



Inguinal microbiome in patients undergoing an endovascular aneurysm repair: Application of next-generation sequencing of the 16S-23S rRNA regions

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ABSTRACT

Background: Surgical site infection (SSI) remains a hazardous complication after vascular surgery. In this pilot study we investigated the inguinal microbiome in skin biopsies using histology and 16S-23S rDNA Next Generation Sequencing (NGS). Our hypothesis was that causative microorganisms of SSI are present in the inguinal microbiome.

Methods: Data on surgical site infections and skin samples from the *Percutaneous in Endovascular Repair versus Open* (PiERO) trial were evaluated. Two patients with SSI were matched for age and comorbidity to eight matching patients of the PiERO trial. All patients were treated for an abdominal aortic aneurysm with endovascular repair. Nasal and perineal cultures were taken preoperatively to detect *Staphylococcus aureus* carriage. After disinfection with chlorhexidine, groin biopsies were taken to identify bacteria in deeper skin layers. All samples were subjected to histological analysis and culture-free 16S-23S rDNA NGS.

Results: *Staphylococcus aureus* species were cultured in 5 out of 20 preoperative nasal and perineal swaps. Histology detected only a few bacteria. NGS of the 16S-23S rRNA regions identified DNA of bacterial species in all biopsies (20/20). Most identified genera and species proved to be known skin flora bacteria. No relation was found between SSIs and the preoperative microbiome.

Conclusion: In this pilot study, an innovative analysis of the preoperative microbiome using 16S-23S rDNA NGS did not show a relation with the occurrence of a surgical site infection. No pathogenic bacterial species were present in the inguinal skin after disinfection with chlorhexidine.

Introduction

Surgical site infections (SSI) after vascular surgery may cause hazardous situations. The incidence of SSI varies from 1–6% after aortoiliac surgery [1] to 7–20% after open peripheral bypass surgery [2–4]. Frequently prosthetic graft material is used in vascular reconstruction, increasing the risk of infection. A prosthetic graft infection is usually preceded by an SSI, risking anastomotic bleeding, sepsis, and even death [5,6]. This stresses the importance to prevent any SSI in vascular surgery.

A diverse population of microorganisms that can be pathogenic,

harmless or even beneficial colonizes the human skin. Exogenous and endogenous factors, together with the topographical location, are responsible for the composition of the microbiome present in different areas of the human skin [7]. Studies focusing on finding the original pathogenic source of SSI could not identify a causative relation between intraoperative cultures or biopsies and agents identified during infection [8–10]. Although a gradual reduction in SSIs is achieved [11], SSI incidence remains relatively high after an inguinal approach in vascular surgery [2,3,12]. This inguinal microbiome needs further exploration, specifically in relation to the shift from an abdominal surgical approach toward a minimally invasive inguinal approach by transfemoral access

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to the abdominal aorta.

Techniques to detect and identify microbes have always focused on visualization through light or electron microscopy or growth on culture media. However, light microscopy shows bacterial shapes but cannot identify SSI pathogens. Conventional bacterial culturing is a sensitive method to identify possible pathogens, but it can take days to weeks to culture bacteria, as some clinically relevant bacteria are slow or difficult to grow [13]. Molecular tests (e.g. polymerase chain reaction (PCR)) that target specific microorganisms have proven to be more rapid and sensitive than culturing [14], yet need a priori knowledge of the likely pathogenic species that could be present in a sample. As a complementary approach to culture, Sanger sequencing of the variable 16S rRNA gene has emerged for identification purposes [15]. The 16S rRNA gene (~1.5 kilobase (kb) in length) has proven to be a useful molecular target as it is present in all bacteria, either as a single copy or in multiple copies, and is well preserved over time [16]. Yet this method fails to identify some bacteria at the species level due to high-sequence similarities between some bacterial species [17]. Although Sanger sequencing of the 16S rRNA gene can be applied directly on clinical samples, it is challenging to identify more than one species simultaneously, which precludes its use in polymicrobial samples [18].

