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MAJOR ARTICLE

Identification of Prosthetic Joint Infection Pathogens Using a Shotgun Metagenomics Approach

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Background. Metagenomic shotgun sequencing has the potential to change how many infections, particularly those caused by difficult-to-culture organisms, are diagnosed. Metagenomics was used to investigate prosthetic joint infections (PJIs), where pathogen detection can be challenging.

Methods. Four hundred eight sonicate fluid samples generated from resected hip and knee arthroplasties were tested, including 213 from subjects with infections and 195 from subjects without infection. Samples were enriched for microbial DNA using the MolYsis basic kit, whole-genome amplified, and sequenced using Illumina HiSeq 2500 instruments. A pipeline was designed to screen out human reads and analyze remaining sequences for microbial content using the Livermore Metagenomics Analysis Toolkit and MetaPhlAn2 tools.

Results. When compared to sonicate fluid culture, metagenomics was able to identify known pathogens in 94.8% (109/115) of culture-positive PJIs, with additional potential pathogens detected in 9.6% (11/115). New potential pathogens were detected in 43.9% (43/98) of culture-negative PJIs, 21 of which had no other positive culture sources from which these microorganisms had been detected. Detection of microorganisms in samples from uninfected aseptic failure cases was conversely rare (7/195 [3.6%] cases). The presence of human and contaminant microbial DNA from reagents was a challenge, as previously reported.

Conclusions. Metagenomic shotgun sequencing is a powerful tool to identify a wide range of PJI pathogens, including difficult-to-detect pathogens in culture-negative infections.

Keywords. metagenomics; prosthetic; joint; infection; diagnosis.

Prosthetic joint infections (PJIs) are devastating complications of up to 2% of total joint arthroplasties and are associated with significant morbidity and cost [1]. A wide range of pathogens can cause PJIs, including aerobic and anaerobic bacteria, and fungi [2, 3]. Polymicrobial infections are also common. Five to 20% of cases that meet clinical criteria for PJI remain culture negative, and despite advances in culture techniques and molecular biology assays, such as panel polymerase chain reaction (PCR) assays, many cases remain in which no microbiological etiology is identified [2]. In the absence of a defined pathogen, culture-negative PJIs are typically empirically treated with a broad-spectrum intravenous antimicrobial regimen to cover the many pathogens commonly associated with PJI.

Aseptic failure refers to arthroplasty failure in the absence of findings of infection. While this can encompass a wide range of etiologies, of particular interest is the phenomenon of aseptic loosening in which arthroplasty components loosen and lead to pain and mechanical failure. Microorganisms have been suspected to play a potential role in a subset of aseptic loosening cases. Previous studies using a variety of methods have demonstrated wide disparity in the frequency of organisms detected in association with aseptic loosening, leaving open the question as to whether microorganisms undetected by culture may play a role in some cases of "aseptic" failure [4, 5].

Metagenomic shotgun sequencing is a method in which all nucleic acid in a sample is extracted and sequenced using next-generation sequencing techniques, after which the resulting sequences are used to identify organisms present in the sample. This approach has been used to characterize a wide range of specimen types, including those in environmental and microbiome studies [6, 7]. Metagenomics is also gaining interest in infectious diseases diagnostics as a method to identify pathogens that are not easily detected using current techniques, such as culture or directed PCR approaches. It offers advantages over 16S ribosomal RNA (rRNA) gene "broad-range" PCR in that it is potentially able to identify any pathogen, especially when DNA- and RNA-based methods are used in combination. Further, resistance-associated genes and mutations can be identified [8]. However, metagenomic shotgun sequencing faces many challenges as well. The often overwhelming presence of human nucleic acids in samples can make detection of

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pathogens challenging [9]. The time and cost associated not only with sequencing but also analysis of large, complicated sets of data can also be barriers. Contaminating microbial DNA in reagents is also often encountered, making it difficult to distinguish true pathogens from background noise [10–12]. Despite these and other limitations, metagenomics research is rapidly growing and offers the potential to change how many unknown or difficult-to-detect pathogens are identified.

