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Investigations on microbiome of the used clinical device revealed many uncultivable newer bacterial species associated with persistent chronic infections

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Abstract

Introduction. Chronic persistent device-related infections (DRIs) often give culture-negative results in a microbiological investigation. In such cases, investigations on the device metagenome might have a diagnostic value. **Materials and Methods**. The 16SrRNA gene sequence analysis and next-generation sequencing (NGS) of clinical metagenome were performed to detect bacterial diversity on invasive medical devices possibly involved in culture-negative DRIs. Device samples were first subjected to microbiological investigation followed by metagenome analysis. Environmental DNA (e-DNA) isolated from device samples was subjected to 16SrRNA gene amplification followed by Sanger sequencing (n=14). In addition, NGS of the device metagenome was also performed (n=12). Five samples were only common in both methods. **Results**. Microbial growth was observed in only nine cases; among these, five cases were considered significant growth, and in the remaining four cases, growth was considered either insignificant or contaminated. Culture and sequencing analysis yielded identical results only in six cases. In culture-negative cases, Sanger sequencing of 16SrRNA gene and NGS of 16SrDNA microbiome was able to identify the presence of rarely described human pathogens, namely *Streptococcus infantis, Gemella haemolysans, Meiothermus silvanus, Schlegelella aquatica, Rothia mucilaginosa, Serratia nematodiphila*, and *Enterobacter asburiae*, along with some known common nosocomial pathogens. Bacterial species such as *M. silvanus* and *S. nematodiphila* that are never reported in human infection were also identified. **Conclusions**. Results of a small number of diverse samples of this pilot study might lead to a path to study a large number of device samples that may validate the diversity witnessed. The study shows that a culture free, a holistic metagenomic approach using NGS could help identify the pathogens in culture-negative chronic DRIs.

Keyword: culture-negative infection, Device microbiome, Persistent infection, 16S rRNA gene sequence analysis, metagenome analysis

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Introduction

Persistent chronic infections are caused mainly by sessile bacterial communities with inherent antibiotic resistance. Infections such as rhinosinusitis, otitis media, asthma, cystic fibrosis, non-CF bronchiectasis, chronic obstructive pulmonary disease, and other invasive medical device-related infections (DRI) often develop into chronic, long-term complications that are difficult to treat with antibiotics due to the formation of recalcitrant biofilms. Respiratory pathogens like *Streptococcus pneumoniae, Haemophilus influenzae, Staphylococcus aureus, Pseudomonas aeruginosa, Acinetobacter baumannii, Moraxella catarrhalis* commonly cause chronic respiratory diseases and such infections are caused due to therapeutic interventions such as mechanical ventilation. Ventilator-associated pneumonia is most common among the patients admitted to the hospital for critical care. After initial host invasion biofilm producing bacteria can attach to living and non-living surfaces, such as prosthetics and indwelling medical devices and start developing biofilms that may trigger antibiotic resistance and inflammation resulting in persistent infections. Multiple incidences have shown that persistent chronic infections are also due to very fastidious or non-culturable microorganisms (1-3).

Many previous research works have hypotheses that the persistent bacteria that cause chronic infections to adhere to the substratum of the medical device, which can cause localized inflammation, ensuring the failure of the implant

device (4). However, the diagnosis of persistent infection is difficult with traditional laboratory culture methods since causal organisms often fail to grow in routine culture media, though much evidence in the literature implicating highly fastidious or non-cultivable microorganisms having a pivotal role in causing implant infections (1, 5). Due to the lack of causal organism growth on the routine laboratory culture medium and related antibiotic susceptibility data, the clinicians of the patient do not like to treat them with antibiotics, and such situations pose more problems. These non-cultivable microbes may not grow on culture media because they lack nutrients for their metabolic needs in the synthetic culture media, which exist in their natural environmental habitat or micro niche (6). The culture-negative results upon culturing of the sample (excised device) on routine bacteriological media at the same time, the failure of indwelling medical devices in bringing the desired endpoint among the patients because of the formation of biofilms on those devices indicates infection-positive but negative culture situation, which is one of the most problematic recurring conditions in a hospital set-up that has to be addressed immediately by the medical community. Metagenomic tools appear to be the most accurate and reliable approach for investigating culture-negative implant-related infections that can reveal causal organisms' identity and their virulence potential developed under stress conditions in the host (7). The objectives of the present study are to use both culture-dependent and culture-independent methods to detect the bacterial diversity present on the surface of implanted medical devices in critically ill patients that were removed periodically for revising the treatment and also to find any new bacterial species that are fastidious, rarely implicated in human infections that are responsible for chronic DRIs.

Materials and Methods

Ethical Clearance

Approvals from the Institutional Ethical Committees of MGMCRI and JIPMER have been obtained for the study.

Transfer Media preparation

According to earlier investigators' observations, contamination of laboratory DNA may result in an abundance of false-positive microorganisms in clinical metagenome samples (8). To avoid contamination of the laboratory DNA, autoclaved Milli-Q water was sterilized by ultraviolet (UV-B) (mainly UV-B: 280-315 nm) under a laminar airflow for 1 hour, then treated with 30µl DNase I (1 U / µl) per liter Milli-Q water and incubated at 37° C for 72 hours and then inactivated by autoclaving at 121° C for 20 minutes. Again, the water was treated with 1 ml/L diethylpyrocarbonate (DEPC) and incubated at 37° C for 24 hours. This treated water was used for the preparation of 1 x phosphate-buffered saline (1 x PBS, pH = 7.4) as a transfer media, which was used for the collection of all the clinical specimens.

A total of 21 device samples were collected from 21 patients in 1X PBS from critical care units of hospitals, namely Mahatma Gandhi Medical College and Research Institute (MGMCRI) and Jawaharlal institute of postgraduate medical education and research (JIPMER), located in Puducherry UT, India. Utilized devices such as endotracheal tubes (ET), Foley catheters (FC), bone implants(OS), central venal catheters (CL), pacemakers, and other miscellaneous devices from each patient were subjected to moderate ultrasonication to dislodge any adherent bacteria present on them, which is followed by processing of these samples for aerobic and anaerobic microbiological culture. Briefly, the used medical device implants were excised wherever necessary under sterile conditions, and the excised device samples were placed in a sterile 50 mL falcon tube (Tarsons, India) filled with DNA free DEPC treated sterile 1X PBS, pH=7.4 (buffer prepared by using DNA free ingredients) and subjected to ultrasonication followed by 30 seconds vigorous agitation by vortexing for the removal of microbial biomass present on used devices. All these samples were transported from the hospital within a few hours to the laboratory at 4° C temperature.

For ultrasonication of samples, this study used a BactoSonic ultrasound water bath, a handy tool for removing cells from surfaces and not usually used for cell lysis. In the laboratory, falcon tube with the rescued implants was vortexed for 60 s using the Vortex-Genie 2 (Merck Scientific Industries, India). Subsequently, it is sonicated (BactoSonic ultrasound bath, Germany) for 5 to 8 minutes at a frequency of 40 ± 2 kHz and power density of 0.22 ± 0.04 W/cm^2, followed again by 45 seconds of vortexing. These samples were divided into two portions. The first portion was used for Gram-staining and microbiological culture, whereas the remaining portion was rescued and preserved at -80° C for extraction of the metagenome later. The first portion was centrifugated (Sigma 6K15) at 17001 RCF (X g) for 15 minutes to concentrate the bacterial biomass. After centrifugation, the supernatant was aspirated, and the pellet was dissolved in 0.1ml of transfer media. Aliquots of 10uL of concentrated sonicated preparative suspensions were then inoculated onto blood agar, nutrient agar, and chocolate agar plates and kept for incubation in a CO2 incubator and the duplicate plates were kept in an anaerobic jar (Hi-Media, India) for anaerobic incubation. Inoculated plates were incubated at 37°C for 24 hours to one week, which could be used to recover slow-growing bacteria. For the cultivation of anaerobic bacteria incubated at 37°C for 14 days. One aliquot of 10uL of this sample was subjected to Gram-staining for microscopy. Plated media were examined daily for seven days for checking any growth. Those samples that grew bacteria were marked, and these isolates were further subjected to standard biochemical tests to confirm their identification phenotypically. Grown isolates were assayed from biofilm production by microtitre plate assays (9) and antimicrobial susceptibility testing (10). Afterward, the rescued sample portions of the processed device samples were retrieved from – 80° C and subjected to direct extraction of metagenomic DNA using two manual methods. One of the methods adopted here for DNA extraction gave the highest DNA yield from the medical device samples described below.