Next-generation sequencing (NGS) offers higher resolution and accuracy in identifying microbial species [19,20]. This technology allows culture-independent testing of polymicrobial samples, detecting multiple species in parallel [21]. A method based on NGS of PCR products obtained from amplification of the 16S-23S rRNA gene internal transcribed spacer regions (~4.5 kb, 16S-23S rRNA NGS) has recently been developed, with higher resolution and faster results. 16S-23S rDNA NGS allows culture-independent detection of bacteria in polymicrobial samples [21,22]. Ribosomal DNA (rDNA) provides the genetic coding from which rRNA molecules are constructed.

Hypothesis

We hypothesized that identifying the inguinal skin microbiome with 16S-23S rDNA NGS may assist daily clinical practice in detection of pathogenic bacteria, aiding the prevention of infectious complications in aortic and peripheral vascular bypass procedures.

Methods and materials

The clinical, multicenter randomized trial *Percutaneous in Endovascular Repair versus Open* (PiERO) addressed the risk of SSI in inguinal endovascular aneurysm repair (EVAR) (trialregister.nl NTR4257) [23,24]. *Staphylococcus aureus* (*S. aureus*) carriage was determined with preoperative cultures of nose and perineum in all patients of the PiERO trial. In this pilot study we examined the skin groin biopsies of 10 selected PiERO patients using regular culture methods, microscopy techniques and NGS of the 16S-23S rRNA region [25].

Patient population

All patients received prophylactic antibiotics according to protocol (1 gr cefazolin intravenously). Culture swabs were taken from the nose and perineal regions (Amies transport medium). After chlorhexidine skin disinfection, two 3 mm biopsies were taken from the right groin. One sample was preserved in a culture medium (Thioglycollate Medium USP, Mediaproducts, Groningen, The Netherlands) and another kept in formalin solution. For feasibility reasons 10 patients were selected for this pilot study. The selection was based on the only two PiERO patients that developed SSI. SSIs were defined according to the Centers for Disease Control and Infection Prevention (CDC) guidelines [26]. Each patient that contracted SSI (case 1 and case 2) was matched for age and smoking habits with four other patients from the trial (Table 1).

Objectives

Our primary objective was to analyze the composition of the inguinal microbiome in patients undergoing an EVAR. Secondary objective was to examine the correlation between *S. aureus* carriage, with 16S-23S rDNA NGS identified bacteria and SSI in the PiERO trial.

Bacterial cultures

The swabs of the nasal and perineal regions were cultured on a 5% sheep blood agar (Mediaproducts BV, Groningen, The Netherlands) and the presence of *S. aureus* was identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS) (VITEK MS, bioMérieux Inc., Durham, NC, USA) and the coagulase test. The swabs of the SSI were cultured on a 5% sheep blood agar, a Media CAP agar with colistin and aztreonam, and a standard MacConkey plate (Mediaproducts BV).

Histological analysis

The formalin-fixed skin biopsies were paraffin-embedded. Subsequently, 4 µm sections of tissue were mounted on pre-coated slides. The tissue was dewaxed with xylene and rehydrated through graded alcohol following standard procedures. Additionally, sections were stained with hematoxylin and eosin stain and Gram stain (PREVI® Color, bioMérieux Inc., Durham, NC, USA) and then coverslipped.

Next-generation sequencing of the 16S-23S rDNA regions

DNA-extraction

The Purelink Genomic DNA purification kit (Invitrogen, Carlsbad, CA, USA) was used for DNA extraction of the skin biopsies. A small piece was digested in 180 µl digestion buffer and 20 µl proteinase K (Invitrogen). Digestion was performed in a thermoshaker at 56 °C until lysis was complete. 200 µl Purelink Genomic lysis/binding buffer was added to 200 µl of lysed sample and vortexed to create a homogenous solution. 200 µl 96% ethanol was added and the DNA purification protocol was followed according to the manufacturer's instructions.

