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Characterizing the Native Microbiome Using Next-Generation Sequencing of Bilateral 'Aseptic' Knees

Tracy Borsinger, MD [a,*], Michael Torchia, MD, MS [b], Bethany Malskis, BS [a], Benjamin A. Levy [c], Paul M. Werth, PhD [a], Wayne E. Moschetti, MD, MS [a]

[a] Department of Orthopaedics, Dartmouth-Hitchcock Medical Center, Lebanon, New Hampshire
[b] Department of Orthopaedics, Essentia Health Duluth Clinic, Duluth, Minnesota
[c] Department of Orthopaedics, Geisel School of Medicine at Dartmouth, Hanover, New Hampshire

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ABSTRACT

Background: Next generation sequencing (NGS) has proven ability to identify organisms beyond those identified through traditional culture-based techniques in cases of suspected prosthetic joint infection. However, there is concern that some microorganisms identified may represent the natural joint microbiome rather than pathogenic agents. This work sought to evaluate the presence of microorganisms identified with NGS in bilateral native, presumed "aseptic" knees with osteoarthritis.
Methods: There were 40 patients undergoing primary unilateral (30) or bilateral (10) total knee arthroplasty enrolled prospectively. During surgery, samples of fluid and tissue were obtained from operative knees, and joint fluid was obtained from nonoperative knees. Samples were sent for NGS analysis and processed according to manufacturer protocols. Patient age, body mass index, comorbidities, prior history of injections, and grade of arthritis were evaluated for association with positive NGS results.
Results: There were 3 of 80 samples (3.8%) that demonstrated positive NGS. There were two of these that had multiple microorganisms identified (1 knee with 4 microorganisms; 1 knee with 2 microorganisms). An additional 2 samples had positive NGS results below the manufacturer's threshold for reporting. The most common organism identified was *Cutibacterium acnes*, present in 2 of the 3 positive samples. No patient baseline characteristics were associated with positive NGS results.
Conclusions: Some native knee joints with osteoarthritis have positive microorganisms identified with NGS. The presence of microorganisms in the native knee has important implications for better understanding the native joint microbiome as well as utilization of NGS in cases of suspected prosthetic joint infection.

Prosthetic joint infections (PJIs) are a source of major patient morbidity and health care cost, and are projected to increase with the increasing frequency of primary total joint arthroplasty [1–3]. Next-generation sequencing (NGS) can identify all DNA present in a synovial sample and thus offers potential for improved diagnosis and subsequent targeted treatment of PJIs [4–6]. While NGS has a proven ability to identify many organisms beyond those identified through traditional culture-based techniques, in cases of suspected PJI, there is a concern that additional organisms identified may represent contamination or a false positive. Alternatively, there has been increasing evidence that these microorganisms represent a naturally existing microbiome of the joint rather than contaminants or pathogenic agents. Specifically, prior work has demonstrated that up to 30% of patients who have osteoarthritis undergoing primary total knee arthroplasty (TKA) have organisms identified in their joint by NGS at the time of surgery [7–11]. Appropriate use of NGS will be predicated on accurate interpretation of these organisms to avoid both under- and over-diagnosis.

Research over the past several decades has elucidated an increasing role for the human body's microbiome in overall health [12,13]. The quantity of microorganisms encompassing the human microbiome is estimated to be approximately 10 times the number

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* Address correspondence to: Tracy Borsinger, MD, Department of Orthopaedics, Dartmouth-Hitchcock Medical Center, One Medical Center Drive, Lebanon, NH 03756.

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of human somatic and germ cells [13]. The intestinal microbiome alone has been implicated in atherosclerotic cardiac disease [14,15], cancer [16,17], and neuro-degenerative and psychiatric disorders [18]. Within the field of orthopedics, prior research has identified a role for the human microbiome in osteoarthritis [19], inflammatory arthritis [20,21], as well as the development of low back pain and sciatic symptoms in patients who do not have a history of prior surgery [22–24]. Limited research has assessed the native joint microbiota [10,25], and to our knowledge, only one prior study systematically examined the microbiota of the native, aseptic knee joint itself in patients undergoing TKA for osteoarthritis [7].

