. All CVCs were ARROWgard Blue® (chlorhexidine acetate and

silver sulfadiazine coated) (Arrow Int, Inc, Reading, PA, USA), and ACs were Vygon Leader Cath brand (Ecouen, France). There was no imposed limitation on dwell time, and resite of catheters always occurred at a new body site. Guide-wire exchange was not performed. Dressings and administration sets were maintained by ICU nurses using unit protocols in accordance with guidelines [11]. If the attending intensive care specialist strongly suspected that the IVC (either AC or CVC) could have been the cause of patient's signs and symptoms, then both IVCs were removed and either replaced at a new site, or an alternative IV access (e.g. peripheral vein) was sought. Diagnosis of IVC-BSI was made using conventional methods [11].

IVC samples were cultured using the roll plate technique, and an IVC sample was considered colonised if >15 cfu (colony forming unit) were isolated [15]. Microorganisms were then isolated and identified according to standard hospital protocol. Data collected included APACHE II score for severity of illness, patient demographics, antimicrobial use, catheter dwell time and reason for removal, ICU and hospital length of stay.

High-throughput sequencing of 16S rRNA

Following processing for culture, catheter tips were suspended in 200 µl of lysis buffer, which contained 20 mg/ml lysozyme, 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1.2% Triton, and Proteinase K at 37 ^{o}C overnight. Bacterial genomic DNA was extracted from all IVCs using the QIAamp DNA mini kit (Qiagen, Australia). For each catheter, a control (unused) catheter was taken from the original packaging and rolled back and forth on blood agar plates, with bacterial DNA extracted as above. 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' CTG CTG CCT CCC GTA G 3') which would cover two variable regions (V1 and V2). Thirty different self correction barcodes were designed and added to primers. For each DNA sample, three replicate PCRs were performed. PCR products were cleaned through 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.

Bioinformatics

Raw 16S rRNA sequence reads were trimmed as follows. The base quality of reads tends to decrease towards the 3'-prime end. To remove low quality 3'-ends, reads with an average base quality below 25 were trimmed from the 3'-prime end until their average quality score was above 25. The base quality of reads also considerably decreases after the first ambiguous character ('N') (data not shown). Therefore, reads containing ambiguous characters were additionally trimmed at the occurrence of the first 'N'. Subsequently, low quality reads were identified and excluded using criteria adapted from Huse *et al.* 2007 [16].

All reads were assigned to Bergey's bacterial taxonomy using the RDP classifier and iteratively grouped into Operational Taxonomic Units (OTUs) based on their best BLAST-hit to full-length reference sequences from the RDP database. All reads with a best-BLAST hit with a sequence-identity > 98%, were assigned to OTUs. Subsequently, all remaining reads with a best-BLAST hit, with a sequence-identity > 95%, were then assigned to OTUs. The remaining reads were assigned to OTUs analogously using identity cut-offs of 90% and 80%. 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. Coordinates analysis (PCoA) was carried out in R based on theta-similarities of the relative abundance of the different genera in each sample. Sequences were chimera checked using ChimeraSlayer. Anosim, Adonis and PERMDISP2 were calculated in Calypso. Anosim was run with Jaccard distance as dissimilarity measure and patient as grouping. PERMDISP2 and Adonis were run

for the OTU relative abundance matrix with patients as grouping. The two tailed *t*-Test was used to evaluate the difference between variances.

Genome sequencing of *Ralstonia pickettii* AU12-08

To maximize the recovery of bacteria, small pieces of IVC samples were cut and washed by PBS and incubated in medium M_{10} for 24 hours [17]. The solutions were then diluted in a series (neat, 1:10, 1:50, 1:100) on Mueller-Hinton plates. As many different colony types as visually distinguishable, were picked up, purified, and stored in glycerol at -80ºc. Bacterial DNA was released from bacterial cells by boiling, and one microliter was used as a template in PCR amplification. Purified PCR products were then sequenced, and compared to NCBI GenBank database using BLAST program. The most predominant bacterial species identified in pyrosequencing were chosen for further study.

Bacterial metagenomic DNA from *Ralstonia pickettii* AU12-08 was extracted and genome sequence of bacterial species was determined on the Genome Sequencer FLX (GS-FLX) system (Roche, Australia) at 454 Life Sciences. Metagenomic contigs were *de novo* assembled using GS De Novo assembler (version 2.3; Roche). Automatic genome annotation was performed on the RAST server [18] and IMG/M-ER [19].

Biofilm growth essay

The assay to grow and quantitate biofilms has been used previously for other bacterial species [20,21]. An overnight broth culture was diluted 1:200 in fresh broth and 200 µl was inoculated into the wells of a 96 well Linbro tissue culture plate. The plates were incubated at 37°C, under 5% CO_2, for 12 days. Growth was then assessed by measuring the optical density at 490 nm (OD_{490}) in a BioRad plate reader. To quantitate biofilm formation, 20 µl of Difco crystal violet (Becton Dickinson, Australia) was added to each well and incubated at room temperature for 15 minutes. Wells were washed vigorously with distilled water and the plate

was air dried. A volume of 230 µl of 95% ethanol was added to each well and the OD_{570} was measured. All strains were tested in quadruplicate. Each plate included 4 wells, which contained sterile broth instead of bacteria, but were treated identically otherwise. The OD_{570} was standardized against these wells.

Accession number

The draft genome of *Ralstonia rickettii* AU12-08 has been deposited at GenBank under accession number ASZV 00000000.

Results

Sample characteristics

The 15 recruited patients had a mean age of 51.3 years, an APACHE II score of 23.6, ICU stay of 16.7 days, and 80% were on systemic antimicrobials at the time of catheter removal (Table 1). The mean duration of catheter placement was 5.6 days, and there was no difference in dwell time between ACs and CVCs ($p>0.1$, two tailed *t*-Test).

According to the results of semi-quantitative cultures, 4 of the 30 removed IVCs were considered colonised, and in these cases both the AC and CVC were colonised from two patients. Isolates are including *Staphylococcus epidermidis* (catheter numbers 2A and 2C), *Staphylococcus hominis* (3A), and mixed enteric and skin bacteria (3C) (Table 1). One of the 15 (6.66%) patients was diagnosed as IVC-BSI (matched tip and blood cultures with no other explanatory source for the symptoms). Both colonisation (semi-quantitative method) and IVC-BSI occurred in patients already receiving multiple antibiotics (see Table 1).

Bacterial community profiles of IVCs

No bacterial DNA was detected or amplified from negative control IVCs. These control results indicate that the contribution to the bacterial community from manufacture of IVCs, DNA extraction procedures and PCR reagents was negligible. Bacterial DNA was extracted

from each IVC sample and individually amplified. A total of 50,364 sequences (18.8%) did not meet the quality control criteria and, thus, were excluded, and 217,488 high-quality sequence reads were used for further analysis. Chimera checks showed that sequences were not chimeric. Overall, microbial communities on both types of IVCs had a surprisingly high diversity and complex community structure. 16S rRNA gene sequences were assigned to sixteen bacterial phyla: Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Deinococcus-Thermus, Firmicutes, Fusobacteria, Gemmatimonadetes, Nitrospirae, OD1, Proteobacteria, Spirochaetes, Tenericutes, Thermotogae, TM7 and unclassified bacteria (Fig. 1). The most dominant phylum was Proteobacteria (79.8% of 16S rRNA reads), which included the taxonomic genus *Ralstonia* (53%), *Escherichia* group (16%), and *Bradyrhizobium* (2%). The term of *Escherichia* group used in this study since the16S rRNA has difficulty on differentiating *Escherichia* and *Shigella*, and it was also suggested that *Escherichia* and *Shigella* were sufficiently similar for placement in a single genus [22]. A high number of OTUs was observed for each sample, ranging from 57 to 168 (Fig. 2). The number of different genera per sample (bacterial richness) ranged from 22 to 59, and the overall diversity (Shannon-Weaver index) was in the range of 3.09 to 4.08.