Extraction of metagenome from devices surface

Metagenomic DNA was extracted from each sonicated sample using the manual method described here instead of the kit method since kit-based DNA isolation methods cannot entirely eliminate DNA contamination (11-12). Currently, there are no efficient commercial DNA extraction-based kits specially designed to extract DNA from a smaller amount of biomass cells of medical devices. We used the manual method to extract metagenomic DNA from sonicated bacterial biomass because the medical devices and biopsies contained fewer bacterial biomass cells than soil, water, and other samples. Briefly, the rescued samples were homogenized 3 times for 30 seconds at 48 Hertz, with cooling on ice between these homogenisations. The preparations were centrifuged at 8,000g for 5 min at 4° C and then carefully decanted the supernatant. After this, the samples were homogenized in TE buffer by vortex, then 50µl lysozyme (40mg/ml), 5µl mutanolysin (25KU/ml), and 3 µl lysostaphin (4KU/ml) (Hi-Media, India) was added, and the suspension was incubated for 1 hour at 37 °C. Later it was treated with 4 µL RNAase (20 mg/mL) for 15 min to remove RNA.

Further, 0.5mL lysis buffer containing (10mM Tris-HCl, 50mM Na_2+EDTA, 1% SDS, 100mM NaCl, pH 8), then 5µL of proteinase K (20 mg/mL) was added to the suspension and incubated for 5 h at 55°C under mild agitation. The mixture was centrifuged at 10,000g for 10 minutes at 4°C, and the supernatant was collected. The aqueous phase was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) after centrifugation at 10,000g, 10 min at 4° C. The aqueous layer was subsequently extracted with chloroform: isoamyl alcohol (24:1). Equal ice-cold isopropanol was added to the supernatant obtained and incubated overnight at -20°C. After overnight incubation, the supernatant was centrifuged at 10,000 X g for 10 min at 4° C to collect the DNA. The DNA pellet was washed twice with 70% ice-cold ethanol and allowed to dry at room temperature for 30 min, followed by re-suspension in 50µL of 1X TE buffer or nuclease-free water. In the above DNA isolation protocol, plain sterile LB broth was used as a negative control sample to check any contamination of DNA extraction reagents during the process of DNA extraction. Finally, the quality of extracted DNA was analyzed on 1% agarose gel, and the DNA quantification and its purity were determined using Nanodrop (Thermo Scientific, USA, UK)

Polymerase chain reaction (PCR) for 16S rDNA

The 16S rRNA gene was amplified using the universal primer pair designed to amplify 16S rDNA from the concentrated metagenome sample using PCR by following the method described elsewhere (13). The primers used in the PCR target the highly conserved regions of the 16S rRNA gene and amplify them. These primers were designed to amplify most bacterial species' DNA. The 16S rRNA gene primers used were 5' -ATCCTGGCTCAGAGCGAACG-3' (16Sr DNA F) and 5' -CCCTACGGCTACCTTGTTACG-3'(16Sr DNA R) (Sigma, Bengaluru, India), and the PCR amplicon generated a nearly complete sequence of the 16S rRNA gene (~1,450 base pairs) (14). All the PCR reactions were performed in a total volume of 50µL, comprising 2 µl of extracted bacterial DNA and 48 µl of PCR reaction mixture containing 1 × PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100), 1.0 U Taq DNA polymerase (Fermentas), 0.2 mM dNTPs (Fermentas) and the concentration of each primer is about 0.2 µM. PCR was conducted in a Veriti thermal cycler (Applied Biosystems, UK). The thermal cycler conditions comprised of an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, 55°C annealing for 1 minute, extension at 72°C for 1.5 minutes, and a final extension step at 72°C for 10 minutes.

While performing PCR, stringent measures were employed to avert all kinds of contamination lucidly described previously in the literature (15). Strict aseptic conditions were maintained while conducting all the experiments to prevent microbial contamination. Positive and negative controls were included with each batch of samples being analyzed through PCR. The positive control is a standard PCR reaction mixture containing 10ng of *Acinetobacter* genomic DNA instead of a sample, and the negative control contained sterile water instead of a sample. Each PCR product (10 µl) was subjected to electrophoresis on a 2% agarose gel, and the DNA amplicon was detected by staining with ethidium bromide (0.5µg/ml) and examined in Gel doc (Bio-Rad, UK).

Metagenome DNA sequencing

The NGS of metagenome samples was custom sequenced by Bionivid, Bengaluru, and Macrogen, South Korea. Metagenome sequencing of the 12 selected samples (those with good e-DNA concentration) was performed on the HiSEQ platform. Because of cost considerations, only 12 samples were included in the final metagenome sequencing analysis. NGS of microbiome primarily analyzed 16S rDNA for profiling the kind of bacterial species present in the device sample. The 16S rRNA gene was amplified from 12 device microbiome samples, and the resultant PCR products were purified using the PCR purification kit following the manufacturer's instructions (Thermo, UK). Metagenome sequencing of 16S rRNA V3-V4 region sequencing was performed using the primers designed for 16S region (v3-v4). 16S V F: CCTACGGGNGGCWGCAG and 16S V R: GACTACHVGGGTATCTAATCC.

Bioinformatics Analysis

To get species-level insight into each sample, we used QIIME2 (Quantitative insight into Microbial Ecology - Version: 2019.7) as a primary tool. Sequence data were denoised for quality control using basic quality-based filtering, phiX reads (commonly present in marker gene Illumina sequence data) filtering, and chimeric sequences filtering. Quality plots were generated through the demux plugin in a QIIME2 for a distribution summary of sequence qualities in forward and reverse read sequences at each position. The Divisive Amplicon Denoising Algorithm (DADA2) plugin in QIIME2 was used for quality control as well as for constructing the ASV (Amplicon Sequence Variants)/OTU (Operational

Taxonomic Units) count table (16). The quality of the initial bases seems to be high. Hence trimming is not required for any bases from the beginning of the sequences in both forward and reverse reads. The quality drops around position 275 in forward reads and at position 190 in reverse reads. Therefore, sequences were truncated at 275 and 190 bases for forward and reverse reads, respectively. The summary of the distribution of sequence qualities at each position in forward and reverse sequences is illustrated in **Fig. 1** and **Fig. 2**, respectively. OTU abundance table is constructed from pre-processed reads by de-replication, singleton removal, and OTU clustering. OTU annotation or taxonomy assignment was done by building taxonomic classifiers. Taxonomic classifiers perform best when trained based on specific sample preparation and sequencing parameters, including the amplification primers and the sequence reads' length.

The taxonomic classification naïve-Bayes model was trained on extracted reference reads from the SILVA database (version-138) based on the primer used (341F/805R) for amplifying selected 16S gene regions (V3-V4). Finally, abstracted consensus sequences (ASVs)/OTU sequences were used as a test set for a trained taxonomy assignment model. Sampling depth was verified using an alpha-diversity rarefaction plot to measure observed OTUs and Shannon index. To make inferences more reliable, we used other widely accepted tools such as UPARSE (uses RDP as reference database) and online server SILVAngs (uses SILVA database) tools. We found that results from all the tools employed were nearly consistent (17-21).