This work sought to use NGS to analyze the potential microbiome of bilateral knee joints in patients undergoing both unilateral and simultaneous bilateral TKA. In doing so, we sought to add to prior literature regarding the potential for presumed 'aseptic' native knees to have microorganisms present on NGS analysis. Furthermore, in evaluating bilateral knees in individual patients, we sought to better understand whether the physiologic niche of the bilateral knee joints is in some way influenced by individual determinants, including the severity of osteoarthritis.

Materials and Methods

Patient Enrollment and Perioperative Protocol

After obtaining institutional review board approval, patients undergoing bilateral simultaneous and unilateral primary TKA were enrolled prospectively at the time of surgery at our tertiary academic hospital. Patients who were less than 18 years old, prisoners, had inflammatory arthritis, or native joint infection were excluded. There were forty patients prospectively enrolled in the present study allowing for NGS analysis of a total of 80 samples. The average age of patients enrolled was 67 years (range 41 to 84 years) with an average body mass index (BMI) of 32.4 (range, 22.4 to 49.5). The average number of prior injections was 1.6 injections with a median of 2 injections (Table 1).

All patients were treated in accordance with institutional universal decolonization protocols and infection prevention measures. Prior to surgical intervention, each patient received 5 days of nasal mupirocin decolonization. Patients underwent chlorhexidine showers both the night prior to and the morning of surgery. In the preoperative holding area, the surgical site was shaved if applicable, and the surgical site was cleaned with chlorhexidine wipes. In the operating room, all patients received antibiotic prophylaxis per institutional standards within 1 hour prior to skin incision and the operative extremities were prepped and draped using a combination of chlorhexidine, alcohol, and Duraprep (3M, St. Paul, M). The skin was then covered with an iodine-impregnated barrier prior to skin incision.

Table 1
Patient and Treatment Characteristics of 40 Patients Enrolled in Prospective Analysis.

N (Number of patients)	40
Mean age (range)	67 (41 to 84)
Sex (%)	
Men	18 (46)
Women	22 (54)
Number of prior injections (mean (SD))	1.6 (1.9)
Elixhauser score (mean (SD))	1.4 (1.4)
Reported income (%)	
Less than 10,000	3 (7.5)
10,000 to less than 15,000	2 (5.0)
20,000 to less than 25,000	2 (5.0)
25,000 to less than 35,000	7 (17.5)
35,000 to less than 50,000	6 (15)
50,000 to less than 75,000	7 (17.5)
75,000 or more	12 (30)
Not reported	1 (2.5)
Mean body mass index (range)	32.4 (22.4 to 49.5)
Race (%)	
White/Caucasian	39 (97.5)
Unknown	1 (2.5)
TKA surgery (%)	
Bilateral	8 (20)
Unilateral	32 (80)

SD, standard deviation; TKA, total knee arthroplasty.

Sample Collections

For the simultaneous bilateral TKA procedures, synovial fluid was aspirated from the knee joint prior to the arthrotomy but after the skin was incised. A minimum of one cubic centimeter of synovial fluid was sent for NGS analysis. After the arthrotomy, 3 synovial tissue samples were obtained: one each from the lateral gutter, medial gutter, and suprapatellar region. Finally, a sterile gauze pad was used to swab the distal femur. These samples were combined with the synovial fluid for the final NGS evaluation. Samples were collected and handled by the operating room staff in a standard, aseptic manner according to our institutional protocols. The samples were placed directly into a manufacturer-provided sterile container. which was then sealed and labeled.