Microbial communities on ACs and CVCs had a similar community composition and diversity. *Ralstonia* were dominant and accounted for 52.1% of examined sequences in ACs, followed by *Escherichia* group (14.5%), *Staphylococcus* (5.2%), and *Propionibacterium* (5.3%). CVCs had a similar community structure at an OTU level. Over 90% of identified OTUs were present on both ACs and CVCs. Further, in terms of overall microbial diversity, no difference was found between AC and CVC samples ─ the Shannon-Weaver diversity index values for both ACs and CVCs were around 3.5 ($p>0.05$, two tailed *t*-Test). All results indicated no statistically significant differences in the bacterial communities examined from CVCs and ACs ($p>0.05$, two tailed *t*-Test). The PCoA (Fig. 3a) suggested that bacterial

communities on ACs and CVCs had similar community profiles, as the AC and CVC microbial communities did not form distinct clusters. Thus, incidence of bacterial colonisation and, therefore, the potential for causing IVC-BSI did not appear to differ between ACs and CVCs. These findings support the assertion that ACs should be considered as an equally likely site, as CVCs, of catheter colonization in critically ill patients [12].

For the majority of patients (11/15) the microbial communities found on the two different catheters were more similar than the average distance of microbial communities from different patients. The average Jaccard distance of communities from different patients was 0.6, the average Jaccard distance of communities from the same patient was 0.39 (p=0.12, t-test) (Fig. 4). According to Anosim, Adonis and PERMDISP2, the intra-patient communities were generally more similar than between-patient communities (Anosim: p=0.002; Adonis: p=0.001; PERMDISP2: 1e-225).

We compared the bacterial communities present on 'colonised' and 'uncolonised' IVCs. The dominant bacteria on both groups were *Ralstonia* and *Escherichia* group**.** No statistically significant differences were found between these two groups in terms of bacterial profiles, OTU distribution, Shannon-Weaver diversity, and PCoA (Fig. 3b). Moreover, it was also noticed that for patient number 2, who had both IVCs 'colonised' by coagulase-*negative staphylococcus* (CNS) and *Staphylococcus* spp. using the roll-plate culture method, *Staphylococcus* spp. in fact represented only 3% of the bacterial community present on both IVCs via molecular methods. Further, for patient number 15, the AC and CVC yielded sub-diagnostic growth (<15 *cfu*) of *Staphylococcus* spp., and for mixed *Staphylococcus* and *Escherichia* group respectively, whereas over 60% of 16S rRNA sequences from the AC were *Staphylococcus spp*. and 72% of 16S rRNA gene sequences from CVC were *Acinetobacter spp.* Bacterial communities on this patient's IVCs were different from the

remaining patients' IVCs, which were dominated by species closely related to *Ralstonia* and/or *Escherichia* group.

Eight IVC samples (4 ACs and 4 CVCs) came from four patients not receiving antibiotic treatment. We compared the bacterial communities on these samples to the 22 IVC samples from 11 patients treated with intravenous antibiotics in the two weeks prior to sample collection. Statistically, there was no significant difference in the bacterial communities on IVCs from patients with, or without, antibiotic treatment, as confirmed by the results of PCoA (Fig. 3c) and OTU distribution.

***Ralstonia pickettii* AU12-08 genome sequencing**

The closest cultured relative of AU12-08 is *R. pickettii*, and it shares 100% sequence identity with the 16S rRNA genes of the predominant bacterial species in pyrosequencing libraries. Therefore, a thorough understanding of *R. pickettii* AU12-08 genome is vital for the systematic understanding of bacterial pathogenesis on IVC-related infections. The sequence data consists of 6,229,152 bp chromosome with G+C content of 63.6%. The *R. pickettii* AU12-08 genome harbours 50 tRNA genes coding for all amino acids, and 5733 predicted protein coding genes consistent with other sequenced *Ralstonia* spp. [23,24]. Comparisons of the general genomic features of *R. pickettii* AU12-08 with nosocomial pathogenic *R. pickettii* strains 12J and 12D, and environmental isolate *R. solanacearum* GM1000, are shown in Table 2.

A seven gene operon coding for *Pel* synthesis machinery was present in *R. pickettii* AU12-08. *Pel* was originally found to be responsible for the production of glucose-rich biofilm matrix exopolysaccharide in strain *Pseudomonas aeruginosa* 14 [25]. The detailed comparison of *Pel* coded by *R. pickettii* AU12-08 genome with *R. pickettii* 12J, *R. pickettii* 12 D and PA 14, showed a high degree of similarity with respect to gene organization and predicted function

(Fig. 1Sa). An independent study by Vasseur et al. showed that glucose-rich polysaccharides are essential for the formation of a surface-associated biofilm; thus, it is a major component of biofilm in strain *Pseudomonas aeruginosa* K [26]. The *pel* operon contains seven genes, *pelA* to *pelG*, which display sequence similarity with genes that encode sugar-processing enzymes. These include oligogalacturonide lyase (*pelA*), glycosyltransferases (*pelC* and *pelF*), sucrose synthase (*pelE*), and transmembrane proteins (*pelD* and *pelG*). Transposon insertion into, or deletion of, these genes resulted in severe defects in biofilm formation [26].

More genes in the *R. pickettii* AU12-08 genome were found to encode resistance to antibiotics and toxic compounds compared to *R. pickettii* 12J, *R. pickettii* 12D and *R. solanacearum* GM1000 (table 2). The *R. pickettii* AU12-08 genome contains 22 putative Multidrug Resistance Efflux Pumps (resistance nodulation division family), which enable bacteria to excrete antibiotics and other toxic compounds. Multidrug Resistance Efflux Pumps have been previously demonstrated to not only confer resistance to drugs used in therapy, but also to have a role in bacterial pathogenicity, i.e., through bacterial colonization and bacterial survival in the host [27]. Seven genes in chromosome 1, and 3 genes in chromosome 2 of *R. pickettii* AU12-08 genome, were coding for beta-lactamase including beta-lactamase (EC 3.5.2.6), beta-lactamase class C, and penicillin binding proteins and beta-lactamase class D. Environmental isolate *R. solanacearum* GM1000 does not encode beta-lactamase (EC 3.5.2.6), beta-lactamase class C or penicillin binding proteins. *R. pickettii* AU12-08 shares a high similarity with PA14 on beta-lactamase which has been reported to resist Beta-lactam antibiotics (Fig. 1Sb). In addition, the presentation of gyrA, gyrB, parC and parE in *R. pickettii* AU12-08 might indicate the resistance of fluoroquinolones. Thirteen genes were coded for components of tripartite multidrug resistance system. Further, 170 genes were coded for resistance toxic compounds, including cobalt-zinc-cadmium, copper homeostasis, mercury operon, arsenic and bile hydrolysis.

Biofilm essay

R. pickettii AU12-08 recovered from IVC tips was studied for biofilm formation. To characterise the kinetics of biofilm formation, the levels of biofilm formation were measured after 3, 6, 9 and 12 days respectively. We also examined the effect of other bacterial species (*E. coli* and *S. aureus*) on the general growth characteristics of *R. pickettii* AU12-08 (Fig. 5a). We compared the biofilm formation of *R. pickettii* AU12-08 to that of other bacterial species by measuring the $OD_{570.}$ As shown in Fig. 4, throughout duration of the biofilm formation, the biofilm formed by *R. pickettii* AU12-08 alone, was less than that when formed in combination with multiple bacterial species (Fig. 5b). These results suggest that *R. pickettii* AU12-08 forms biofilms independently, and also in a greater volume of biofilms when present with other bacterial species.

