Article focus

This study investigated the use of 16S rRNA metagenomics for detecting bacterial pathogens in synovial fluid (SF) from patients with hip or knee prosthetic joint infection (PJI).

This study compared the performance of bacterial detection using different methods, including 16S metagenomics, traditional cultures, and targeted Sanger sequencing. Data highlighted 16S rRNA metagenomics as a suitable and promising method to detect and identify infecting bacteria, most of which may be uncultivable.

Key messages

This study demonstrates that 16S metagenomics is a method with high potential for PJI diagnosis in the future. This method could detect very low levels of bacterial infection in SF, even when the bacteria are dead, i.e. after the patient has received antibiotic treatment.
method reduces the time required for bacterial identification and also improves polymicrobial detection in PJI diagnosis.

- Based on the performance against traditional cultures and targeted Sanger sequencing, the data highlight the potential of 16S metagenomics to diagnose PJIs, especially mixed infections. We provide a foundation for further development towards the 16S metagenomic diagnosis of PJI.

**Strengths and limitations**

- 16S rRNA metagenomics detects very low levels of bacterial infection but does not detect fungi or viruses in the body fluids of patients. Moreover, genus-level identification and quantification are generally more reliable than species-level identification.
- 16S rRNA metagenomics is strongly susceptible to contamination from reagents and sample processing, which may generate false positives (contaminated bacteria) or false negatives (underestimated infectious bacteria). Thus, all materials, reagents, and procedures should be strictly managed and standardized.
- The future goals for application of 16S metagenomics to PJI diagnosis are established in a standardized protocol including specimen collection, DNA extraction, 16S polymerase chain reaction (PCR), next-generation sequencing (NGS) criteria setting, bioinformatic analysis, and final reports.

**Introduction**

Total joint arthroplasty is one of the most successful surgical procedures in modern medicine. The demand for primary total knee and total hip arthroplasty has been projected to grow by 673% and 174%, respectively, from 2005 to 2030 in the United States. Prosthetic joint infection (PJI) is the most common cause of knee arthroplasty failure, and accounts for 25.2% of failed total knee arthroplasties. It is also the third most common indication (14.7%) for revision hip arthroplasty. The diagnosis of PJI mainly depends on the combination of clinical tests, including serum C-reactive protein (CRP), peripheral blood leukocytes, synovial fluid (SF) white blood cells, bacterial cultures of preoperative SFs and intraoperative tissues, radiological, and other tests such as positron emission tomography. Identification of the bacteria is not only the benchmark for PJI diagnosis but also provides guidance for antibiotic choice in PJI treatment. However, the culture-negative rate of PJI is around 23% to 35%. Furthermore, prior antimicrobial use has been shown to decrease the sensitivity of culture in PJI, and 53% of patients received an antimicrobial agent before the diagnosis of culture-negative PJI. Accordingly, detection of the bacteria remains a challenge for the diagnosis of PJI.

Polymerase chain reaction (PCR)-based methods may improve diagnosis of microorganism infection by reducing turnaround time and eliminating the requirement for culture. PCR assays of SF using pathogen-specific primers were reported to be 70% to 96% sensitive. However, this method detects only organisms that are tested for, and therefore will miss atypical or unexpected pathogens. High-throughput sequencing overcomes this issue by quantitative detection of unculturable, unsuspected, and non-viable pathogens without sacrificing speed. 16S rRNA metagenomic analysis has been used successfully to analyze bacteria in clinical specimens. However, it is very rare for bacterial detection in PJI. In this study, we investigated the roles of 16S rRNA metagenomics in the detection of bacterial pathogens in SFs from patients with hip or knee PJI. We hypothesized that the bacterial V3 and V4 fragments would be amplified efficiently with all bacteria to generate amplicons for sequencing. We compared 16S rRNA metagenomics results before (first-stage surgery) and after debridement (second-stage surgery) in order to explore whether there is a difference in bacterial detection. We believe that this method can detect not only very low-level infections but also antibiotic-killed bacteria during preoperative antibiotic treatment. Accordingly, only live pathogenic bacteria can give positive results after bacterial culture-based methods. In the event of pathogenic bacteria being killed by preoperative antibiotics, bacterial culture-based methods may yield false-negative results. However, the 16S rRNA metagenomics method is capable of detecting bacterial nucleic acid regardless of whether the bacteria is alive or dead. We further attempted to optimize the protocol through comparison of the results from different database analyses including SILVA, Greengenes, and The National Center for Biotechnology Information (NCBI). This study also compared the performance of bacterial detection from different methods including 16S metagenomics, traditional cultures, and targeted Sanger sequencing.