Figure 1

Summary of the distribution of sequence qualities at each position in forward sequences

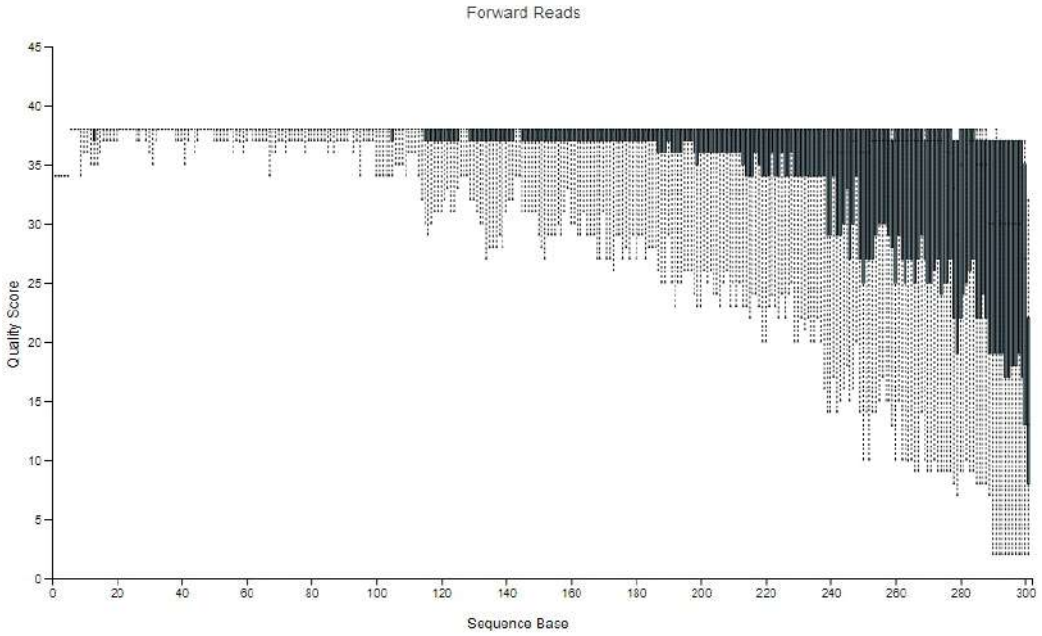
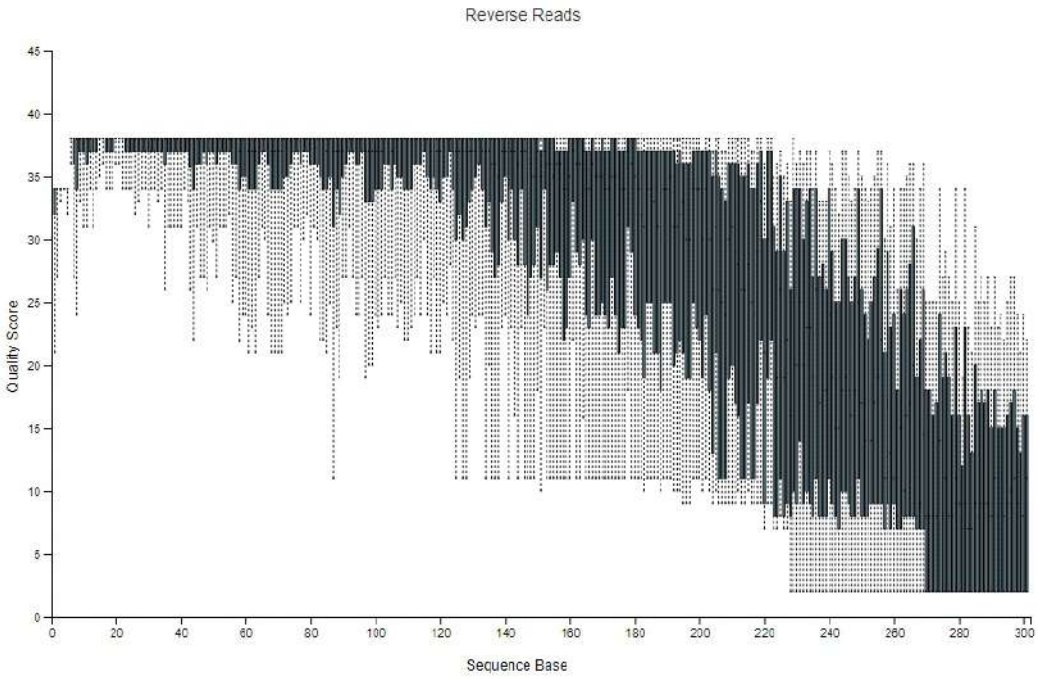


Figure2

Summary of the distribution of sequence qualities at each position in reverse sequences



Results

Bacteriology results for the various used devices removed from each of the patients enrolled in the study are given in Table 1. Bacteria were grown from only four of the patient's samples. Cultivable bacteria such as *Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella* spp, and *E. coli* were isolated from four samples. These four cases from which bacteria were identified correlated well with the diagnosis of clinical infection. Biofilm assay revealed these four bacteria were positive for biofilm production and classified as either moderate or firmly adherent (Table 1) as described elsewhere (9). A very scanty growth with only one or two colonies from the primary specimen is considered insignificant or contaminant when there is no clinical correlation. Such observation was recorded from four samples (viz., F-18, ET-7, ET- 36 & OS-9). The remaining samples did not grow any bacteria. Those samples that showed no bacterial growth were subsequently analyzed through a culture-independent method such as 16S rDNA amplification and sequencing, which revealed the presence of diverse bacterial species.

Initially, 14 samples were processed through culture-independent methods wherein samples were obtained from critically ill patients with life-saving invasive medical devices concerning their daily patient care needs. A total of 14 removed/used invasive devices were included in the metagenome study. Samples were taken for metagenome isolation, followed by the amplification of 16S rRNA gene from that extracted DNA. The Sanger sequencing method for detecting the 16S rRNA gene of the predominant species present in those samples revealed a maximum of two species in most of the samples except one sample (ET-8) with

Table 1

Bacterial culture results for the various used devices from 15 patients had only four samples yielding bacterial growth. 16S rDNA sequencing by Sanger method revealed a maximum of two species are present in most of the samples except one sample that had 16S rDNA sequences belong to three species

Different species (1 or 2) observed through Sanger sequencing of 16S rDNA sequencing	Sample ID details*	Aerobic/anaerobic culture status	Antimicrobial susceptibility testing			Biofilm Production‡
			R	I	S #	
Rothia sp						
Pseudomonas spp (Unc)* †	ET-8	No growth				
Sphingobacteriales bacterium (Unc)* †						
Pseudomonas aeruginosa	ET-9	*Pseudomonas aeruginosa*	R to All	-	-	+++
		Acinetobacter baumannii	AMP,COT	AK	CIP,CTX,IMP,MRP, PB,CL,CFS,GEN	++
Achromobacter xylosoxidans,	ET-11	*Klebsiella spp.*	AMP, GEN, COT	CIP	AK,CTR,TZP,MRP, PB,IMP,COL,CFS	++
Acinetobacter baumannii	FC-2	*Pseudomonas aeruginosa,*	GEN	-	CAZ,TOB,CIP,AK,C L,PB,CFS,IMP,MRP	++
Acinetobacter bouvetii		*Acinetobacter baumannii*	R to All	-	-	+++
Lactobaccilus spp.	FC-5	No growth				
Pseudomonas sp	FC-8	No growth				
Cronobacter sakazakii,	FC-10	No growth				
Serratia marcescens	FC-18	*Stenotrophomonas* spp † (considered as insignificant)	R to All			++
Escherichia coli	FC-25	*E. coli*				
Herbaspirillum sp	FC-47	No growth				
Staphylococcus spp	OS-7	No growth				
Comomonas spp. (Unc)* †	OS-1	No growth				
Enterococcus faecalis	OS-2	No growth				
Proteus mirabilis, *Stenotrophomonas maltophilla*	OS-4	Non-fermentative Gram-negative bacteria †				