Discussion

Using high-throughput 16S rRNA sequencing, we demonstrated the complex diversity of the microbiological flora on commonly used intravascular catheters. An understanding of the composition of bacterial communities on IVCs is essential for the prevention and treatment of IVC-BSI. Many identified bacterial species in this study were known pathogens or opportunistic pathogens such as *Ralstonia spp*., *Escherichia* group *spp*., *Staphylococcus spp*., *Streptococcus spp*., *Acinetobacter spp*., *Chryseobacterium meningosepticum*, *Corynebacterium spp*., *Stenotrophomonas spp*. and *Pseudomonas spp*.

The predominant bacterial species on examined IVCs were closely related to *R. pickettii* (over 50% of examined sequences). *R. pickettii* (*Pseudomonas pickettii*) is a non-fermentative, gram-negative bacterium isolated from environmental and clinical samples. *R. pickettii* has the ability to survive in 0.05% chlorhexidine solutions [28] and penetrate 0.2 mm filters [29]. Therefore, many of the cases of infection with *R. pickettii* were due to contaminated water or aqueous solutions given intravenously [30]. Even ‘‘sterile’’ medical

products including ranitidine and saline solution had been reported to be contaminated with *R. pickettii* [31,32]. Several nosocomial catheter-related outbreaks have been described [30,33,34].

R. pickettii growth is slow and unfavourable on the blood agar plates currently used for routine IVC culture, which may explain why clinical results are generally negative for this organism. Our results challenge the belief that *R. pickettii* is of low virulence and incidence in IVCs, and suggest that it is, in fact, embedded in a biofilm and thus escaping detection using current methods. It may be of virulence itself, or it may be important in the subsequent colonisation or activity of other microbes. *R. pickettii* AU12-08 contains genes important for biofilm formation. A seven gene operon coded for *Pel* synthesis machinery. *Pel* was originally found to produce glucose-rich biofilm matrix exopolysaccharide in *Pseudomonas aeruginosa* 14. The biofilm assay used in this study demonstrated that *R. pickettii* AU12-08 is capability of forming biofilms.

Patients recruited within this study were treated with vancomycin, meropenem and piperacillin/tazobactam (Table 1). Vancomycin is used to treat infections caused by gram-positive bacteria, meropenem is a beta-lactam and piperacillin/tazobactam combines the extended-spectrum penicillin piperacillin and β-lactamase inhibitor tazobactam. The genome of *R. pickettii* AU12-08 harboured numerous genes coding for resistance of antibiotics and toxic compounds. *R. pickettii* AU12-08 has 22 putative multidrug resistance efflux pumps to enable bacteria to excrete antibiotics and toxic compounds in catheter coatings. *R. pickettii* AU12-08 are gram-negative bacteria, therefore they are not sensitive to vancomycin. *R. pickettii* AU12-08 also contains β-lactamase, which can break the β-lactam (meropenem) structure. *R. pickettii* AU12-08 possesses genes encoding proteins involved in biofilm formation, that also contribute to increased antimicrobial drug resistance. Considering that most patients were receiving antibiotic treatment, yet most IVCs had *R. pickettii* present, it

would appear that it is able to resist current therapy. Whilst the origin of the detected bacteria cannot be ascertained from our study, it is possible that some organisms may have been environmentally acquired. Since all controls were negative, it is unlikely that contamination occurred in the laboratory. Our study did not include specimens from patients who were not suspected of infection at the time of catheter removal, and this could be a focus of future studies, as long as the dwell time of catheters was matched between groups. Colonization of the indwelling IVC can occur via health care workers hands if they contaminate the hub or infusates while giving medications and fluids, or when drawing blood. This is plausible since these IVCs are typically accessed for therapy many times per day - staff hand hygiene and administration set decontamination prior to access may not always be optimal. Other organisms we identified may be resident on patients' skin – gram positives but also gram-negative bacteria, which increase in density on the skin and heightened the increased acuity of illness and duration of hospitalization typical in ICU patients. To verify with certainty the source of these bacteria would require a comprehensive assessment beyond the scope of the current study.

Conclusions

This study demonstrates the utility of high-throughput sequencing for examining bacterial communities on medical devices. Many microorganisms that were encountered were closely related to known human opportunistic or true pathogens and some have been commonly involved in IVC-related infections. Description of these bacterial communities will improve our understanding of IVC-related infections since bacterial colonization is the first step leading to IVC-related infections. In addition, better understanding of these bacterial communities may aid the development of more efficient diagnostic and therapeutic approaches for IVC-related infections, both of which remain difficult in modern medicine.

Acknowledgments

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Conflict of Interest

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

Table 1. Patient demographics and roll-plate intravascular catheters culture results.

Patient number	ICU stay (days)	Diagnosis	APACHE II score	Antibiotic usage	'Roll-plate' culture result
1	14	Subdural hematoma	27	Meropenem and Vancomycin	NG
2	17	Sepsis pneumonia	27	Vancomycin, Piperacillin and Tazobactam	2A: 100 cfu *Staphylococcus epidermidis* 2C: 10^3 cfu mixed CNS
3	12	Peritonitis	22	Piperacillin and Tazobactam	3A: 50-100 cfu *Staphylococcus hominis* 3C: 50-100 cfu mixed *Staphylococcus* and *Escherichia*
4	8	Cardiac arrest	29	None	NG
5	7	Sepsis	18	None	NG
6	9	Respiratory failure	19	None	NG
7	14	Subdural hematoma	27	None	NG
8	38	Respiratory failure	30	Piperacillin and Tazobactam	NG
9	10	Burn	16	Piperacillin and Tazobactam	NG
10	8	Fecal peritonitis	13	Meropenem and Vancomycin,	NG
11	32	Subarachnoid hemorrhage	19	Vancomycin, Meropenem, Piperacillin and Tazobactam,	NG
12	12	Craniotomy	30	Meropenem, Piperacillin and Tazobactam	NG
13	38	Multi-trauma	21	Piperacillin and Tazobactam	NG
14	19	Cardiac arrest	36	Piperacillin and Tazobactam	NG
15	13	Liver failure	20	Meropenem, Vancomycin, Piperacillin and Tazobactam,	NG

Table 2. General features of genomes of *R. pickettii* strains AU12-08, 12J, 12D and *R. solanacearum* GM1000.

	R. pickettii **AU12-08**	***R. pickettii*** **12J**	***R. pickettii*** **12D**	***R. solanacearum*** **GM1000**
Origin	Clinical isolate	Nosocomial pathogen	Nosocomial pathogen	Environmental isolate
Length (Mb)	6.23	5.33	5.69	5.81
GC%	63.7	63.6	63.3	67
#CDs	5733	5100	5361	5635
tRNA	50	57	54	57
Virulence factors				
Multidrug resistance efflux pumps	22	18	18	13
Beta-lactamase	10	4	4	3
Multidrug resistance tripartite systems	13	3	3	11
Resistance to fluoroguinolones	4	4	5	4
Resistance to toxic compounds	168	128	112	55
Quorum sensing and biofilm formation	9	12	13	12

Figure Legends

Fig. 1. Microbial genera detected in intravascular catheters. A represent arterial catheters and C represent central venous catheters.

Fig. 2. Heat map depicting relative abundance of bacterial genera across intravascular catheters samples of 15 patients. A represent arterial catheters and C represent central venous catheters.