Next-generation sequencing of the 16S-23S rRNA encoding regions

The 16S-23S rRNA region was amplified by PCR using forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), specific for the 16S rRNA gene [27], and reverse primer 2490R (5'-GACATCGAGGTGCCA AAC-3'), specific for the 23S rRNA gene and slightly modified by truncation of a single nucleotide compared to the original publication [28]. Amplification of the 16S-23S rRNA regions was carried out in a 25 µl reaction consisting of 1x Phire hotstart buffer (Thermo Fisher Scientific, Breda, The Netherlands), 5 mM dNTPs (Roche), 0.5 µl Phire hotstart II DNA polymerase (Thermo Fisher Scientific), 600 nM of each primer and 5 µl of DNA template. PCR was performed using a Biorad PTC-200 thermocycler. PCR fragment detection was performed with the Agilent 2100 Bioanalyzer and the DNA 7500 kit (Agilent, Santa Clara, CA, USA). PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany). A negative process control consisting of a sample without human skin was used to access the presence of process-related contaminants.

For library preparation, the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) was used according to the manufacturer's instructions. The purified PCR amplicons quantified with a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) were diluted to 0.2 ng/µl and a total of 1 ng DNA was tagmented at 55 °C for 5 min followed by PCR amplification to introduce Illumina index sequences. The library DNA fragments were size-selected and purified using AMPure XP beads (Beckman Coulter, Brea, CA, USA.). The indexed libraries were normalized, pooled and loaded onto an Illumina MiSeq

Table 1
Patient characteristics, pre-operative cultures and SSI-result.

	Case 1	Case 2	Comp.Case 1		Comp.Case 2		Comp.Case 1		Comp.Case 2	
			Ctrl 1	Ctrl 2	Ctrl 3	Ctrl 4	Ctrl 5	Ctrl 7	Ctrl 6	Ctrl 8
SSI	+	+	–	–	–	–	–	–	–	–
Age	72	72	72	72	73	70	72	75	72	70
Smoker	No	Yes	No	No	Yes	Yes	No	No	Yes	Yes
Diabetes mellitus	–	+	–	–	–	–	–	–	–	–
SA nasal culture	–	–	–	–	+	+	–	–	–	+
SA perineal culture	–	–	–	–	–	+	–	+	–	–

SSI: surgical site infection, + present/positive, – absent/negative. Ctrl: Control, SA: *Staphylococcus aureus*. Comp.Case: comparison with case.

reagent cartridge using MiSeq reagent kit v3 and 600 cycles. The paired-end 2x300 bp sequencing was run on an Illumina MiSeq sequencer.

Data analysis of the 16S-23S rDNA NGS

NGS generated 700,000–1,000,000 sequencing reads per sample. The FASTQ files containing the 300-nucleotide paired-end reads were de novo assembled into contigs with CLC Genomics Workbench software (Qiagen). *De novo* assembly of the reads was performed with a minimum contig length of 1500 bp using CLCbio. Resulting contigs were filtered to a subset using total thousand read count and an average of hundred read coverage.

Bacterial species identification

Following the *de novo* assembly the generated contigs were assigned a taxonomic classification by alignment using the nucleotide Basic Local Alignment Search Tool (BLAST) against the nucleotide collection database (NCBI database, December 2016). The alignment on NCBI was manually performed by submitting contigs' sequences via the website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Results were sorted for the best Per.ident.score (percent identity score): bacteria were assigned to a species or genus when the identity score was $\geq 98.6\%$ or between 90% and 98.6%, respectively. An identity score of $< 90\%$ was interpreted as an unidentified microorganism. If more than one contig was generated for the same species, the reads of all contigs belonging to the same species were added up and the relative abundance of that particular bacterial species in each sample was calculated by dividing the total read count of the corresponding contigs by the total number of mapped reads in the sample.

Statistical analysis

Patient characteristics were expressed as mean and standard deviation (SD) for continuous data. Categorical data were expressed as frequencies and percentages. The Statistical Package for the Social Sciences, v 24 was used to make calculations (SPSS Inc., Armonk, NY, USA).

Ethics statement

All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki for medical research. The Institutional Review Board approved the study protocol UMCG research no. 2013 365. All patients provided written informed consent. Data were analyzed anonymously.