‡ - Unc* - uncultivated; * ET- Endotracheal tube; FC- Foly's Catheter; OS- Bone implants # CAZ- Ceftazidime; CFS- Cefoperazone sulbactam; CIP - Ciprofloxacin (CIP); CL - Colistin; COT - Co-trimoxazole; CTR- Ceftriaxone; GEN - Gentamicin; IMP - Imipenem; MRP - Meropenem; PIP - Piperacillin; TZP - Piperacillin-tazobactam; PB - Polymyxin B; TOB- Tobramycin. † Not identified as it is considered insignificant. ‡ ++ = Moderately adherent; +++ = strongly adherent bacteria

Table 2

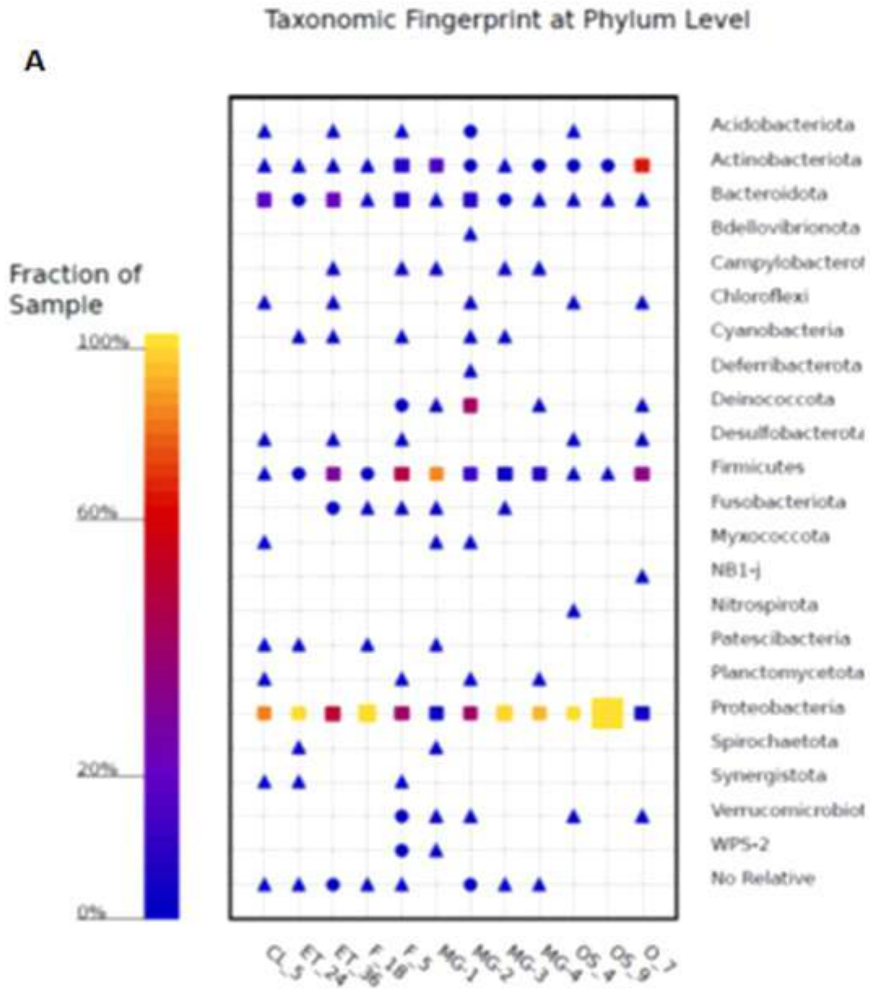
The most predominant species with their OTU values identified through analysis of NGS of Twelve metagenomic samples, which are associated with the device related infection and morbidity

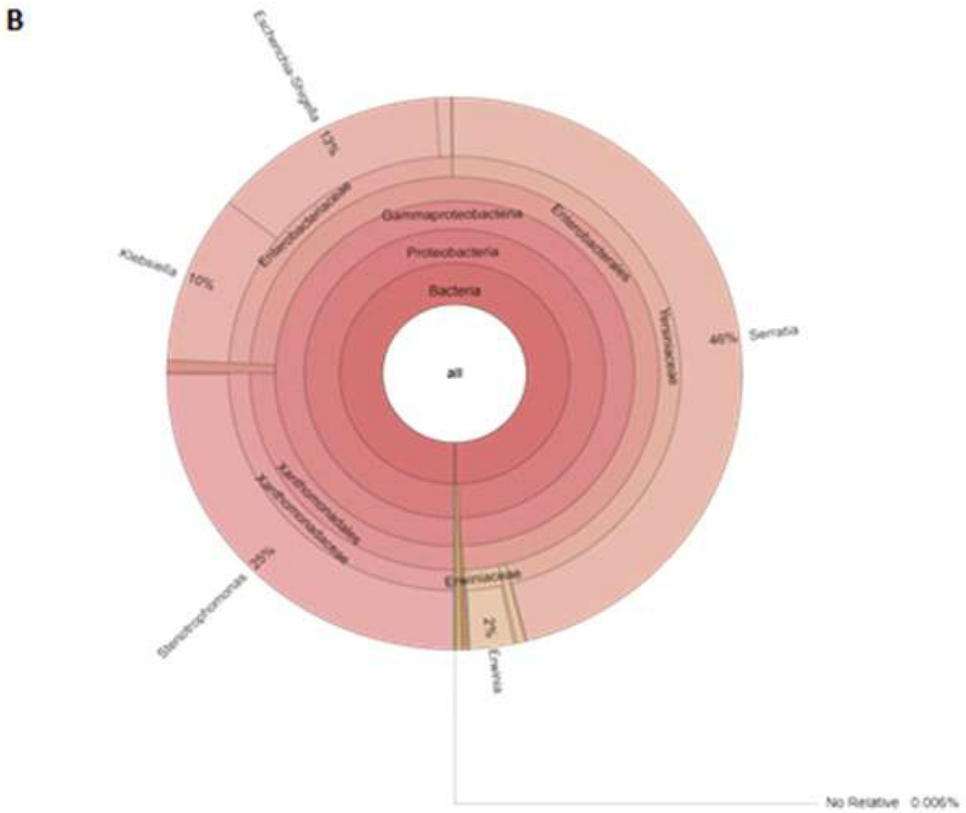
Age/Sex of the Patient	Device Sample ID Details (/Alternative NGS ID$^{\Phi}$)	Primary diagnosis patients back ground and wh device is used‡ and how long it is used	Species identifiec through NGS of metagenome of medical device	Abundance of OTU (HiSEQ)	Different species (1 or 2) detected through Sange sequencing of 16S rDNA sequencing	Microbiologica culture (aerobic) statu	Co-morbidity, antibiotics prescribed and corresponding complication developed with the device
72/M	ET-7/MG3	Encephalitis, Sepsis, 5 days	*Serratia nematodiphila* *Stenotrophomonas maltophilia* *E. coli*	47977 18956 2502	ND	*E. coli* (considered as contaminant)	DM, Meropenem, VAP
81/M	ET8 /MG1	Pulmonary Edema, Acute kidney injury, 11days	*Gemella haemolysins* *Streptococcus infantis* *Rothia mucilaginosa*	35210 34199 25754	*Rothia sp* *Pseudomonas spp (Unc*)* *Sphingobacteria es bacterium (Unc*)*	No growth	HT, Type 2 DM, Meropenem, LRI
44/M	ET-24	Septic Encephalopath Bilateral SDH , 6 days	*Stenotrophomonas maltophilia* *Acinetobacter spp.*	64710 20760	ND	*Acinetobacter spp.*	Colistin, Septic Encephalopathy, VAP
43/M	ET-36	Sepsis, Septic Encephalopath 10days	*Acinetobacter spp.* *Staphylococcus spp.* *Elizabethkingia*	64172 45414 26443	ND	*E. coli* (considered as contaminant)	Type 2 DM, Vancomycin, Bed sores
35/F	FC-5	UTI, 7 days	*Shigella spp.* *Lactobaccilus spp.*	22140 18994	*Lactobaccilus spp.*	No growth	UTI
45/M	FC-12 /MG4	UTI, 8 days	*Acinetobacter baumannii* *Enterobacter asburiae*	27564 22777	ND	No growth	UTI
75/M	FC-18	UTI, 10 days	*Serratia nematodiphila* *Stenotrophomonas spp*	21886 9907	*Serratia marcescens*	*Stenotrophom nas* spp (considered insignificant)	UTI
60/M	OS - 4	Prosthetic joint implant, >14 days	*Stenotrophomonas spp.*	105372	*Proteus mirabilis*, *Stenotrophomc nas maltophilla*	Non-fermentative Gram-negative bacteria‡	Prosthetic joint infection
47/F	OS-7	Bone Infection, implant ,>12 day	*Corynebacterium spp.* *Staphylococcus spp.*	69576 14867	*Staphylococcus spp*	No growth	Amoxycillin/ clavulanate Bone Infection
70/M	OS-9	Bone Implantation,	*Stenotrophomonas spp.* *Proteus spp.*	10609 9093	ND	Aerobic spore forming Gram-positive bacteri (considered as contaminant)	Implant Infection
75/M	CL -5	Bacteraemia, 7 days	*Stenotrophomonas maltophilia* *Stenotrophomonas spp.* *Elizabethkingia*	76421 19238 15559	ND	No growth	Meropenem, Septic Encephalopathy
35/M	JIP2 /MG2	Infected Bone implant , 16 day	*Schlegelella aquatica* *Meiothermus Silvanus* *Vulcaniibacterium (Unc*)*	36424 32389 6978	ND	No growth	Prosthetic joint infection