**Materials and Methods**

**Patients and sampling.** Between November 2016 and March 2017, 11 hip/knee PJI patients (three female patients, eight male patients) were enrolled in this study. PJI was defined by fulfilling one of the following three criteria: 1) sinus tract communicating with the prosthesis; 2) pathogen isolated from two or more samples obtained from the infected prosthetic joint; 3) four of the following six criteria exist: a) elevated serum erythrocyte sedimentation rate (ESR) and serum CRP concentration; b) elevated synovial leucocyte count; c) elevated synovial polymorphonuclear neutrophil percentage (PMN%); d) presence of purulence in the affected joint; e) isolation of a microorganism in one culture of prosthetic tissue or fluid; and f) greater than five neutrophils per high-power field in five high-power fields observed from histological analysis of prosthetic tissue at ×400 magnification. All of the PJI patients were treated...
with two-stage exchange arthroplasty. In brief, resection arthroplasty for PJI included radical debridement, removal of prosthesis, implantation of antibiotic-loaded bone cement, and administration of systemic antimicrobial agents for the control of joint infection (first-stage surgery). Delayed reimplantation of the prosthesis was performed after successful antimicrobial therapy, which was defined by the absence of signs of infection and the return of ESR and serum CRP levels to normal (second-stage surgery). During the same enrolment period, three patients (two hip and one knee) with aseptic loosening who were scheduled for revision arthroplasty were enrolled as a control group. Specimens of joint fluid measuring at least 2 ml were collected by needle aspiration prior to entering the joint to minimize contamination by blood. Patients with malignant tumours, those who had received immunosuppressive agents, and those who had a history of allergy to vancomycin or ceftazidime were excluded. The study was approved by the local institutional review board, and was compliant with accepted ethical standards at our hospital. Informed consent was obtained from all patients before initiating this study.

**Specimen preparation, sequencing, and bacterial culture.** Synovial fluid specimens were delivered to the laboratory immediately after aspiration and centrifuged at 10000 ×g. The resulting pellet was extracted with EasyPrep HY Genomic DNA Extraction Kit (TE-GD01; BIOTOOLS Co., Ltd., New Taipei City, Taiwan). Bacterial V3 and V4 fragments were amplified with primer 341F (CCTAYGGGGBGCASCAG) and primer 806R (GGACTACNNGGATCTAAT) to generate amplicons of 466 bp. Paired-end reads from the amplicons were assembled into tags in FLASH v.1.2.7, clustered into operational taxonomic units (OTUs) at 97% similarity using Uparse v7.0.1001 (http://drive5.com/uparse/), and identified with regard to genus and species using Ribosomal Database Project (RDP) classifier v2.2 against SILVA (v128), Greengenes (13_8), and NCBI databases. These analyses were conducted on 15 August 2017.

Specimens were analyzed by 16S rDNA metagenomic analysis on an Illumina HiSeq 2000 Sequencing system (Illumina, Inc., San Diego, California) and the results were compared with 16S rDNA amplification and Sanger sequencing results, as well as with bacterial culture results, which represent the current benchmark for the diagnosis of PJI. Patient characteristics are summarized in Table 1. Of note, we initially tested SFs from three uninfected patients to obtain control results. However, 16S PCR products were not obtained from these three specimens, and these specimens were therefore excluded because they were unable to be used for 16S rRNA metagenomic analysis.