For unilateral TKA procedures, the operative knee sample was obtained as previously described. For the nonoperative knee, the skin at the knee was prepped with Chlorapep (Becton Dickinson, Franklin Lakes, NJ) or Duraprep solution, and the sample was obtained through aseptic aspiration in the operating room. The sample was transferred to the manufacturer-provided sterile containers, sealed, and labeled.

Next-Generation Sequencing Technique

All samples were then shipped at ambient temperature in a preaddressed box to MicroGen Diagnostics (Lubbock, Texas) per the company's specifications and subsequently underwent processing as per the company protocol below.

Each sample subsequently underwent DNA extraction per protocol (kit Zymo Research, Tustin, California, USA). Total DNA was extracted, which included bacterial, fungal, and human material. In order to control for potential contamination during the extraction process, 3 negative controls are run for every 92 samples. The polymerase chain reaction amplification was selective for the 16S rRNA hypervariable regions V1-V2 and ITS region (Applied Biosystems, Carlsbad, CA). At this point, the bacterial and fungal DNA were amplified selectively through targeted primers, and the human DNA was not amplified. The polymerase chain reaction products were combined based on qualitative band strength to form the amplicon library and size selection on the library was performed using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN) and Qiagen Minelute Kit (Qiagen, Venlo, Netherlands). The library was quantified using the Qubit 4.0 fluorometer (Life Technologies, Waltham, MA). Downstream library preparation and sequencing followed the Illumina MiSeq manufacturer protocols (San Diego, CA).

The sequencing reads were then analyzed for quality and length during the data analysis pipeline. The data analysis pipeline consists of 2 major stages: the denoising and chimera detection stage and the microbial diversity analysis stage. MicroGen^{DX} performs chimera detection using the de novo method built into UCHIME. Any read that falls below the quality score, quality metric, or appropriate length are discarded due to the poor quality. Only high-quality reads are interpreted and reported. The high-quality

sequencing reads that were left were compared to the internally maintained and curated database during the data analysis step. The corrected sequences were demultiplexed using an internally developed algorithm that ensures the barcode for each sequence matches 100%, any sequence that did not contain a valid barcode was removed. These demultiplexed sequences then went through operational taxonomic unit (OTU) selection processes. The OTU clusters were globally aligned using USEARCH against a database of high-quality sequences. The output was then analyzed using an internally developed algorithm that assigns taxonomic information to each sequence and then computes and writes the final analysis files.

Threshold reporting was set at 2% according to the manufacturer's standard, with species below this threshold reported as number of reads. Synovial fluid biomarkers, including synovial C-reactive protein, synovial white blood cell count, and synovial percentage polymorphonuclear cells (%PMNs), were also reported.

Data Analyses

After collection of NGS data, the information was deidentified and recorded in a secure database. Patient data were then linked with our institutional data repository. All primary TKA patients at our institution undergo a standard preoperative pathway which includes collection of basic demographic information, presence of comorbidities, and severity of osteoarthritis using the Kellgren–Lawrence scale [26]. For those undergoing unilateral TKA, radiographs of the contralateral knee were reviewed by a single orthopaedic surgeon with grading of the osteoarthritis using the Kellgren–Lawrence scale. Statistical analyses were performed in the R environment (version 4.2.0; Vienna, Austria) [27]. *T*-test and *Chi*-squared analyses for continuous and categorical variables were respectively utilized to compare patient factors including age, sex, race, BMI, laterality, smoking status, Elixhauser score, number of prior injections, and Kellgren-Lawrence grade, as well as laboratory characteristics among those who did and did not have microorganisms identified with NGS.

Results

There were 3 of the 80 samples (3.8%) that demonstrated positive NGS results. There were 2 of these 3 knees that had multiple microorganisms identified with NGS (one knee with 4 microorganisms and one knee with 2 microorganisms, Figure 1). The most common organism identified was *Cutibacterium acnes*, which was present in 2 of the 3 positive samples. An additional 2 samples had positive NGS results that were below the manufacturer's threshold for reporting (*Cutibacterium acnes* and *Staphylococcus capitis*). None of the patients included underwent any reoperation for infection at 1-year follow-up.