Results

The study population of the PiERO trial consisted of 137 patients, 10 of whom were selected for the current study (Fig. 1). Patient characteristics are presented in Table 1. Mean age was 72 years (range 70–75). All patients were male and five were smokers (1/2 with SSI and

4/8 controls). One patient suffered from diabetes mellitus. Preoperative nasal and perineal culturing yielded 5 out of 20 cultures positive for *S. aureus*. Two patients (2/10) had nasal *S. aureus* colonization, one patient (1/10) had perineal colonization, and one patient (1/10) was colonized in both nose and perineum. Both SSI cases had neither nasal nor perineal *S. aureus* colonization (Table 1).

Histological analysis

Although standard histological analysis by light microscopy detected few bacteria in the skin, identification of bacterial species is impossible. If bacteria were recognized, they were localized adherent to skin adnexa – hairs and sebaceous and sweat glands (Figs. 2 and 3). Histological differences in skin tissue were not detected between subjects for blood supply, skin thickness or number of sebaceous glands.

16S-23S rDNA NGS analysis

Bacterial identification results obtained by BLASTN analysis using the NCBI database are shown in Fig. 4. *Staphylococcus* species were identified in eight biopsies. Seven samples contained *Propionibacterium* species in small relative abundance, and *Corynebacterium* species were identified in five samples. In one sample *Streptococcus* species was identified, but this patient did not develop SSI. For Case 1, who developed SSI, a large relative abundance of *Staphylococcus haemolyticus* was identified using NGS. However, wound culture results from Case 1 yielded *Escherichia coli*, *Enterococcus* spp. and *S. aureus*. None of the species identified by NGS from the preoperative biopsies are known as pathogens, able to initiate SSI.

In some samples NGS identified bacteria suggestive for contamination (e.g. *Sphingomonas* spp., *Paracoccus* spp. and *Hermimonas* spp.). NGS non-template control samples – samples processed without human tissue – were positive for the same species, as well. When these species were identified in samples they were classified as a contaminant.

Bacterial species identified with 16S-23S rDNA NGS were compared to patient characteristics and results of preoperative *S. aureus* cultures of patients with and without SSI. In 4 out of the 8 control cases, which did not develop SSI but carried *S. aureus* in either nose or perineum, NGS did not detect *S. aureus* in the groin biopsies.

Discussion

This pilot study shows the identification of the inguinal skin microbiome of vascular patients undergoing EVAR by combining standard techniques, culture and histology, and a novel technique consisting of 16S-23S rDNA NGS. We have demonstrated three findings. First, the 16S-23S rDNA NGS technique was able to identify a variety of bacterial species in skin biopsies, even after disinfection. Second, we did not find a correlation between preoperative nasal or perineal *S. aureus* carriage, deep-skin bacterial flora and SSIs. And third, in this small sample only skin-colonizing non-pathogenic bacteria were identified in the biopsies, suggesting that disinfection removed pathogenic superficial (transient)