For Sanger sequencing, the 16S rDNA amplicons were cloned using T&A Cloning Kit and DH5a competent cells (both Yeastern Biotech Co. Ltd, Taipei, Taiwan). For each sample, at least ten clones were picked, sequenced, and compared against the genetic sequence database, GenBank, using the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information [NCBI], U.S. National Library of Medicine, Bethesda, Maryland). Specimens of periarticular tissue and joint fluid were sent for bacterial culture in both the PJI and control groups. In brief, SFs and deeper layers of the synovial membrane were cultured in BD BACTEC Peds Plus or BD BACTEC Plus Aerobic (Becton, Dickinson and Company [BD], Sparks, Maryland) and incubated at 37°C under aerobic and anaerobic conditions. The patient’s deep tissue was placed directly into the bacterial culture container, and then sterile normal saline was added to avoid tissue drying. Cultures were examined daily for two weeks, and isolated bacteria were identified by matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF) mass spectrometry on an Ultraflex III TOF/TOF system (Bruker Corporation, Billerica, Massachusetts).

**Data analysis.** Data are reported as the mean (SD), and were analyzed in GraphPad Prism (GraphPad Software Inc., San Diego, California).

### Results

#### Distribution of bacterial taxa over two-stage exchange arthroplasty.

We identified ten major pathogen species in 11 infected patients, including *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Serratia*, *Escherichia*, *Pseudomonas*, *Bacteroides*, *Acinetobacter*, *Propionibacterium*, and *Sphingomonas* (Fig. 1). The polymicrobial composition of SF from PJI revealed a significant change between the first- and second-stage surgeries. Changes in relative abundance between the first- and second-stage surgeries were assessed prior to, and three months after, the first-stage surgery. These data demonstrate that first-stage resection

| Parameter | Aseptic | First-stage surgery | Second-stage surgery |
|-----------|---------|---------------------|----------------------|
| Number of patients | 3 | 11 | N/A |
| Sex, male:female, n (% | 0:3 (0:100) | 8:3 (73:27) | N/A |
| Mean age at surgery, yrs (sd; range) | 68 (6; 63 to 77) | 63 (10:2; 40 to 76) | N/A |
| Type of joint prosthesis, knee:hip, n (%) | 1:2 (33:67) | 8:3 (73:27) | N/A |
| Mean serum CRP, mg/dl (sd) | 3.3 (1.3) | 65.2 (72.2) | 24.9 (40:9) |
| Mean synovial fluid white blood cells, cells/dl (sd) | 333 (451) | 22.393 (18133) | 5967 (8110) |

CRP, C-reactive protein; N/A, not applicable

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**Table I.** Patient characteristics

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**Figure 1.** The polymicrobial composition of synovial fluid from patients with PJI before (at the end of first-stage surgery) and after (after second-stage surgery) surgery. The distribution of bacterial taxa is shown as the ratio of each bacterial species to the total number of bacterial sequences identified. The black and red bars represent bacterial species identified in the first- and second-stage surgeries, respectively. The gray bars indicate bacterial species that were identified in both surgeries. The ratio of bacterial species is expressed as a percentage of the total number of sequences identified. **Letters** indicate the ratio of each bacterial species to the total number of bacterial sequences identified. "A. N. B. C. D. E. F. G. H. I. J." represent the ten major pathogen species identified in this study. The number of sequences used to identify each bacterial species is shown in the inset. **Distribution of bacterial taxa over two-stage exchange arthroplasty.** We identified ten major pathogen species in 11 infected patients, including *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Serratia*, *Escherichia*, *Pseudomonas*, *Bacteroides*, *Acinetobacter*, *Propionibacterium*, and *Sphingomonas* (Fig. 1). The polymicrobial composition of SF from PJI revealed a significant change between the first- and second-stage surgeries. Changes in relative abundance between the first- and second-stage surgeries were assessed prior to, and three months after, the first-stage surgery. These data demonstrate that first-stage resection
Two-stage exchange arthroplasty bacteria genus in knee abundance (%)