In prior studies involving a small number of clinical cases, we reported the use of metagenomics to identify PJI pathogens as well as the application of tools to optimize these methods [9, 13, 14]. Since then, additional studies have provided further evidence of the value of this approach for detection of PJI pathogens as well as organisms in other infection types [15–17]. Here, we report the results of shotgun metagenomic analysis of 408 sonicate fluid samples generated from resected prostheses, providing the largest cohort to date of metagenomic analysis of clinical specimens of any type. In addition, compared with recently published smaller studies, we used different sample processing methods, particularly different microbial DNA enrichment methods and sequencing techniques, which allowed for greater depth of coverage of detected organisms. With the inclusion of a large number of aseptic failure cases, we also sought to further investigate the potential role of unidentified microorganisms in the failure of these otherwise seemingly uninfected samples.

MATERIALS AND METHODS

Samples

Samples were collected under the Mayo Clinic Institutional Review Board protocol 09-00808. Sonicate fluids were prepared from resected prosthetic hip and knee components between 2011 and 2016 using previously described vortexing/sonication methods [18]. Sonicate fluids from resected antibiotic spacers and polyethylene inserts were not included. Complete clinical microbiological data were available for all samples. Pathology findings were determined by the consulting pathologist at the time of surgery. Four hundred eight samples, including culture-positive PJIs, culture-negative PJIs, and aseptic failures, were selected sequentially from a database of resected arthroplasties evaluated using sonication-based methods for culture. Use of sonication was at the operative surgeon's discretion and therefore not performed on all resected arthroplasties at our institution during the period of sample collection. Twenty samples (from 2 preparation batches) were removed from analysis due to high levels of contaminant bacterial DNA across samples as all samples were positive for high levels of *Streptococcus mitis* DNA.

Sample Classification

Samples were classified as aseptic failure or PJI based on Infectious Diseases Society of America (IDSA) PJI diagnostic criteria [19]. Sonicate fluid cultures with ≥20 colony-forming units (CFU)/10 mL were considered culture positive. Two or more cultures growing the same organism were required for intraoperative tissue cultures to be considered positive.

Sample Preparation, Sequencing, and Analysis

Full details regarding sample preparation, sequencing methods, tools used for metagenomic data analysis, and interpretation of results are available in the Supplementary Methods.

Statistical Analysis

Differences in age between aseptic failure and PJI subjects were calculated using an unpaired *t* test. A χ^2 test was used to test for differences in proportions of arthroplasty location and sex.

RESULTS

Metagenomic Shotgun Sequencing

A total of 408 sonicate fluid samples from resected hip and knee arthroplasties were analyzed using metagenomic shotgun sequencing. This included 213 samples classified by IDSA criteria as having PJI, and another 195 classified as having aseptic failure [19]. Subject demographics, classification criteria, and clinically relevant findings, including laboratory testing, are summarized in Table 1, with individual sample details available in Supplementary Table 1. An average of 28 592 988 paired-end reads were sequenced from each sample (range, 15 133 370–45 274 168 reads).

Analysis of Aseptic Failure

To test whether microorganisms were present in prosthetic joints that failed for reasons other than infection (aseptic loosening, adverse local tissue response, instability, periprosthetic fracture, etc), 195 sonicate fluids generated from uninfected prostheses were analyzed by shotgun metagenomics. Of these, 7 (3.6%) contained sufficient sequences and reference genome coverage to indicate the possible presence of bacteria (Tables 2 and 3). The detected species are all known to cause PJI (Table 4). In 6 of 7 cases, the organism was detected with both analytic tools, with 1 case identified with Livermore Metagenomics Analysis Toolkit (LMAT) alone (Supplementary Table 1). Large numbers of microbial reads were present in many of the uninfected samples, reflecting contaminant DNA sequences common to metagenomics, particularly in samples with low-input DNA after removal of human DNA (Supplementary Table 1). Alignment of these reads to reference genomes invariably resulted in high coverage depth of short genome fragments rather than being distributed across a genome, a pattern often present in negative controls with reagents only and no input sample.