We did not identify a relationship between positive NGS results in one knee compared to the contralateral within individual patients. In fact, no patients were found to have positive NGS results in their bilateral knees. Interestingly, all of the knees with microorganism(s) identified with NGS (3 of 3) in the present study had advanced arthritis (Kellgren–Lawrence Grade IV), while 83.8% of those without positive NGS results had advanced arthritis.

None of the patient characteristics or treatment factors were noted to be significantly different between the 2 groups in our 40 patients with bilateral knee samples evaluated (Table 2). Average age was 68 years for those who had no microorganisms on NGS and 64 years for those who had a microorganism(s) identified ($P = .709$). Although not statistically different, the mean number of injections was higher in those who had positive NGS results (2.33 injections, SD 2.52) compared to those who had negative NGS results (1.35 injections, SD 2.21; $P = .468$).

Evaluation of the synovial fluid cell count and biomarkers from the knee samples did not demonstrate a difference in C-reactive protein, white blood cell count, or % of PMNs between those knees with microorganisms identified with NGS compared to those with negative NGS results. Of note, in 13 samples, there was insufficient fluid for synovial biomarker analyses. Although not statistically significantly different with the numbers available, the % of PMNs was higher in those who had microorganisms identified in their knee samples compared to those who had no microorganisms identified with NGS (68.7 versus 42.1%, $P = .081$, Table 3).

Discussion

Due to the major morbidity and mortality as well as healthcare costs associated with PJI, research regarding appropriate diagnosis and targeted therapy is important for orthopaedic surgeons, policymakers, and patients [1–3]. Although traditional culture-based methods remain the gold standard in clinical guidelines for PJI

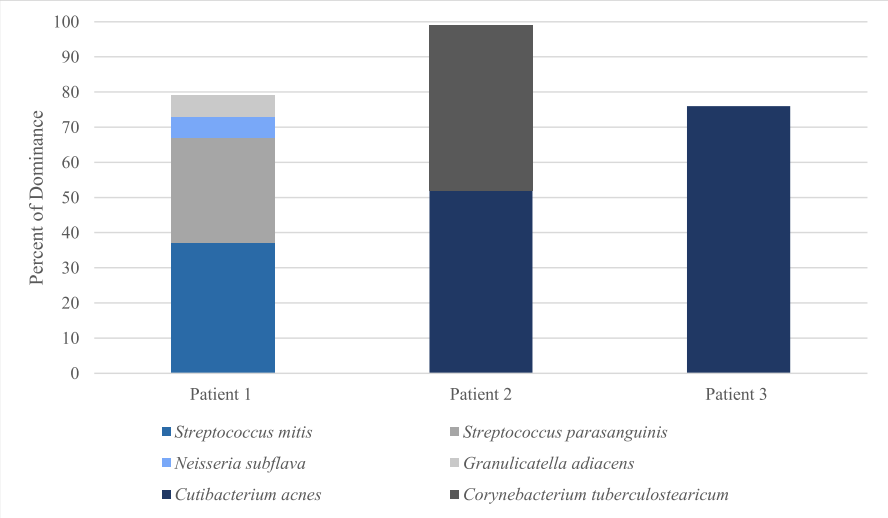

Fig. 1. Microbial strains identified with next-generation sequencing and percent dominance in 3 of the 80 native knee samples.

Table 2
Patient and Preoperative Characteristics Stratified for NGS Positive Results.