| Patient | 1st Sample | 2nd Sample |
|---------|------------|------------|
| 1       | Staphylococcus 98% | Sphingomonas 4% |
| 2       | Sphingomonas 4% | Bacteroides 6% |
| 3       | Streptococcus 98% | Escherichia 4% |
| 4       | Sphingomonas 4% | Streptococcus 6% |
| 5       | Staphylococcus 2% | Strp. 0.2% |
| 6       | Bacteroides 2% | Staphylococcus 18% |
| 7       | Escherichia 0.2% | Staphylococcus 6% |
| 8       | Staphylococcus 2% | Strp. 0.6% |
| 9       | Sphingomonas 9% | Staphylococcus 67% |
| 10      | Bacteroides 8% | Streptococcus 5% |
| 11      | Escherichia 0% | Strp. 0.6% |

Two-stage exchange arthroplasty bacteria genus in hip abundance (%)

| Patient | 1st Sample | 2nd Sample |
|---------|------------|------------|
| 1       | Staphylococcus 98% | Sphingomonas 3% |
| 2       | Bacteroides 2% | Acinetobacter 4% |
| 3       | Sphingomonas 4% | Staphylococcus 7% |
| 4       | Bacteroides 5% | Staphylococcus 84% |
| 5       | Streptococcus 0.2% | Pseudomonas 3% |
| 6       | Escherichia 0.6% | Pseudomonas 7% |
| 7       | Sphingomonas 8% | Klebsiella 56% |
| 8       | Acinetobacter 4% | Pseudomonas 30% |

Relative abundance of bacterial taxa in individual samples. a) Samples from first- or second-stage knee surgery, and b) first- or second-stage hip surgery are plotted along the horizontal axis. Relative abundances inferred from 16S metagenomic sequencing are plotted on the vertical axis. ND, not detected.
arthroplasty and sequential antibiotic treatment provide a very effective way to eliminate the microorganism that caused the PJIs. This observation may help to guide patient management and treatment selection between second-stage surgery and additional debridement.

Although SILVA is a comprehensive, up-to-date, and quality-controlled database of 16S rRNA genes, Greengenes and NCBI are also often used. Thus, we matched individual patient data against all three databases, and obtained similar results, with variability observed only among less abundant genera. For example, *Serratia* were detected in patients 2, 4, and 8 only against Greengenes. Genera with relative abundances higher than 0.5% are listed in Table II, in which those with abundances of 0.6% to 5.0%, and 6% to 100%, are marked by a dagger symbol and an asterisk, respectively. The results were essentially the same across databases for genera with an abundance higher than 5%, implying that analysis based on major genera is robust.

Comparison of bacterial cultures, targeted Sanger sequencing, and 16S metagenomics. As bacterial cultures are the benchmark test to detect infections, deeper layers of the synovial membrane or SF were also collected during surgery and inoculated into blood culture bottles. Bacteria from positive cultures were identified by MALDI-TOF mass spectrometry and are listed in Table III. Bacteria were not detected by mass spectrometry in two cultures, resulting in a positive rate of about 82% to 85%. One species was detected in each of the remaining cultures, including *Staphylococcus aureus* in two patients, *Staphylococcus caprae* in three patients, *Klebsiella pneumoniae* in three patients, and *Staphylococcus epidermidis* and *Streptococcus dysgalactiae* in one patient each (Table III).

The data highlight 16S rRNA metagenomics as sufficient to identify mixed pathogens in a single specimen. Indeed, this method identifies not only the same genera detected in bacterial cultures, but also others that were not detectable in the bacterial cultures. For example, patients 1, 3, 5, 9, and 10 were infected with only one strain based on an abundance cutoff of 5% (Table III), whereas mixed infections with one to seven genera were detected in the other six patients. At a cutoff of 0.5%, only patients 9 and 10 can be considered infected with a single strain (Table II).