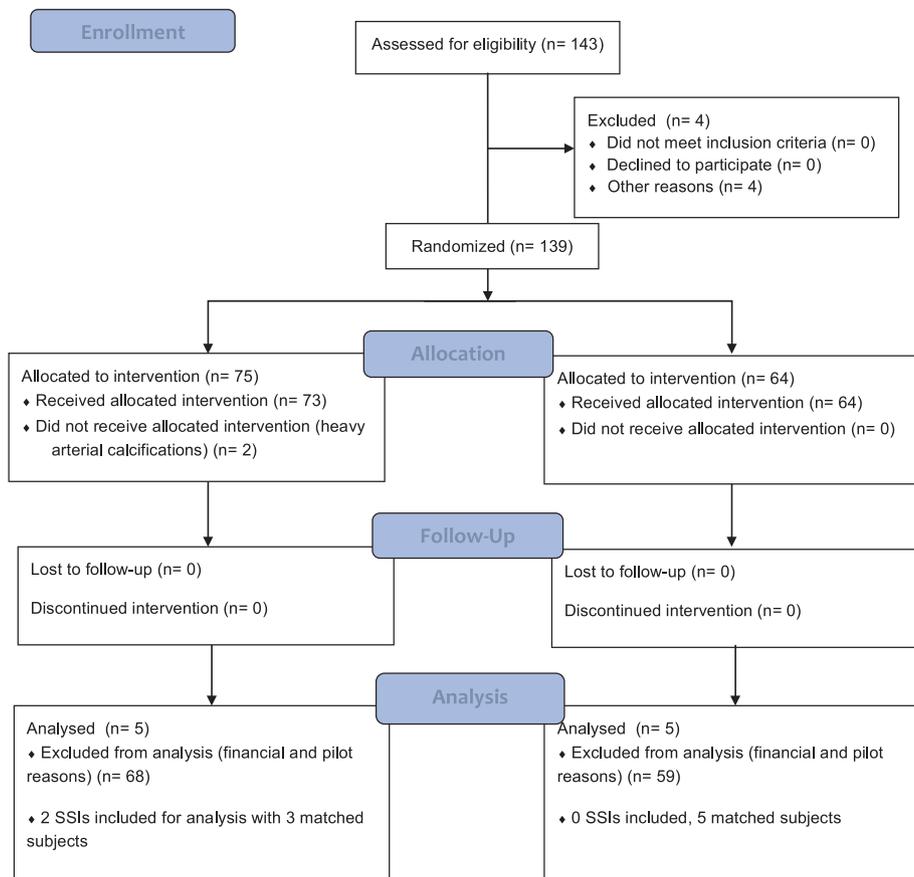


Fig. 1. Flow chart of inclusion in the PiERO trial and selection of 10 matched patients.

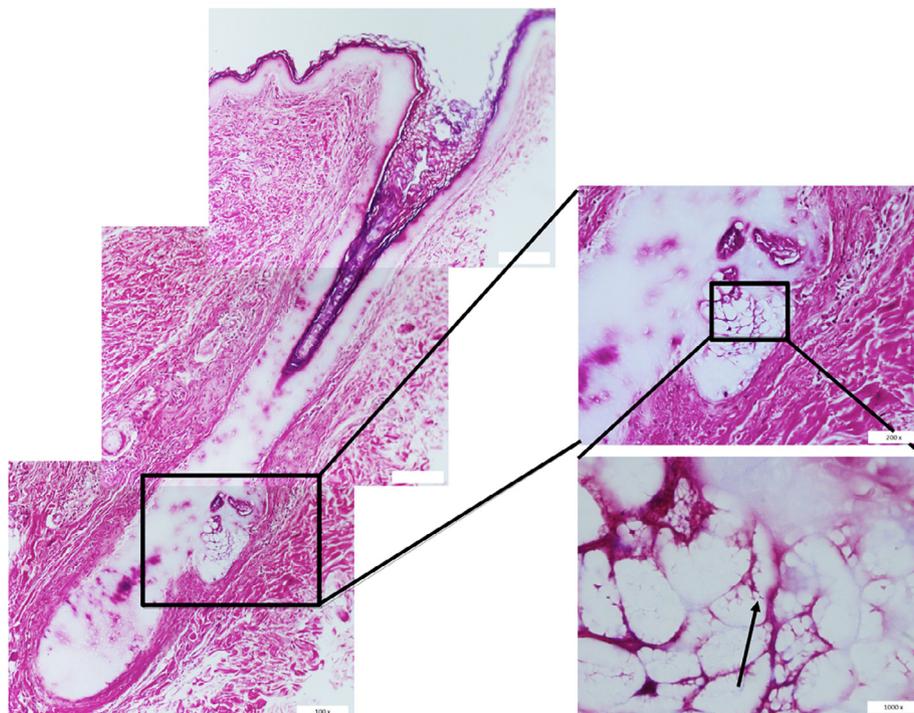


Fig. 2. Microscopy of a sebaceous gland of a PiERO patient, stained with H&E and Gram stain. Enhanced 100x, 200x and 1000x. Bacteria are positioned in the depth and are hardly visible using standard microscopy (arrow).