Characteristic	Negative NGS	Positive NGS	*P*-Value
N	37	3	
Age (mean (SD))	67.5 (9.5)	69.7 (7.1)	.709
Sex (%)			>.999
Men	17 (45.9)	1 (33.3)	
Women	20 (54.1)	2 (66.7)	
Number of injections (mean (SD))	1.4 (2.2)	2.3 (2.5)	.468
Elixhauser score (mean (SD))	1.3 (1.34)	1.3 (0.6)	.937
Body mass index (mean (SD))	32.7 (6.3)	35.9 (4.3)	.489
Race (%)			>.999
White/Caucasian	36 (97.3)	3 (100.0)	
Unknown	1 (2.7)	0 (0.0)	
Laterality (%)			.414
Bilateral	7 (18.9)	1 (33.3)	
Left	14 (37.8)	0 (0.0)	
Right	16 (43.2)	2 (66.7)	
Kellgren–Lawrence (%)			.751
2	3 (8.1)	0 (0.0)	
3	3 (8.1)	0 (0.0)	
4	31 (83.8)	3 (100.0)	
Smoking (%)			.413
Never	11 (29.7)	2 (66.7)	
Quit	24 (64.9)	1 (33.3)	
Yes	2 (5.4)	0 (0.0)	

NGS, next-generation sequencing; SD, standard deviation.

diagnosis, increasing emphasis has been placed on augmenting this with other techniques including NGS, particularly in cases where cultures are negative but clinical suspicion for infection is elevated [4,28]. The high sensitivity of NGS makes it powerful for the detection of microorganisms, but it raises concerns that a portion of the organisms identified by this technique may represent the intrinsic joint microbiome rather than pathogenic species. The present work suggests that in a small portion of patients, microorganisms may be present on NGS analysis even in the 'aseptic,' native knee with arthritis. Therefore, the additional information provided by NGS beyond that of classic culture-based methods requires appropriate and accurate interpretation to avoid both under and overdiagnosis.

The findings of this study align with those of prior work demonstrating positive NGS results in presumed 'aseptic' joints; however, the relative prevalence of this was lower in the present study [7,29]. In fact, the concept of a native microbiome is well-established in other areas of medicine including gastrointestinal, cardiac, oncologic, and neurodegenerative medicine [14–18]. Prior work has implicated the naturally occurring microbiome of the spine in cases of low back pain and disc degeneration [24,30]. Similarly, the gut native microbiome has been implicated with respect to the severity of osteoarthritis [19]. However, there are limited studies examining the relationship of the knee joint microbiome to preoperative factors in patients undergoing TKA for osteoarthritis [7,31]. One of the initial studies evaluating the role of NGS in hip and knee PJI reported that 35% of control patients undergoing primary total joint arthroplasty had positive NGS results

Table 3
Comparison of Synovial Fluid Characteristics in Knees With and Without Positive Next-Generation Sequencing Results.

Synovial Fluid Biomarker	No	Yes	*P*-value
n	77	3	
CRP (mean (SD))	1.14 (4.25)	0.34 (0.48)	.748
WBC (mean (SD))	153.88 (215.64)	143.67 (139.31)	.936
ESR (mean (SD))	42.13 (25.65)	68.70 (12.90)	.081

CRP, C-reactive protein; WBC, white blood cells; ESR, erythrocyte sedimentation rate.

[10]. While this population of controls with microorganisms reported by NGS likely included both hips and knees, it does suggest the existence of a natural microbiome in native, 'aseptic' joints. Similarly, another study evaluated NGS results in patients undergoing revision total hip arthroplasty and TKA and found that a microorganism was identified by NGS in 56 (65.9%) of culture-negative patients [32]. In conjunction with the present work, these findings highlight the potential for more comprehensive identification of microorganisms with NGS, but also the importance in understanding which microorganisms may represent pathogens versus native microbiota.