The most common Gram-positive infecting genus was *Staphylococcus* (82%; Fig. 3), whereas *Escherichia* spp., *Klebsiella* spp., *Pseudomonas* spp., and *Sphingomonas* spp. were the most common Gram-negative pathogens. Finally, we assessed the impact of antibiotic treatment, and found that infection had recurred after three months of debridement in patient 1. Patients 2 and 5 tested positive in intraoperative cultures from second-stage surgery; patients 3, 4, 6, 9, and 10 were infection-free; and patient 11 was infected with fungus, while patients 7 and 8 had died due to sepsis from PJIs (Table IV). Collectively, the data demonstrate that 16S metagenomic analysis detects low-abundance 16S rRNA genes in specimens such as SF.

Discussion

Current methods in PJI diagnosis. Bacterial cultures of SF and prosthetic tissue are the benchmark test for PJI diagnosis, however some bacteria are difficult to grow or are even uncultivable. In addition, cultures have high false-negative rates and are time consuming, as they might need one to two weeks for bacteria growth, especially in patients with low-grade infections. Targeted cloning and subsequent Sanger sequencing of 16S rDNA has also been used in the past decade, for example to identify disease-associated bacteria in clinical specimens, such as oral tongue and bronchoalveolar lavage fluid, enamel and dentin lesions, and pus from brain abscesses. Remarkably, this approach identified many bacteria that were not previously detected in these specimens, some of which were unculturable. Although this method generally overcomes the limitations of bacterial cultures,
it is also time consuming and expensive because many bacteria, typically 46 to 125 per subject, have to be analyzed. In contrast, next-generation sequencing (NGS) and 16S metagenomics can now be used to characterize mixed infections and to identify infecting pathogens. The first commercial NGS platform was released in 2005, and metagenomics analysis of human and environmental microbiota has since flourished.41-43 However, its application in clinical diagnosis is not as well developed, although it has already been used to detect antimicrobial resistance genes in septic patients.44 Metagenomic analysis also detected varicella zoster virus in cerebrospinal fluids from patients with multiple sclerosis, even though the virus was never previously associated with the disease.45
We have now used 16S rRNA metagenomics to investigate bacteria in the SF of infected prosthetic joints. To this end, we have also developed a new protocol to assess pathogen composition and eliminate contaminating signals.

16S rRNA metagenomics as a potential method for PJI diagnosis. Based on our data, 16S rRNA metagenomics appears to be more sensitive than bacterial cultures in detecting pathogens at the genus and species level. It also reduces turnaround time from approximately one week (at least five days) for bacterial cultures to two days, where the procedure consists of one hour of DNA extraction, two hours of PCR, 40 hours of NGS, and four hours of bioinformatics analysis. Universal bacterial primers also detect very low-abundance pathogens, especially in patients who received antibiotics before surgery. In addition, 16S metagenomics detects polymicrobial infections and quantifies infecting pathogens based on...
true abundance, and not culturability, growth in culture, antibiotic resistance, and dominance. According to our experimental results (Fig. 1), the 16S rRNA-based method also tested some atypical pathogens in PJI such as *Acinetobacter* and *Sphingomonas*. Although we detected *Acinetobacter* in specimens from our patient, it is one of many bacterial infections and accounts for less than 4% of total bacteria. Therefore, we reasonably speculate that, in the case of multiple bacterial infections, presence of *Acinetobacter* is not common because earlier technology has not detected them. On the other hand, the sample from patient 11 did not show growth of bacteria, but instead growth of *Candida albicans*. Moreover, the 16S rRNA-based method identified *Sphingomonas* from the samples of this patient. Based on these results, we reasonably speculated that patient 11 may have both *Sphingomonas* and *C. albicans* infections. Taxonomic analysis of 16S reads following metagenomic sequencing is typically based on SILVA, Greengenes, and RDP.\(^{46}\) SILVA is a comprehensive database of bacterial rRNA genes and is the largest and most widely used of the three.\(^{46}\) Remarkably, we obtained similar results by matching patient data with SILVA, Greengenes, and NCBI databases,\(^{47}\) especially for the major genera detected.