** CL – Central venous catheter line; ET- Endotracheal tube; FC- Foly's Catheter; OS - Bone implants; JIP – Bone implant (joint); HT- Hypertension; DM- Diabetes mellitus; VAP- Ventilator Associated pneumonia. Synonymous IDs -ET 7= MG3, ET8=MG1, FC-12 =MG4 and JIP2 =MG2. ‡ Not identified as it is considered insignificant; ND- Not done. † - Development of infection is the main reason for removal of the device/implant.*

Figure 3 A

Taxonomy fingerprint at the phylum level obtained in the SILVAngs pipeline and **Figure 3 B** *Taxonomy krona chart at the species level obtained in the SILVAngs pipeline*





sequences that belonged to three species (Table 1). Along with some commonly encountered nosocomial pathogens such as *Acinetobacter baumannii, Enterococcus faecalis, Stenotrophomonas maltophilla, Pseudomonas aeruginosa* etc., many rare bacterial species namely *Achromobacter xylosoxidans, Acinetobacter bouvetii, Comomonas spp., Cronobacter sakazakii, Herbaspirillum* spp, *Rothia* spp and *Sphingobacteriales* bacterium that are seldom encountered in the clinical samples were identified from these samples (Table 1).

Subsequent sequencing analysis performed using the Illumina NGS platform for 12 metagenome samples extracted from the used device samples identified and detected the most abundant genera/species present in those samples more precisely. Five samples initially analyzed through Sanger sequencing were also repeated in NGS analysis (ET8, F-18, F-5, O-7, and OS-4). The abundant OTU information obtained through multiple analytic tools such as UPARSE, QIIME 2, and SILVAngs analysis pipeline identified the predominance of 24 bacterial genera/groups identified across the 12 samples. Taxonomy fingerprints at the phylum level obtained in the QIIME 2 and SILVAngs) analysis pipeline showed limited diversity concerning overall samples except for F5 and MG-2 (**Fig. 3A** & **B** and **Fig. 4**, respectively). Analysis of individual samples revealed the presence of limited numbers of OTUs ranging from a meager five up to a maximum of 40 except for two samples such as MG2 and F5, which had an increasing number of OTUs like 67 and 109, respectively. MG2 and F5 samples harbor biomass comprising more microbes belonging to diverse species. The remaining nine samples showed a high abundance of only one or two particular kinds of OTUs belonging to particular species more likely responsible for causing infection (Table 2). Overall top ranked bacterial genera/species, and the relative abundance of different species classified based on the OTUs at the taxonomic level of species present in the 12 metagenome samples were given in **Fig. 5**.

Analysis of NGS of 16S rRNA DNA with the QIIME2 tool helped us to know the required sampling depth for enumerating all the available microbial diversity in the sample. Rarefaction plots are generated through the QIIME2 tool, which is mainly used as a measure of sampling depth required to get maximum diversity information about the sample. The sampling depth appeared adequate to cover the maximum diversity existing in our device samples. The box plots in **Fig. 6** and **Fig. 7** represented the distribution of the selected alpha diversity metric for each of the samples studied and their sampling depth. The sequencing output for each sample is 0.2 million reads. The box plot's lower and upper whiskers are the distribution's 9th and 91st percentiles (respectively). In contrast, the box's lower and upper extents are the distribution's 25th and 75th percentiles (respectively). The horizontal bar through the middle of the box is the median of the distribution (i.e., the 50th percentile). Outlier points of these distributions are not shown. The line chart in the upper figure connects the median values of the alpha diversity metric distribution across the sampling depths. If a sampling depth is higher than the number of sequences in a sample, that sample is not included in the rarefaction plot at that sampling depth.

Correct identification was possible when there were optimal identity thresholds that were 100% for the V4 hypervariable region. The most abundant OTUs predominantly present that had optimal identity thresholds of 100% for the V4 hypervariable region from each of these samples are observed to be limited to only two or a maximum of three species, which appears to be very significant in the