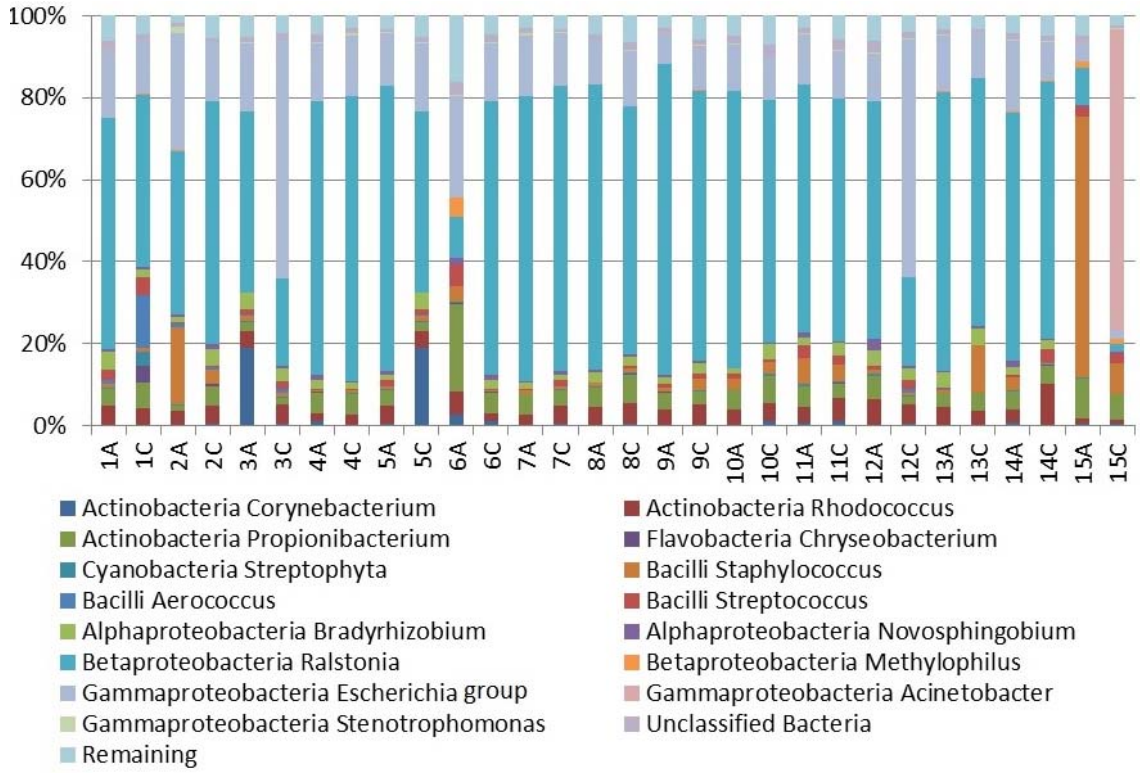
Fig. 3. Principal coordinate analysis of bacterial communities of intravascular catheters samples based on UniFrac distances. (a) 15 samples; (b) colonised IVCs (grey balls) and uncolonised IVCs (black balls); (c) antibiotic treatment (red balls) and without antibiotic treatment (blue balls). Each point corresponds to each IVC sample.

Fig. 4. Jaccard distance measuring dissimilarity between and intra 15 patients.

Fig. 5. (a) Time course of biofilm formation of five groups of bacteria. (b) Biofilm formation of five groups of bacteria. The data represent the average absorbance at 570 nm (± s.e.m.). A, *Ralstonia pickettii*; B, *Pseudomonas aeruginosa*; C, *Escherichia coli*; D, *Staphylococcus aureus*; E, *Ralstonia pickettii* together with *Escherichia coli* and *Staphylococcus aureus*.

Fig. 1S. (a) Comparison of extracellular matrix proteins (*Pel)* maps reconstructed from *R. pickettii* AU 12-08 and other five bacterial strains. 1, *Pel* B; 2, *Pel* C; 3, *Pel* A; 4, *Pel* D; 5, *Pel* E (987 bp); 6, *Pel* F; 7, *Pel* G. **(b) Beta-lactamase from *R. pickettii* AU 12-08. (c) Multiple drug efflux pumps from *R. pickettii* AU 12-08.**

Fig. 1.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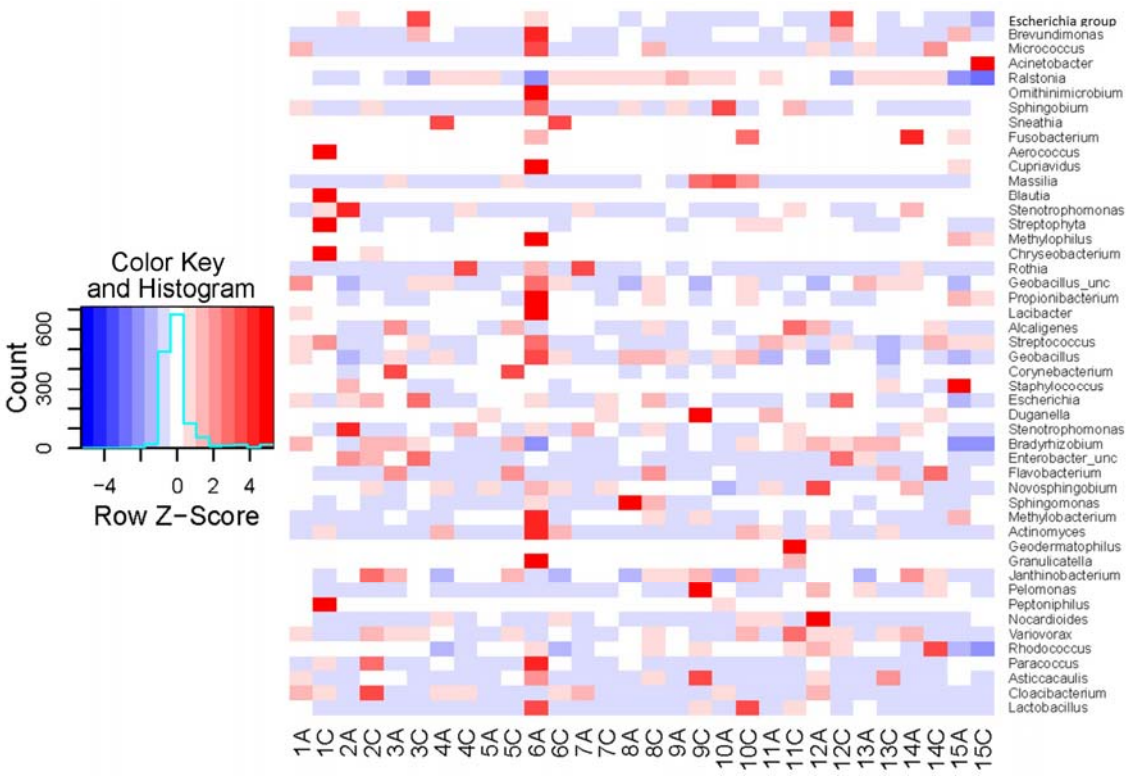
Actinobacteria Corynebacterium
Actinobacteria Rhodococcus
Actinobacteria Propionibacterium
Flavobacteria Chryseobacterium
Cyanobacteria Streptophyta
Bacilli Staphylococcus
Bacilli Aerococcus
Bacilli Streptococcus
Alphaproteobacteria Bradyrhizobium
Alphaproteobacteria Novosphingobium
Betaproteobacteria Ralstonia
Betaproteobacteria Methylophilus
Gammaproteobacteria Escherichia group
Gammaproteobacteria Acinetobacter
Gammaproteobacteria Stenotrophomonas
Unclassified Bacteria
Remaining

Fig. 2.