To date, there is little literature on the diagnosis of PJI using NGS. We tried to compare the differences between a few research methods. Our experimental method uses 16S RNA primer to amplify the bacterial gene, and then analyze bacterial infections by the Illumina HiSeq Sequencing platform. We also compared the sequencing results of the infections by the comparison of three databases (SILVA, Greengenes, and NCBI). Tarabichi et al\(^{29}\) combined the primer for the 16S rRNA gene and internal transcribed spacer gene to amplify bacterial and fungal genes simultaneously, and then analyze microorganism infections using the Ion Torrent PGM sequencing platform (Thermo Fisher Scientific, Waltham, Massachusetts). For Street et al’s\(^{48}\) strategy, after sonication fluid is collected,
the Illumina MiSeq Sequencing platform analysis is performed directly without amplification of the PCR. Although the experimental design and analysis methods of each study are different, establishing a suitable NGS standard procedure for joint fluids in patients with PJI is a future method for PJI diagnosis. **Points to consider in 16S rRNA metagenomics.** Nevertheless, some issues need to be resolved. First, 16S rRNA metagenomics is specific for bacteria, and will not detect fungi or viruses. Second, genus-level identification and quantification are generally more reliable than species-level identification. Indeed, although we detected four Gram-positive genera (*Staphylococcus*, *Streptococcus*, *Propionibacterium*, and *Corynebacterium*) and six Gram-negative genera (*Escherichia*, *Klebsiella*, *Pseudomonas*, *Bacteroides*, *Serratia*, and *Sphingomonas*), we identified only ten species, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus capre*, *Streptococcus dysgalactiae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Bacteroides fragilis*, *Serratia marcescens*, *Sphingomonas aquatilis*, and *Acinetobacter johnsonii*. Third, 16S rRNA metagenomics is strongly susceptible to contamination from reagents and sample processing, which may generate false positives or false negatives.\(^{24,50}\) For instance, analysis of sonicated samples may be more sensitive than analysis of whole tissues,\(^ {36,51}\) but additional procedures and reagents for sonication and inefficient DNA extraction also increase the risk of contamination.\(^ {21,23,52}\) Indeed, removal of interference from contaminating DNA is a major challenge. Thus, all materials, reagents, and procedures should be strictly managed and standardized.\(^ {49}\) Finally, we amplified 16S rDNA directly from SFs to minimize contamination from human DNA, which may account for > 90% of reads even if microbiome DNA is enriched prior to sequencing.\(^ {48}\) Selection of a suitable cutoff value is also a serious issue. For example, low cutoff values such as 0.5% or 0.1% identify too many genera as being present, probably including spurious or irrelevant taxa. However, a strict cutoff value, such as 5%, may eliminate too many genera and prevent detection of mixed infections. In this study, our data indicate that if we were to select 5% as the cutoff value, the results would coincide with clinical observations of PJI. If we were to select 0.5% as the cutoff value, we would be able to identify a very low level of bacterial infection with species unculturable in bacterial culture. According to the comparison of 16S metagenomics and bacterial culture, 16S metagenomics could distinguish the genus when the specimens exhibit a very low level of bacterial infection. The future goals for application of 16S metagenomics to PJI diagnosis are not only to set a suitable cutoff value but also to establish a standardized protocol including specimen collection, DNA extraction, 16S PCR, NGS criteria setting, bioinformatic analysis, and final reports. NGS holds great promise in detecting pathogenic bacteria in clinical samples. We believe that this study highlights the potential of 16S metagenomics to diagnose PJs, especially mixed infections. Understanding the bacterial composition of polymicrobial infection is of great benefit for the future development of novel antimicrobial metal orthopaedic implants.\(^ {33-35}\) A comprehensive understanding of the composition of infectious bacteria can also provide appropriate protective measures for the surgeon before the surgical procedure is performed.\(^ {56}\)

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Ethical review statement

The study protocol was approved by our institutional review board (IRB number, 105-1046C), and was compliant with accepted ethical standards at Chang Gung Memorial Hospital. The written informed consent was obtained from all patients prior to their participation in the study. This study was carried out in accordance with the ethical standards in the 1964 Declaration of Helsinki.

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