Figure 4

Taxa plot based on the 16S amplicon sequencing, processed using QIIME2 pipeline

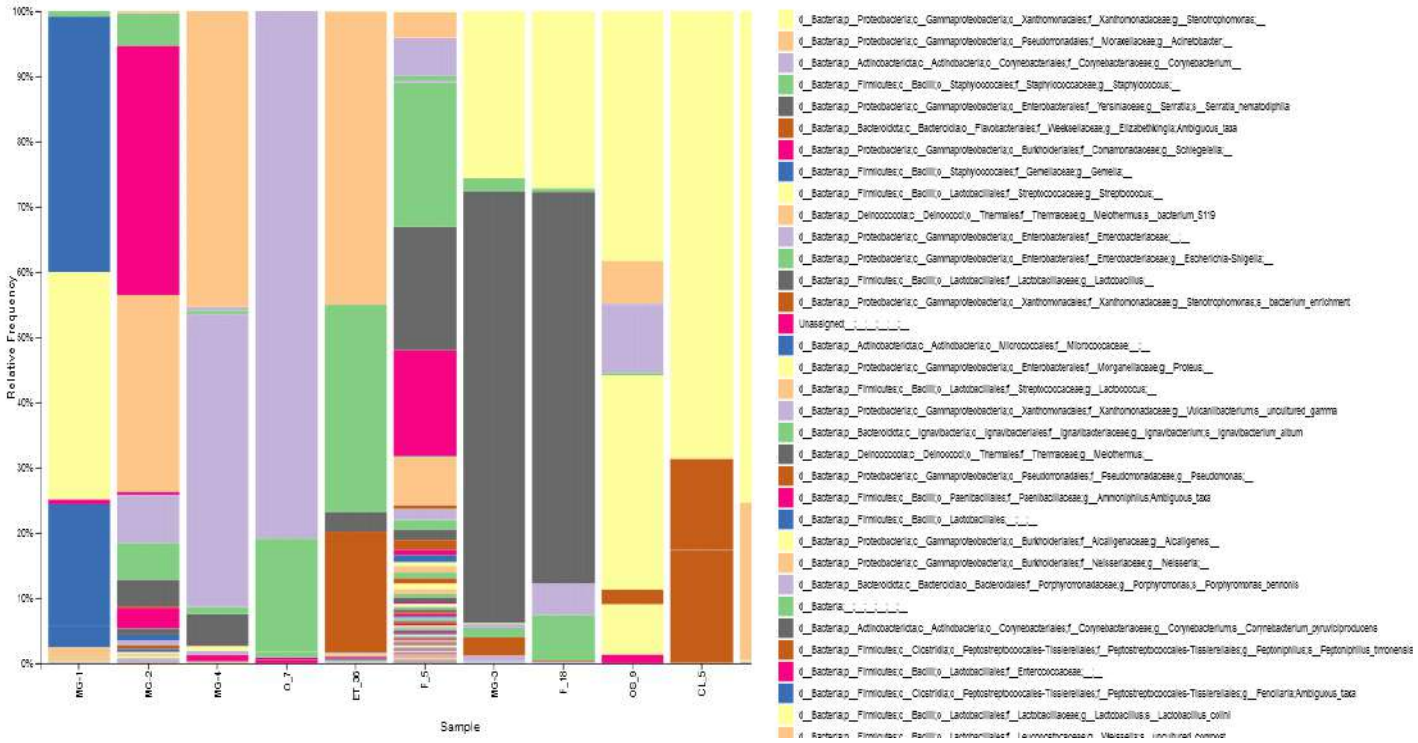


Figure 5

Overall relative abundance of different genera (top ten) classified based on the OTUs at taxonomic level of genus in all the devise metagenome samples analyzed

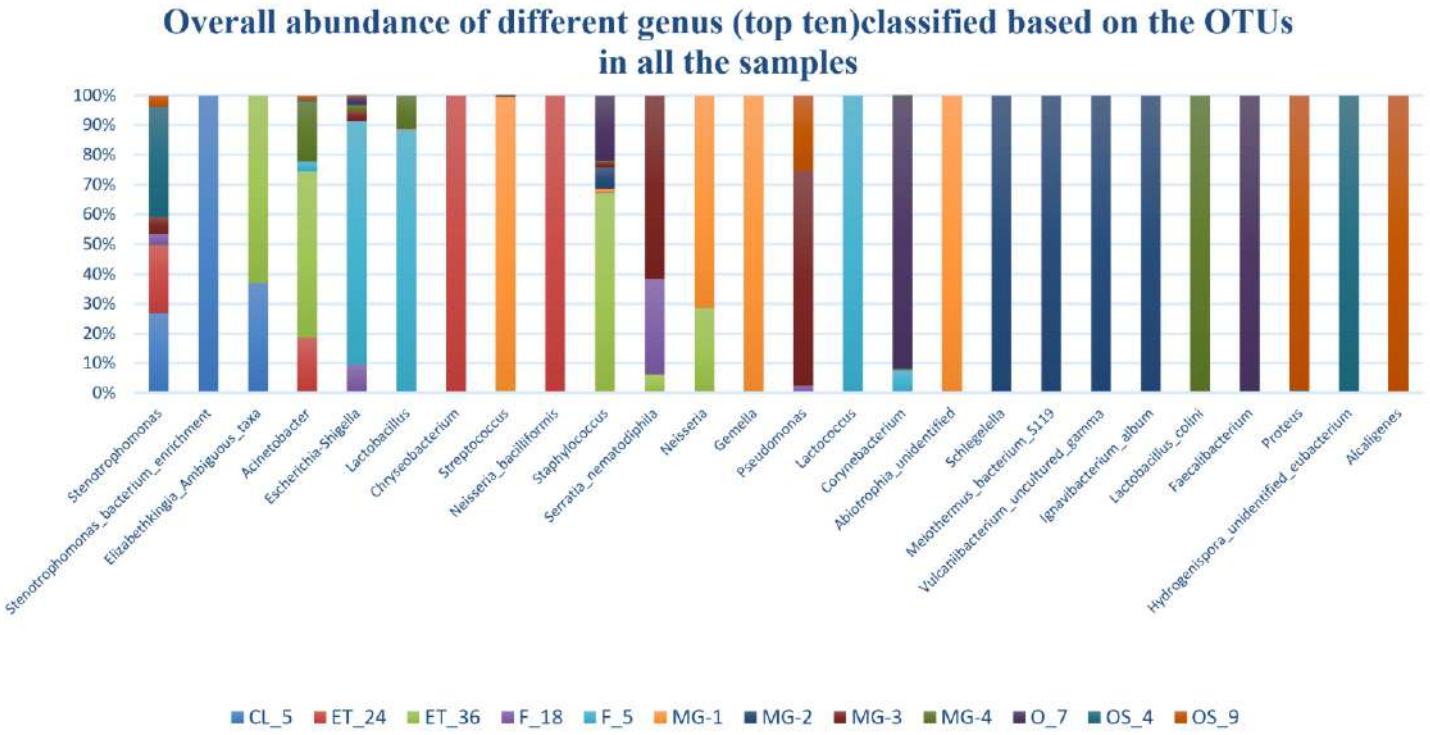
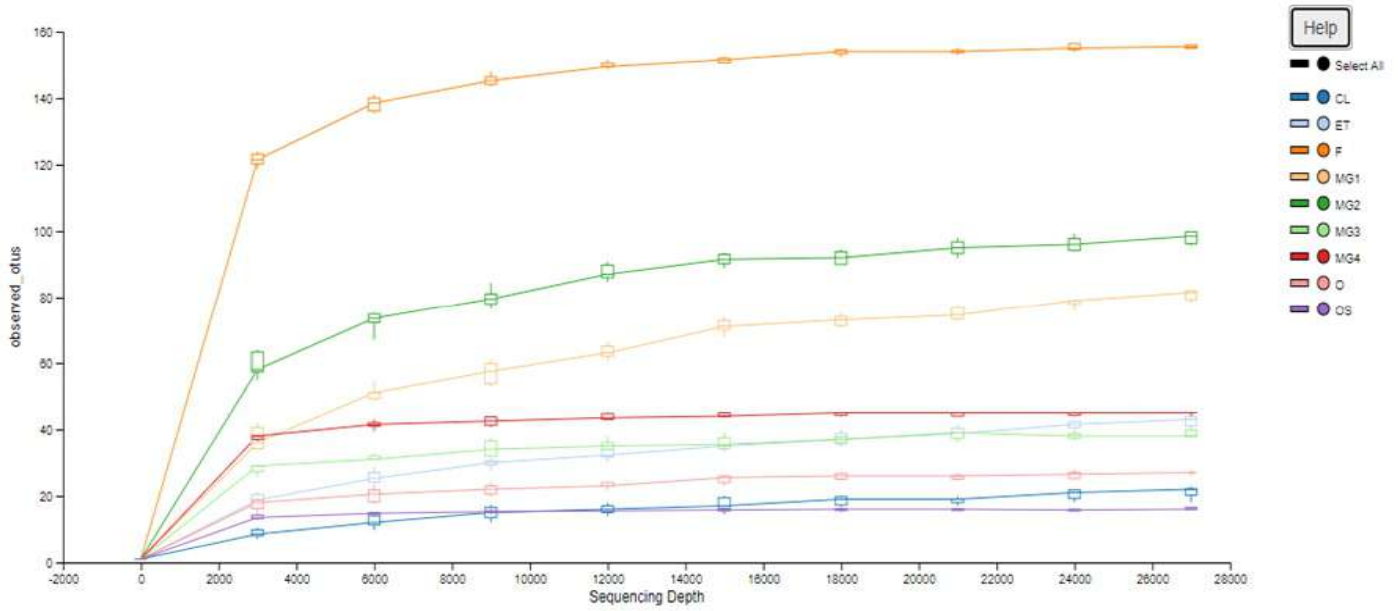