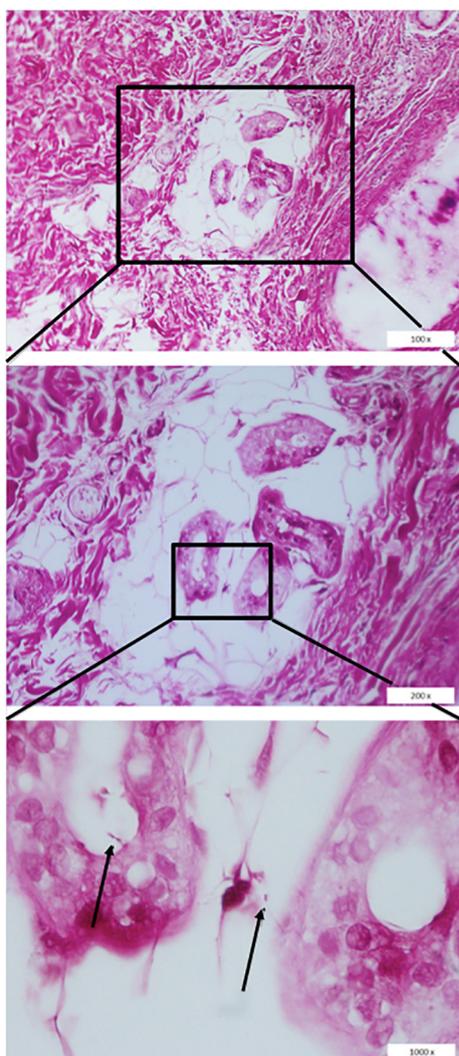


Fig. 3. Microscopy of a sweat gland of a PiERO patient, stained with H&E and Gram stain. Enhanced 100x, 200x and 1000x. Sweat-producing cells are visible and the bacteria are positioned intraluminally. Some bacteria are visible extraluminally too (right arrow).

bacteria, such as *S. aureus* [29].

It appears that the bacterial species identified were not remnants of the superficial skin flora, as in most species bacterial DNA is damaged by the biocidal effect of chlorhexidine [30]. The use of 16S-23S rDNA NGS enabled a very sensitive detection of all bacterial DNA in a small tissue sample and provided relative quantitative information on the detected species and genera [27,28]. No conventional methods can provide these sensitive and specific results from a biopsy.

The current selection of 10 PiERO patients (10/137) was made on preoperative and postoperative characteristics, and was small for feasibility and financial reasons. Though non-pathogenic, the bacterial species identified in this small cohort with NGS, are known to be able to colonize implanted foreign body materials. Neither patients who contracted SSI nor those without SSI carried pathogenic bacteria that cause SSI in their skin, according to NGS analyses. Hence NGS was unable to predict SSI outcome. Conversely, absence of *S. aureus* carriage or pathogenic bacteria did not prevent SSI.

It may be that the bacteria identified with NGS were relatively tolerant to chlorhexidine [30], but our results show that pathogenic bacteria were absent in the skin when initiating the operation. Post-operatively cultured bacteria in case of an SSI were not identified in the skin preoperatively.

How do we explain the original pathogenic source of the pathogens that caused an SSI in two patients? Inguinal regions are moist and contain high densities of bacterial species with pathogenic fecal bacteria closely available to contaminate the skin [29], and preoperative chlorhexidine baths do not reduce the risk of SSI [9]. Could the resident deep-skin flora protect the host against pathogenic species? This has been described before for resident intestinal bacterial flora [31], and recently suggested for resident skin flora [7].

Our study results are of interest because inguinal vascular procedures are complicated by a relatively high percentage of SSI, varying from 7 to 20% in peripheral bypass surgery [4,32,33]. It is thought that abundant bacterial flora in the groin is responsible for primary contamination of the wound, a hypothesis that could not be reproduced in our study. Perhaps damage to lymphatic drainage increases the risk of SSI in the groin [5], and ischemic tissue seems more prone to develop SSI [34]. Our findings anyhow suggest that the superficial and deep-skin bacterial flora is not responsible for SSIs after EVAR.

The cost aspect should be discussed too. A conventional culture with identification and resistance profiling costs €25-€125, and histologic research €121.52 [35]. NGS costs are dependent on the platform used; in this study 16S-23S rDNA NGS Illumina Nextera XT MiSeq sequence material costs were calculated at €100-150 per sample without analytical staff wages. Assuming an SSI incidence in vascular groin incisions of 7% [2], one in 15 groin incisions become infected. So, if NGS would be able to prevent SSI, the microbiome of 15 patients would have to be tested to prevent one SSI (number needed to screen is 15). If the total costs of full knowledge of the microbiome added up to approximately €200 – culture and NGS – the gain could weigh against the supposed mean costs of a single vascular graft infection, at an extra €20,000 [36]. Further research towards the clinical applicability and subgroup identification of patients susceptible to vascular graft infection with these techniques is needed.