Prior work at our institution has demonstrated that 30% of patients undergoing primary TKA for osteoarthritis had at least one microorganism identified on NGS of samples obtained from the native joint at the time of surgery [7]. With respect to the NGS analysis, the manufacturer reports utilizing a different extraction method prior to the start of the present study with integration of host depletion for the more recent 80 samples, reporting microorganisms below the 2% threshold, and addition of synovial fluid biomarker reporting that makes direct comparison of the incidence of NGS positivity challenging. The samples sizes available and changes in NGS protocol may contribute to the lower percentage of positive samples in this study, 3.8 versus 30%. This potential for variability in NGS assay protocols represents a limitation when comparing studies utilizing this technology [33]; however, additional measures such as host depletion should theoretically increase the sensitivity of the assay [34]. Notwithstanding these changes, if we combine our NGS analysis with this prior work the overall positive NGS rate in 120 native knees was 12.5% (15 knees). Evaluation of this larger cohort also demonstrates polymicrobial NGS colonization in 91.7% of the positive samples.

The method of sample collection may also contribute to the identification of microbial samples. In the present study, 50 of the 80 samples included tissue obtained at the time of surgery. Since 30 patients were undergoing unilateral TKA, only synovial fluid was obtained from the contralateral knee. That being said, in the prior 40-patient series, 8 of the 12 positive NGS knees had microorganisms identified with synovial fluid aspiration [31]. In 4 of the 12, there were no microorganisms identified with the aspiration, but at least one of the tissue or swab samples was positive. Alternatively, prior work by Fernandez–Rodriquez et al evaluated only synovial fluid samples from a series of 65 knees, including normal knees, osteoarthritic knees, aseptic revisions, and those undergoing revisions for PJI, identifying 77 OTUs [29]. Their study demonstrated the highest number of species in the osteoarthritis group. *Cutibacterium*, *Staphylococcus*, and *Paracoccus* species were most common in native nonosteoarthritic knees, while *Proteobacteria* was observed more frequently in the osteoarthritic joints. Also, Tsai et al evaluated synovial tissue samples from 14 knees with osteoarthritis and 10 nonarthritic knees and reported *Pseudomonas* species to be 1 of 9 key microbes that correlated to osteoarthritis and immune pathways [11]. Interestingly, no *Pseudomonas* species or *Staphylococcus* species were identified in native knees in the present study, suggesting that there may be factors that influence the microbiome, such as patient comorbidities or geography, that have yet to be elucidated.

Potential Limitations

We acknowledge several potential limitations of the present study. While it is to the best of our knowledge the largest study systematically evaluating NGS results in native knee joints, the outcome of interest is rare, and therefore significant statistical analyses regarding the influence of patient or other factors (e.g, prior injection) are limited. Additionally, we acknowledge that the

change in the manufacturer protocol compared to the prior work by our group makes direct comparison to the prior work challenging. Importantly, the microorganisms identified with NGS could represent contamination; however, we propose a low likelihood of this given our standardized protocol of NGS extraction and processing according to the manufacturers' standards. Additionally, prior work from our institution utilized sterile water controls collected at the time of synovial fluid and tissue sampling and demonstrated that all 4 sterile water controls for the study of 40 patients had negative NGS result [7]. Furthermore, as discussed above, the presence of *Cutibacterium, Streptococcus, Neisseria,* and *Corynebacterium* species has been reported in other studies [7,29]. *Granulicatella* represents a traditionally difficult-to-isolate, nutritionally-variant streptococci most known for its role in endocarditis but has been reported to be a rare cause of septic arthritis and PJI [35–37]. Furthermore, while we report there were no infections in any of the 80 knees at 1 year, the present study did not include longer follow-up to evaluate the potential clinical implications of a positive NGS result in a native knee undergoing primary TKA. Moreover, the rate of microorganisms identified was low such that detecting a potential increased risk of PJI would require larger numbers and represents an important area of future investigation.

Conclusions

To our knowledge, this is the first study to evaluate NGS results in bilateral native knees. While a proportion of native knees have microorganisms identified with NGS analysis, the proportion may be lower than previously reported. This supports the previously established premise of a native knee microbiome and should be taken into consideration in the appropriate application of NGS techniques, specifically for the diagnosis and treatment of infection.

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