Figure 6

Alpha-diversity rarefaction plot as a measure of observed_OTUs

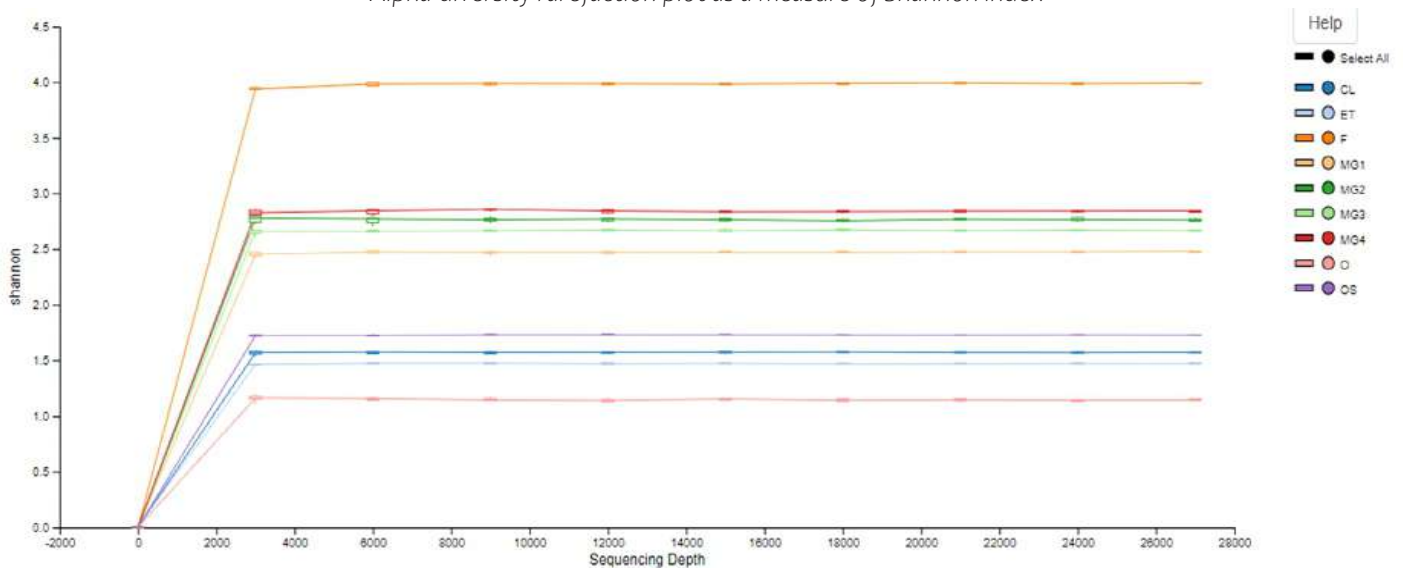


present study. Consequently, so identified species through such OTUs have to be studied for reasons behind their success in causing chronic device-related infection and yet manifested themselves as responsible for culture-negative infections. The most predominant species identified through their OTU abundance from each of these 12 metagenomic samples, which are likely responsible for the device-related infections and morbidity, is given in Table 2. Apart from common nosocomial pathogens such as *Staphylococcus spp, Acinetobacter baumannii, Stenotrophomonas maltophilia, and Elizabethkingia spp.*, most of the bacteria that are identified in the metagenomic samples are either uncultivable or they need a special media with unique ingredients to culture them in routine clinical microbiological laboratory. Many species, include *Streptococcus infantis, Gemella haemolysans, Meiothermus silvanus, Schlegelella aquatica, Rothia mucilaginosa, Serratia nematodiphila, and Enterobacter asburiae* that are encountered in the study are rarely found in the clinical specimens.

For the sample, the ET-8 results of the Sanger method differed somewhat from the NGS results. Rothia spp (Micrococci) and Sphingobacteriales bacterium identified through Sanger were not predominant species in NGS,

Figure 7

Alpha-diversity rarefaction plot as a measure of Shannon index



wherein *Streptococcus infantis, Gemella haemolysins* were predominantly observed in terms of abundance and OTU measure. Some of the rare bacterial species, such as M. silvanus and S. *nematodiphila* encountered in the device microbiome have never been reported as a cause of human infection.

Discussion

The risk of development of infection is unacceptably very high in Indian hospitals when invasive medical devices are used after the surgery. Therefore, a greater understanding of which type of microbes are involved in the development of infection will be essential for better management and devising new and improved methods for treating such infections. Moreover, there needs to be more knowledge of the prevalence of device/implant-related infections in the country. Most of the time, there will be controversies regarding the source of infection, which is debatable. The skin microbiota of the patients and/or hospital staff has been presumed to be a likely reservoir for many such infections. Moreover, many implant-associated infections do not yield microbial growth in routine bacteriological culture, yet all the clinical signs indicate the infection. Chronic persistent infections tend to give culture-negative results while using routine microbiological media, indicating that there is a special requirement for growing certain fastidious organisms responsible for such infections.

The present investigation tested the used clinical devices that were removed during the clinical management of infection for detecting the presence of unique microbiota. As the microbiome of the used clinical device is less complex since it is retrieved from the sterile interior of humans, one can expect only a few types of microorganisms or none. As expected, our pilot analysis by Sanger sequencing of 16S rDNA amplicons obtained from the device biome showed the presence of very few species of bacteria in each of these clinical samples. Among the total of 21 samples analyzed, five common samples that were subjected to both direct sequencing by the Sanger method and NGS of 16S rDNA metagenome had a common finding wherein they found the presence of the same predominant species in each of these samples except ET-8, as its Sanger method results were not completely identical with the NGS results. Interestingly, Sanger sequencing and NGS of 16S rDNA metagenome results obtained from the device biome could identify many rare bacterial species in the device samples, namely *Achromobacter xylosoxidans, Comomonas spp., Cronobacter sakazakii, Herbaspirillum spp, Rothia spp, Gemella haemolysins, Acinetobacter bouvetii, Meiothermus Silvanus, Schlegelella aquatic, Serratia nematodiphila, Enterobacter asburiae* and *Sphingobacteriales* bacterium that are seldom encountered in the clinical samples. This investigation used a small number of clinical samples for NGS because of cost concerns, which is the study's main limitation. However, as the samples taken in this study are diverse and representative, the results of this pilot study might lead a path to study a large number of device samples, and such an increase in samples may validate and reconfirm the diversity of device microbiome as well as newer bacterial species that were identified in this study.

In the following few paragraphs, we looked into individual bacterial species grown on the devices and their association with infection by performing a literature survey. *Achromobacter xylosoxidans* is one bacterium identified in this study from the metagenome of an endotracheal device removed from the patient suspected of suffering from lower respiratory tract infection, whose endotracheal aspirate had mild growth of *Klebsiella* spp, which was susceptible for most of the antibiotics possibly indicating a polymicrobial infection. In recent years, *Achromobacter* spp. particularly A. *xylosoxidans* is rapidly emerging as critical opportunistic bacteria responsible for various nosocomial or community-acquired infections. Although the predominance of A. xylosoxidans was previously reported only in respiratory infections among the patients suffering from cystic fibrosis, this bacteria is now known to affect multiple organs and their systems, which include the respiratory tract, urinary tract and, less frequently, the cardiovascular and central nervous

systems (22 -23). Despite an increasing number of cases published in the last decade suggesting a global increase in diseases caused by *Achromabacter* spp., most clinicians still remain uncertain of the organism's significance since its prevalence has been underestimated by clinicians. The present study reemphasizes the importance of *A. xylosoxidans* in chronic lung infections.

Comamonas species are Gram-negative rods that grow as pink-pigmented colonies in various environments. Despite their common occurrence in nature, they seldom cause human infection. However, it is shown that *Comamonas* infections are mostly seen in patients with predisposing factors such as cancer and continuous use of devices such as central venous catheters and contact lenses (24-25). The occurrence of *Comamonas* species in our sample correlates with the clinical findings and patient-related details of earlier research observations wherein the patients were immunocompromised and with central venous catheters.