Color Key
and Histogram
Count
0
300
600
−4
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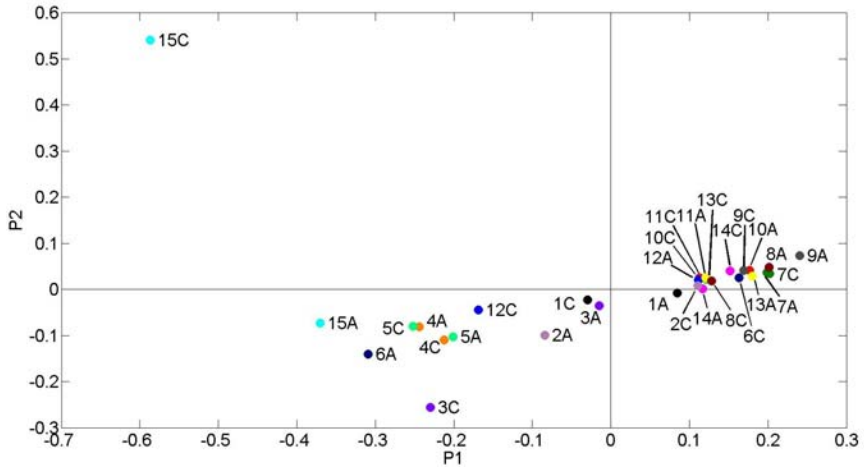
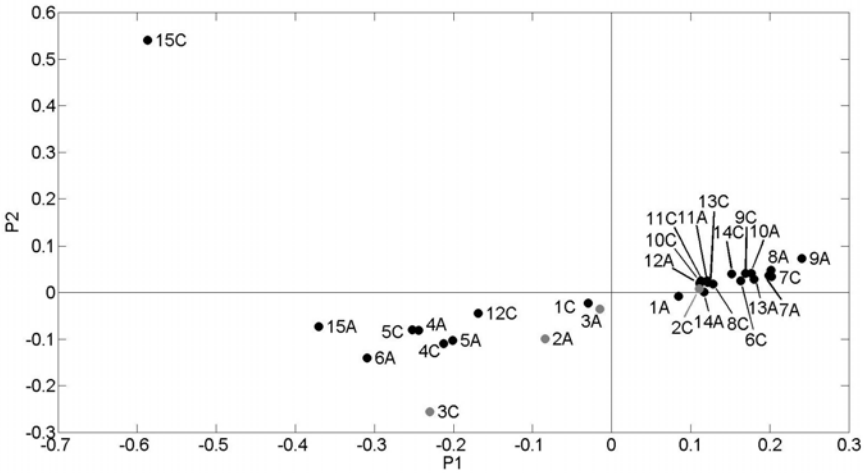
2
4
Row Z−Score
Escherichia group
Brevundimonas
Micrococcus
Acinetobacter
Ralstonia
Ornithinimicrobium
Sphingobium
Sneathia
Fusobacterium
Aerococcus
Cupriavidus
Massilia
Blautia
Stenotrophomonas
Streptophyta
Methylophilus
Chryseobacterium
Rothia
Geobacillus_unc
Propionibacterium
Lacibacter
Alcaligenes
Streptococcus
Geobacillus
Corynebacterium
Staphylococcus
Escherichia
Duganella
Stenotrophomonas
Bradyrhizobium
Enterobacter_unc
Flavobacterium
Novosphingobium
Sphingomonas
Methylobacterium
Actinomyces
Geodermatophilus
Granulicatella
Janthinobacterium
Pelomonas
Peptoniphilus
Nocardioides
Variovorax
Rhodococcus
Paracoccus
Asticcacaulis
Cloacibacterium
Lactobacillus
1A
1C
2A
2C
3A
3C
4A
4C
5A
5C
6A
6C
7A
7C
8A
8C
9A
9C
10A
10C
11A
11C
12A
12C
13A
13C
14A
14C
15A
15C

Fig. 3.

(a)


P2
P1
15C
15A
5C
4A
12C
1C
3A
2A
5A
4C
6A
3C
13C
11C
11A
9C
10C
14C
10A
12A
8A
9A
7C
1A
13A
7A
2C
14A
8C
6C

(b)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P1
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4C
6A
3C
13C
11C
11A
9C
10C
14C
10A
12A
8A
9A
7C
1A
13A
7A
2C
14A
8C
6C

(c)

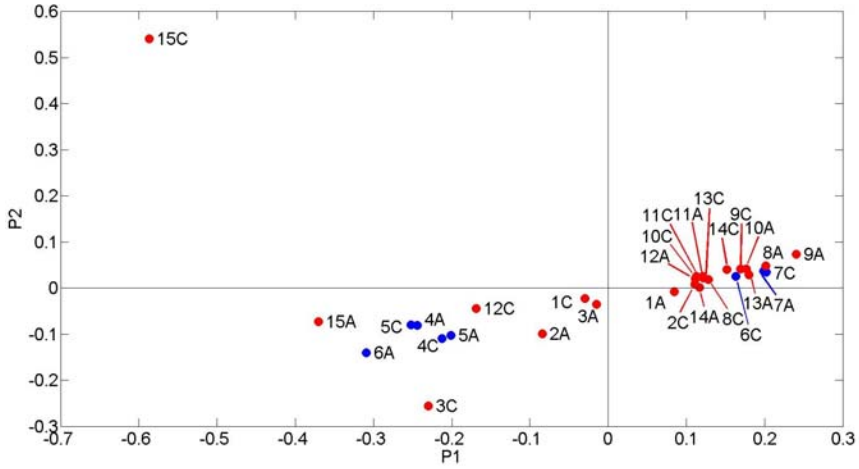

15C
13C
11C
11A
9C
10C
14C
10A
12A
8A
9A
7C
1A
13A
7A
2C
14A
8C
6C
12C
1C
3A
15A
5C
4A
5A
4C
2A
6A
3C
P1
P2

Fig. 4.

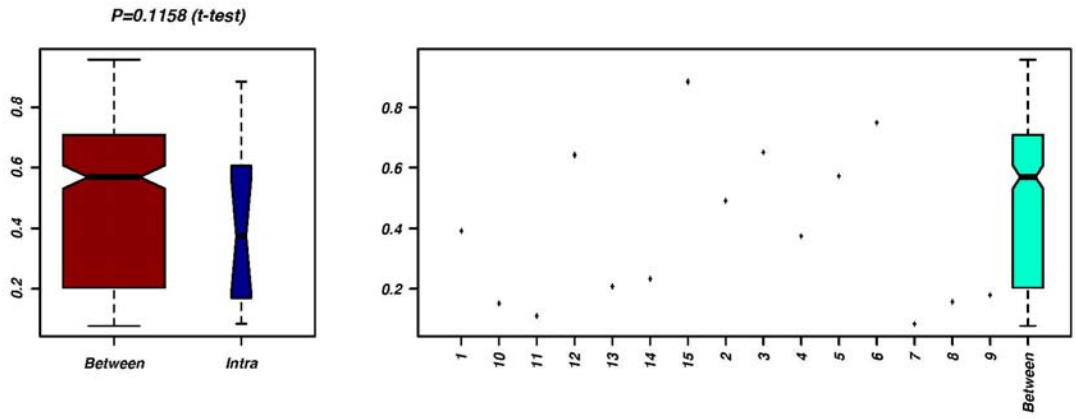
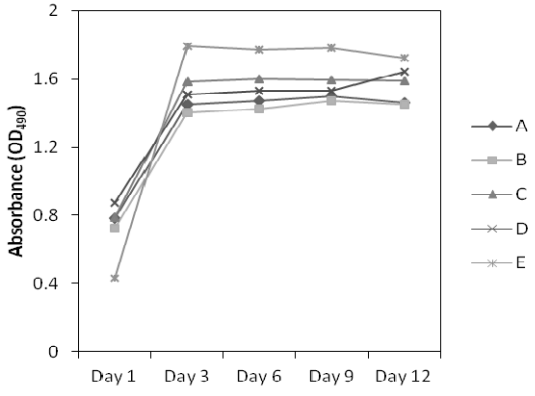

P=0.1158 (t-test)
0.2
0.4
0.6
0.8
Between
Intra
0.2
0.4
0.6
0.8
1
10
11
12
13
14
15
2
3
4
5
6
7
8
9
Between

Fig. 5.