This study has limitations that need to be addressed. Firm conclusions cannot be drawn due to the small sample size. Furthermore, the exemplification for the entire inguinal microbiome may be questionable because of the small amount of tissue used for amplification of bacterial DNA. But we consider this study as a pilot showing that 16S-23S rDNA NGS can detect bacterial species in disinfected skin. The original pathogenic source of an SSI remains unknown and further research on deeper tissues may be of added value.

Another limitation is the presence and detection of possible contaminants. DNA of some contaminants like *Paracoccus* and *Sphingomonas* spp. was detected in the reagents. Thanks to the sensitivity of the NGS technique, even very small amounts of residual DNA in reagents will be detected. The presence of contaminating DNA in reagents is a challenge when analyzing samples containing a low microbial biomass [37]. Contaminating DNA also interferes with the detection of very small numbers of bacteria, making the data analysis complex.

Conclusion

In this pilot study the intradermal microbiome of inguinal skin only contained bacterial species that unlikely cause SSI. Larger studies analyzing the intradermal microbiome and deeper structures of patients for vascular surgery could contribute to a better understanding of the development of SSI, and ultimately prevention of graft infections in susceptible patients.

Declaration of Competing Interest

None.

Acknowledgements

None.

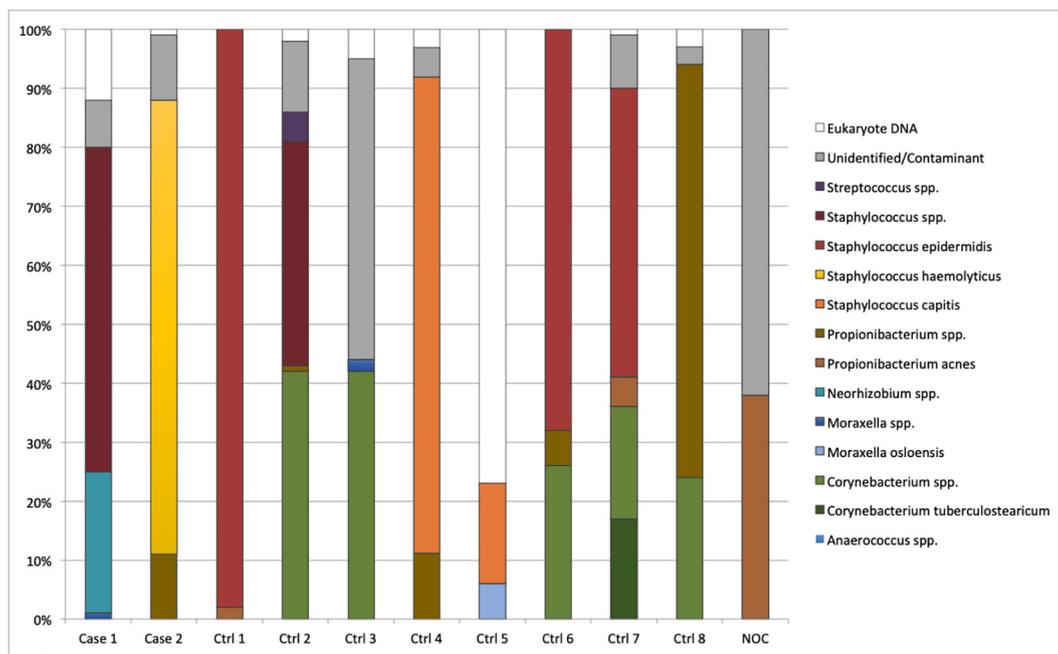


Fig. 4. Preoperative microbiome found in 10 PiERO patients. Cases 1 & 2 contracted SSI, Ctrl (= control) are matched PiERO participants.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mehy.2019.109358>.

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