Cronobacter sakazakii, a Gram-negative rod-shaped bacterium, is shown to be a rare cause of invasive infections such as *sepsis*, meningitis, and necrotizing enterocolitis with very high death rates, primarily affecting newborns. One recent report confirmed a post-surgical osteomyelitis of the femur caused by *C. sakazakii* in a young, otherwise healthy man (26). In our study, this organism was detected in the catheter sample of urinary tract infection cases affecting an older male patient. Although rare, *C. sakazakii* is known to cause urinary tract infections in elderly patients (27). *Herbaspirillum* spp. are Gram-negative bacteria that inhabit soil and water. Infections such as pneumonia and bacteremia caused by this organism have been reported in immunocompromised hosts (28). The present study identified *Herbaspirillum* spp. in the clinical sample of a patient suffering from osteomyelitis.

Previous works have demonstrated that the microbiota of the oral cavity is the common source of dental infection in certain patients (29-31). In the present study, two important species that are implicated in causing VAP, namely *Rothia mucilaginosa* and *Gemella haemolysins*, are Gram-positive cocci that are shown to be normal inhabitants of the human oral cavity and the mucous membranes of the oropharynx in earlier investigations (32), which could be the source of infection. However, among these bacterial species, *G. haemolysans* though uncommon can cause invasive diseases such as meningitis in humans as described elsewhere (33-34). Besides, it is known to cause device-related invasive brain disease. Therefore, we cannot rule out the possibility of *R. mucilaginosa* and *G. haemolysins* causing lung infection in our investigation.

Meiothermus silvanus species is another important bacteria on the bone implant in the present study. It was shown earlier that *M. silvanus* is a pertinent biofilm former on medical implants or present in the wet end of paper machines. Field emission scanning electron microscopic analysis revealed that *M. silvanus* can grow on stainless steel using thread-like organelles for adhesion and biofilm formation (35). The present study also detected the presence of *Serratia nematodiphila, Elizabethkingia spp, Stenotrophomonas maltophilia, Enterococcus feacalis* on endotracheal tubings and central venous catheters, which are the most possible causal organisms for the chronic persistent infections predominantly affecting lungs and other organs. In this regard, the surrounding environment may act as a reservoir for certain kinds of pathogens that are capable of causing nosocomial infections (36). Here, bacteria contamination levels are directly associated with humidity on the device surfaces; cleansing tap water might be the point of dispersion of potentially pathogenic bacteria.

Schlegelella aquatica is a Gram-negative, motile, non-pigmented, rod-shaped bacterium that has been shown to be associated with microflora obtained from cervical lesions in HIV-positive women (37), which was not usually part of normal cervical microflora of healthy women. *Schlegelella* presence in changed microflora was also observed in the cutaneous microbiota obtained from psoriasis patients (38). The Presence of *S. aquatica* in bone implantation case in the present study indicates possible bone infection by this organism. However, the implant device sample was culture-negative in this case, indicating the fastidious nature of this bacterium. Earlier studies have shown that *S. aquatica* is a part of dysbiosis induced by a disease that brings in physiological changes at the lesion site and the whole body level leading to selection for specific and differential microbiota (38). Therefore, it is crucial to emphasize such generation of differences in microbial community structure in diseased patients that could be of potential pathophysiologic and diagnostic significance.

One important study on clonal diversity and dynamics of Streptococcal species (39) revealed that *Streptococcus mitis, S. oralis* and *S. infantis* are the most abundant members of the commensal microbiota of the upper respiratory tract (URT) as evidenced by their investigation that used culture-independent tools based on cloning and sequencing of housekeeping gene *gdh*, which is species-specific and yet it is remarkable in detecting the genetic polymorphism that has helped them to find the origin of frequently emerging clones in that habitat (39). Our investigation also detected *S. infantis* growing on the endotracheal tubing used to treat one of the patients, which could be device contamination since this bacterium is a commensal bacterium of URT.

The present investigation identified the presence of *Enterobacter asburiae* on the Foleys catheter that could not be cultivated. One previous work has described the emergence of *E. asburiae* in the Czech Republic, which can produce IMI-2 carbapenemase, which is of much concern (40). Further, outer membrane proteins (OMPs) namely, OmpC and OmpW were also detected in the colistin-susceptible *E. asburiae* in that study, which demonstrated the OMP differences between colistin-susceptible and -resistant *Enterobacter* strains. It is presumed that the altered Gram-negative cell wall may contribute to the acquired colistin resistance in Enterobacteriaceae. Though resistant *E. asburiae* has been

implicated in many kinds of infections, the clinical significance of its isolation from a urinary catheter in our study is not clear, though their implication for UTI cannot be ruled out.

Many earlier investigations have shown that PCR assays involving the amplification of the 16S rRNA gene, a highly conserved region within the bacterial genome, is the most valuable tool in detecting the bacterial types involved in culture-negative device/implant-related infection (41-43). In our investigation, Sanger sequencing of the 16S rRNA gene was able to detect a maximum of three bacterial species in a single sample based on its predominance in DNA content. In contrast, the NGS method could detect more species and all the unique species of bacteria that are present in minor quantities, which could be responsible for an infection that is not readily detected by the former method. Metagenome sequencing of 16S rRNA, particularly V3-V4 region sequencing of our samples, led to the identification of diverse microflora. UPARSE, QIIME 2, and SILVAngs analysis pipeline revealed 24 predominant bacterial genera and up to 109 kinds of OTU in our samples. Certainly, one can presume that the microbiome present in clinical devices may be less complex than microbiomes in other environmental niches. It is natural to expect that only a few species capable of resisting host immune components may survive in such environments. Besides, taxonomic units identified are usually reduced substantially by the UPARSE algorithm used in the present study as a result, the data generated suggest a robust estimate of the taxa present in a given sample. However, UPARSE tends to discard singletons by default which might reduce the sensitivity of the analysis by eliminating a few rare taxa, especially for samples with low read coverage (17). As the coverage is very high in this study and the samples are less complex than other kinds of metagenomic samples studied, species belonging to rare taxa may not have been missed in our samples. Besides, rarefaction plots generated by the QIIME2 tool revealed that the sampling depth is adequate enough to cover the maximum diversity existing in the device samples used in the study. In addition, SILVAngs 1.4 analysis module was also used for detecting taxonomic units, and this module checks the sequence quality of each read and selects high quality, and filters out all the low-quality reads, wherein reads shorter than 50 aligned nucleotides and reads with more than 2% of ambiguities, or 2% of homopolymers, respectively, were excluded in the analysis (20). Therefore high sequencing coverage of less complex samples studied here has not missed the identification of rare species due to the tools used to analyze the metagenome.

Conclusion

To conclude, metagenome sequencing of 16S rRNA V3-V4 region through NGS has identified the existence of a diverse range of cultivat and un-cultivable bacterial species, and some of these constituted very rare species that grew as biofilms on the surface of various clinically infected devices that were removed from the patients during the treatment management. The results of our investigation revealed that a culture-free, holistic metagenomic approach through the usage of NGS could be a better method to identify the diverse and rare pathogens in culture-negative cases of chronic device-related infection.

Data Submission

The study and generated metagenomic (16S) NGS reads have been submitted to European Nucleotide Archive (ENA), European Molecular Biology Laboratory, European Bioinformatics Institute (EMBL-EBI). However, the study has been assigned with a bioproject accession number (**PRJEB43468**) and samples assigned with accession number (**ERS5890955-ERS5890966**).

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Conflicts of interest

The author(s) declare that there are no conflicts of interest.

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Author Contribution Statement

The authors confirm contribution to the paper as follows: Study conception and design: KP, KV & JM; Data collection: KP, ARS, ARA, KV, JM & BT; Analysis and interpretation of results: KP, ARS, KA, JM, ASA & BT; Draft manuscript preparation: KP, ARS, AKA. All authors reviewed the results and approved the final version of the manuscript. All authors agreed to be responsible for all aspects of the work to ensure the accuracy and integrity of the published manuscript.

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