(a)


Absorbance (OD490)
2
1.6
1.2
0.8
0.4
0
Day 1
Day 3
Day 6
Day 9
Day 12
A
B
C
D
E

(b)

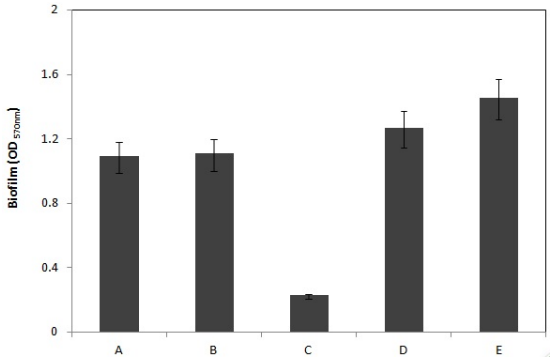
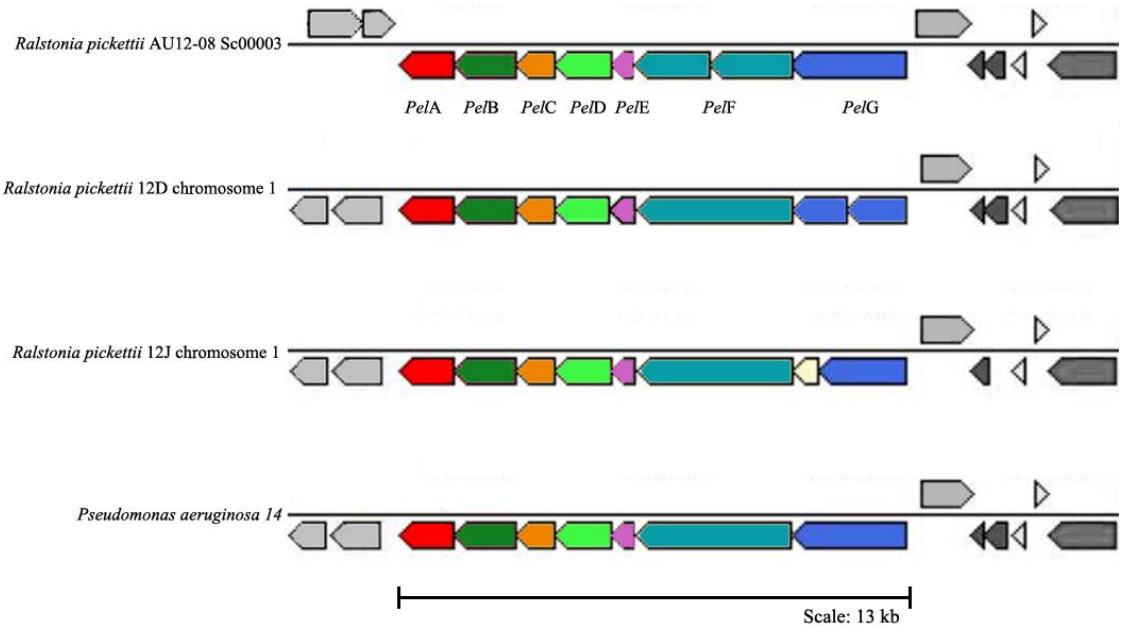
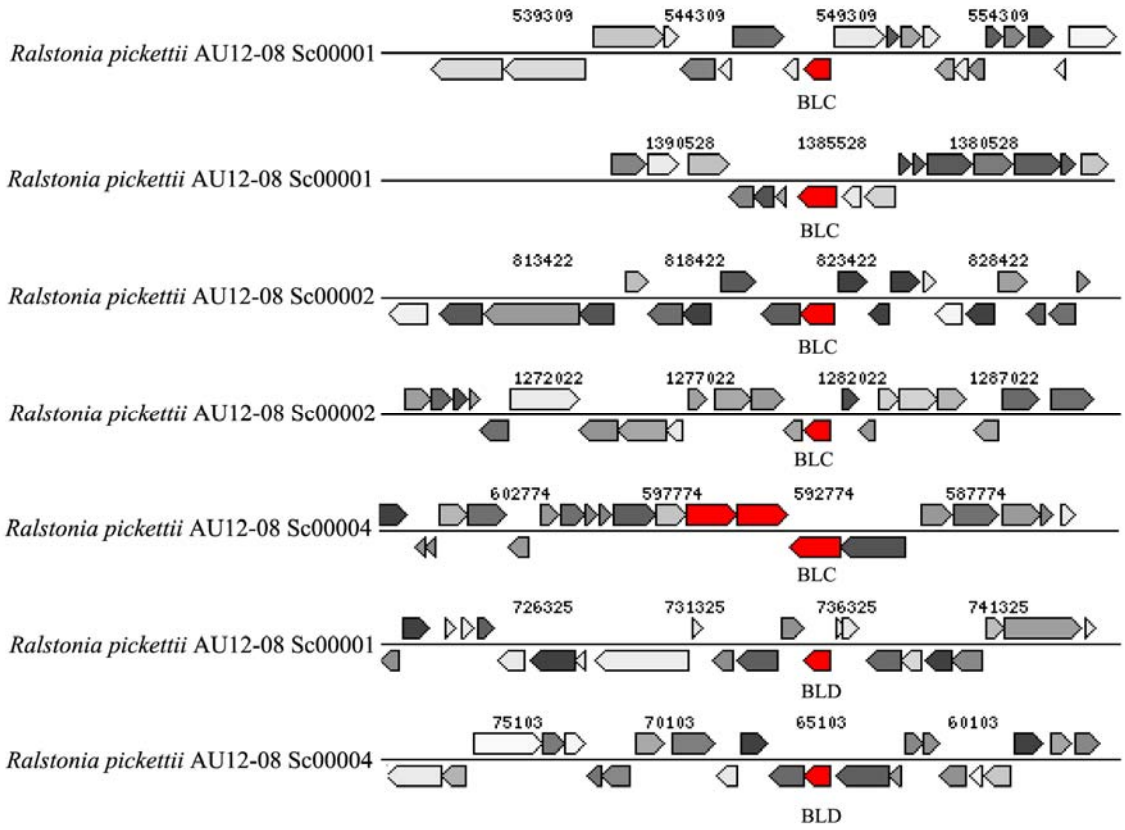

Biofilm (OD 570nm)
2
1.6
1.2
0.8
0.4
0
A
B
C
D
E

Fig. 1S.

(a)

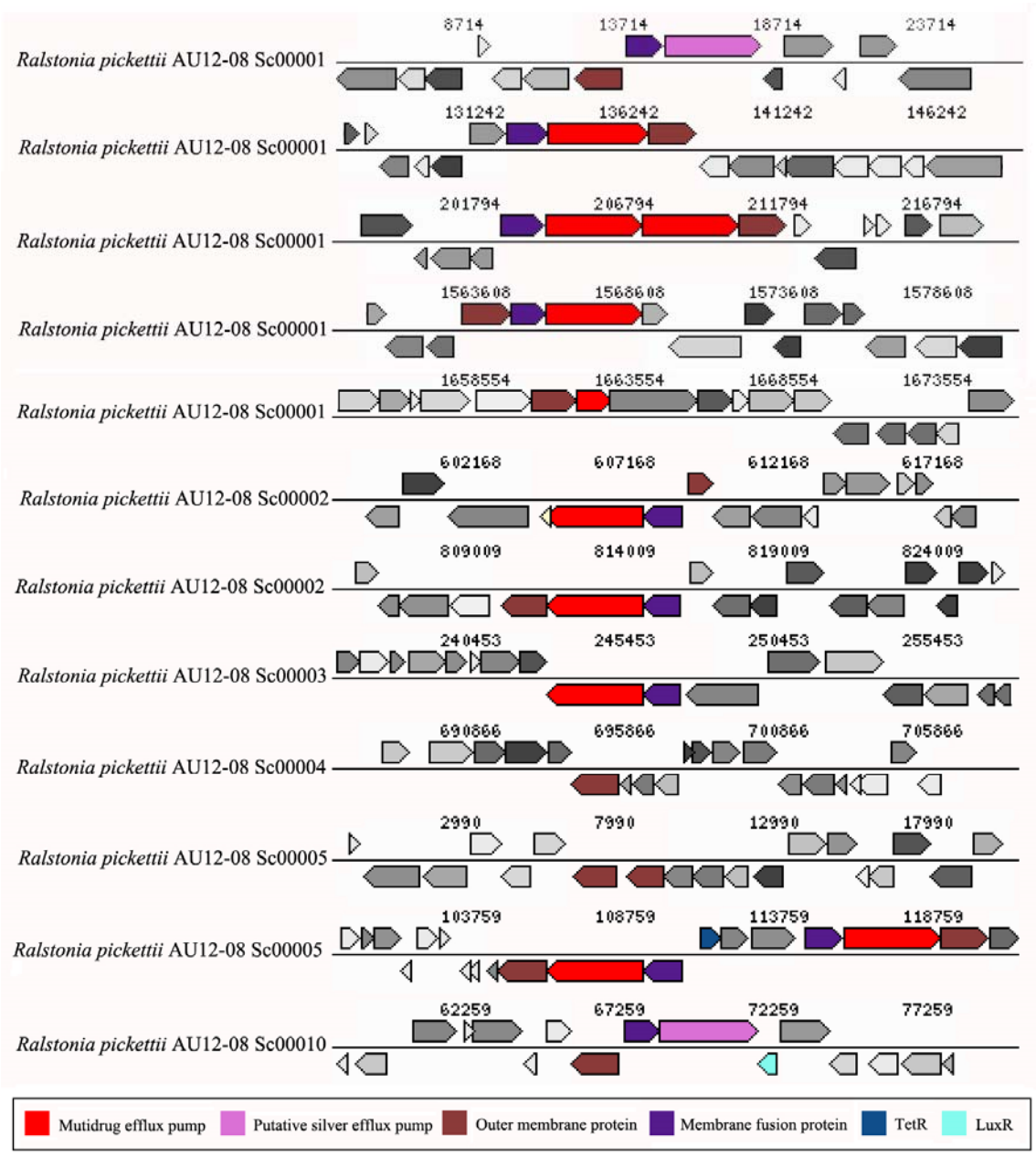

Ralstonia pickettii AU12-08 Sc00003
PelA PelB PelC PelD PelE PelF PelG
Ralstonia pickettii 12D chromosome 1
Ralstonia pickettii 12J chromosome 1
Pseudomonas aeruginosa 14
Scale: 13 kb

(b)



BLC represents beta-lactmase class C and penicillin binding protein; BLD represents beta-lactmase class D.

(c)


8714
13714
18714
23714
Ralstonia pickettii AU12-08 Sc00001
131242
136242
141242
146242
Ralstonia pickettii AU12-08 Sc00001
201794
206794
211794
216794
Ralstonia pickettii AU12-08 Sc00001
1563608
1568608
1573608
1578608
Ralstonia pickettii AU12-08 Sc00001
1658554
1663554
1668554
1673554
Ralstonia pickettii AU12-08 Sc00001
602168
607168
612168
617168
Ralstonia pickettii AU12-08 Sc00002
809009
814009
819009
824009
Ralstonia pickettii AU12-08 Sc00002
240453
245453
250453
255453
Ralstonia pickettii AU12-08 Sc00003
690866
695866
700866
705866
Ralstonia pickettii AU12-08 Sc00004
2990
7990
12990
17990
Ralstonia pickettii AU12-08 Sc00005
103759
108759
113759
118759
Ralstonia pickettii AU12-08 Sc00005
62259
67259
72259
77259
Ralstonia pickettii AU12-08 Sc00010
Mutidrug efflux pump
Putative silver efflux pump
Outer membrane protein
Membrane fusion protein
TetR
LuxR

References

1. Mermel LA, Allon M, Bouza E, Craven DE, Flynn P, O'Grady NP, Raad, II, Rijnders BJ, Sherertz RJ, Warren DK (2009) Clinical practice guidelines for the diagnosis and management of intravascular catheter-related infection: 2009 Update by the Infectious Diseases Society of America. Clin Infect Dis 49 (1):1-45. doi:10.1086/599376

2. Allegranzi B, Bagheri Nejad S, Combescure C, Graafmans W, Attar H, Donaldson L, Pittet D (2011) Burden of endemic health-care-associated infection in developing countries: systematic review and meta-analysis. Lancet 377 (9761):228-241. doi:S0140-6736(10)61458-4 [pii]
10.1016/S0140-6736(10)61458-4

3. Gogos CA, Leonidou L (2010) Catheter-related bloodstream infections: catheter management according to pathogen. *Int J Antimicrob Agents* 36:S26-S32. doi:10.1016/j.ijantimicag.2010.11.004

4. Timsit JF (2007) Diagnosis and prevention of catheter-related infections. Current Opinion in Critical Care 13 (5):563-571

5. Zhang L, Xu ZH (2008) Assessing bacterial diversity in soil. Journal of Soils and Sediments 8 (6):379-388

6. Zhang L, Sriprakash KS, McMillan D, Gowardman JR, Patel B, Rickard CM (2010) Microbiological pattern of arterial catheters in the intensive care unit. BMC Microbiology 10:266-275

7. Rogers GB, Carroll MP, Bruce KD (2009) Studying bacterial infections through culture-independent approaches. J Med Microbiol 58 (Pt 11):1401-1418. doi:jmm.0.013334-0 [pii]
10.1099/jmm.0.013334-0

8. Mancini N, Carletti S, Ghidoli N, Cichero P, Burioni R, Clementi M (2010) The Era of Molecular and Other Non-Culture-Based Methods in Diagnosis of Sepsis. Clinical Microbiology Reviews 23 (1):235-+. doi:10.1128/Cmr.00043-09

9. Pratt RJ, Petlowe CM, Wilson JA, Loveday HP, Harper PJ, Jones SRLJ, McDougall C, Wilcox MH (2007) epic2: National evidence-based guidelines for preventing healthcare-assosciated infections in NHS hospitals in England. J Hosp Infect 65:S1-S64

10. Marschall J, Mermel LA, Classen D, Arias KM, Podgorny K, Anderson DJ, Burstin H, Calfee DP, Coffin SE, Dubberke ER, Fraser V, Gerding DN, Griffin FA, Gross P, Kaye KS, Klompas M, Lo E, Nicolle L, Pegues DA, Perl TM, Saint S, Salgado CD, Weinstein RA, Wise R, Yokoe DS (2008) Strategies to prevent central line-associated bloodstream infections in acute care hospitals. Infect Control Hosp Epidemiol 29 Suppl 1:S22-30. doi:10.1086/591059

11. O'Grady NP, Alexander M, Burns LA, Dellinger EP, Garland J, Heard SO, Lipsett PA, Masur H, Mermel LA, Pearson ML, Raad, II, Randolph AG, Rupp ME, Saint S (2011) Guidelines for the Prevention of Intravascular Catheter-related Infections. Clin Infect Dis 52 (9):e162-e193. doi:cir257 [pii]
10.1093/cid/cir257

12. Traore O, Liotier J, Souweine B (2005) Prospective study of arterial and central venous catheter colonization and of arterial- and central venous catheter-related bacteremia in intensive care units. Critical Care Medicine 33 (6):1276-1280

13. Lucet JC, Bouadma L, Zahar JR, Schwebel C, Geffroy A, Pease S, Herault MC, Haouache H, Adrie C, Thuong M, Francais A, Garrouste-Orgeas M, Timsit JF (2010) Infectious risk associated with arterial catheters compared with central venous catheters. Critical Care Medicine 38 (4):1030-1035

14. Gowardman JR, Lipman J, Rickard CM (2010) Assessment of peripheral arterial catheters as a source of sepsis in the critically ill: a narrative review. J Hosp Infect 75 (1):12-18. doi:10.1016/j.jhin.2010.01.005
15. Maki DG, Weise CE, Sarafin HW (1977) A semiquantitative culture method for identifying intravenous catheter-related infections. New England Journal of Medicine 296:1305-1309
16. Sogin ML, Huse SM, Huber JA, Morrison HG, Mark Welch D (2007) Accuracy and quality of massively parallel DNA pyrosequencing. Genome Biology 8 (7). doi:10.1186/gb-2007-8-7-r143
17. Rafii F, Franklin W, Cerniglia CE (1990) Azoreductase activity of anaerobic bacteria isolated from human intestinal microflora. Appl Environ Microbiol 56 (7):2146-2151
18. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O (2008) The RAST Server: rapid annotations using subsystems technology. Bmc Genomics 9:75. doi:10.1186/1471-2164-9-75
19. Fridkin SK, Welbel SF, Weinstein RA (1997) Magnitude and prevention of nosocomial infections in the intensive care unit. Infectious disease clinics of North America 11 (2):479-496
20. Kaplan JB, Meyenhofer MF, Fine DH (2003) Biofilm growth and detachment of Actinobacillus actinomycetemcomitans. J Bacteriol 185 (4):1399-1404
21. Musken M, Di Fiore S, Romling U, Haussler S (2010) A 96-well-plate-based optical method for the quantitative and qualitative evaluation of Pseudomonas aeruginosa biofilm formation and its application to susceptibility testing. Nat Protoc 5 (8):1460-1469. doi:10.1038/nprot.2010.110
22. Sabat G, Rose P, Hickey WJ, Harkin JM (2000) Selective and sensitive method for PCR amplification of Escherichia coli 16S rRNA genes in soil. Appl Environ Microbiol 66 (2):844-849. doi:Doi 10.1128/Aem.66.2.844-849.2000
23. Zhu B, Liu H, Tian WX, Fan XY, Li B, Zhou XP, Jin GL, Xie GL (2012) Genome Sequence of Stenotrophomonas maltophilia RR-10, Isolated as an Endophyte from Rice Root. Journal of Bacteriology 194 (5):1280-1281. doi:Doi 10.1128/Jb.06702-11
24. Crossman LC, Gould VC, Dow JM, Vernikos GS, Okazaki A, Sebaihia M, Saunders D, Arrowsmith C, Carver T, Peters N, Adlem E, Kerhornou A, Lord A, Murphy L, Seeger K, Squares R, Rutter S, Quail MA, Rajandream MA, Harris D, Churcher C, Bentley SD, Parkhill J, Thomson NR, Avison MB (2008) The complete genome, comparative and functional analysis of Stenotrophomonas maltophilia reveals an organism heavily shielded by drug resistance determinants. Genome Biol 9 (4):R74. doi:10.1186/gb-2008-9-4-r74
25. Colvin KM, Gordon VD, Murakami K, Borlee BR, Wozniak DJ, Wong GCL, Parsek MR (2011) The Pel Polysaccharide Can Serve a Structural and Protective Role in the Biofilm Matrix of Pseudomonas aeruginosa. Plos Pathogens 7 (1). doi:ARTN e1001264
DOI 10.1371/journal.ppat.1001264
26. Vasseur P, Vallet-Gely I, Soscia C, Genin S, Filloux A (2005) The pel genes of the Pseudomonas aeruginosa PAK strain are involved at early and late stages of biofilm formation. Microbiology-Sgm 151:985-997. doi:DOI 10.1099/mic.0.27410-0
27. Piddock LJV (2006) Multidrug-resistance efflux pumps - not just for resistance. Nature Reviews Microbiology 4 (8):629-636. doi:Doi 10.1038/Nrmicro1464
28. Maroye P, Doermann HP, Rogues AM, Gachie JP, Megraud F (2000) Investigation of an outbreak of Ralstonia pickettii in a paediatric hospital by RAPD. J Hosp Infect 44 (4):267-272

29. Sundaram S, Lewis M, Eisenhuth J, Howard G, Larson B (2002) Method for qualifying microbial removal performance of 0.1 micron rated filters - Part IV: Retention of Hydrogenophaga pseudoflava (ATCC 700892) and Ralstonia pickettii (ATCC 700591) by 0.2 and 0.22 mu m rated filters. Pda Journal of Pharmaceutical Science and Technology 56 (3):150-171
30. Ryan MP, Pembroke JT, Adley CC (2006) Ralstonia pickettii: a persistent gram-negative nosocomial infectious organism. J Hosp Infect 62 (3):278-284. doi:S0195-6701(05)00371-3 [pii] 10.1016/j.jhin.2005.08.015
31. Labarca JA, Trick WE, Peterson CL, Carson LA, Holt SC, Arduino MJ, Meylan M, Mascola L, Jarvis WR (1999) A multistate nosocomial outbreak of Ralstonia pickettii colonization associated with an intrinsically contaminated respiratory care solution. Clin Infect Dis 29 (5):1281-1286
32. Fernandez C, Wilhelmi I, Andradas E, Gaspar C, Gomez J, Romero J, Mariano JA, Corral O, Rubio M, Elviro J, Fereres J (1996) Nosocomial outbreak of Burkholderia pickettii infection due to a manufactured intravenous product used in three hospitals. Clin Infect Dis 22 (6):1092-1095
33. Stelzmueller I, Biebl M, Wiesmayr S, Eller M, Hoeller E, Fille M, Weiss G, Lass-Floerl C, Bonatti H (2006) Ralstonia pickettii - innocent bystander or a potential threat? Clinical Microbiology and Infection 12 (2):99-101. doi:10.1111/j.1469-0691.2005.01309.x
34. Moreira BM, Pellegrino FLPC, Schirmer M, Velasco E, de Faria LM, Santos KRN (2008) Ralstonia pickettii bloodstream infections at a Brazilian cancer institution. Current Microbiology 56 (3):219-223. doi:10.1007/s00284-007-9060-1