Shotgun Metagenomics Compared to Sonicate Fluid Culture

During clinical evaluation of a potentially infected arthroplasty, there are typically multiple cultures from various sources including synovial fluid, intraoperative tissue specimens, and sonicate fluid, the last generated in an attempt to dislodge bacteria adherent to the prostheses after removal. Sonicate fluid was the only sample tested by metagenomics herein; therefore,

Table 1. Characteristics of Subjects

Characteristic	Aseptic Failure (n = 195)	PJI (n = 213)
Age[a], y, mean (range)	64.8 (18–89)	65.8 (30–93)
Sex[b], female, No. (%)	106 (54)	98 (46)
Site of arthroplasty[c], No. (%)		
Knee	155 (79)	126 (59)
Hip	40 (21)	87 (41)
IDSA PJI criteria, No.		
Purulence visible intraoperatively	0	130
Presence of sinus tract	0	52
Identical organism identified with 2 separate cultures	0	111
Acute inflammation on histopathology	1 of 170	113 of 144
Antibiotics within 4 wk prior to surgery	10	131
Laboratory parameters, median (range)		
Hemoglobin, mg/dL	13.6 (7.2–17.7)	11.7 (5–15.7)
WBC count, 10^3 cells/μL	6.8 (3.0–48.6)	7.8 (3.1–40.6)
ESR, mm/h (reference range, 0–22)	9 (0–109)	40 (1–130)
CRP, mg/L (reference range, 0–8.0)	3.8 (1–245)	31.8 (3–400)

Abbreviations: CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; IDSA, Infectious Diseases Society of America; PJI, prosthetic joint infection; WBC, white blood cell.

[a] $P = .355$.

[b] $P = .092$.

[c] $P < .00001$.

metagenomic results were initially compared directly to culture results from sonicate fluid. In these analyses, cases where <20 CFU/10 mL of bacteria were detected were considered negative as is the case in our routine clinical practice.

Of the 213 sonicate fluid specimens from subjects with PJI, 115 (54%) were culture positive and 98 (46%) were culture negative (Table 2). Of the 115 culture-positive cases, the pathogens identified by culture were also detected by metagenomics in 109 (94.8%) cases. Metagenomics provided identical findings to those of culture in 99 (86.1%) cases, with additional potential pathogens detected in 11 (9.6%) cases. Four of these 11 were known polymicrobial infections in which additional potential pathogens were detected. The other 7 cases appeared to be monomicrobial by culture but were found to have at least 1 new potential pathogen. In 1 case (sample 588), the species identified by both metagenomic analytic tools (*Peptoniphilus harei*) was different from that determined by conventional techniques, including 16S rRNA gene sequencing and quick indole testing (*Peptoniphilus indolicus*).

In 6 cases, a pathogen detected by culture was not detected using metagenomics. Two of these (samples 838 and 1031) were polymicrobial infections and only 1 pathogen was detected by metagenomics. In 2 cases (sample 793, a *Candida albicans* PJI, and sample 1041, a *Mycobacterium abscessus* PJI), sonicate fluid cultures were not quantified, so it is unknown if these would have met the ≥20 CFU/10 mL threshold for being considered culture-positive by sonicate fluid culture. In 2 other samples (samples 559 and 813), the undetected pathogen was *Pseudomonas aeruginosa*. Subsequent testing revealed that certain *P. aeruginosa* strains may be more susceptible to DNA degradation during the MolYsis microbial enrichment steps, whereas other tested species including *M. abscessus*, *Escherichia coli*, and *Enterobacter cloacae* isolates were not lysed during MolYsis treatment (data not shown).

Of the 98 cases considered to be culture negative by sonicate fluid culture, potential pathogens were detected in 43 (43.9%) using metagenomics. The microorganisms identified largely consisted of those previously reported as PJI pathogens (Table 4). One novel PJI pathogen, *Mycoplasma salivarium*, was identified, as previously reported [14]. Of these new identifications, 19 had been found by culture in specimen types other than sonicate fluid, whereas 24 cases reflected novel detections (ie, not otherwise found) through metagenomics.

Shotgun Metagenomics Compared to All Culture Sources

To better reflect all data available in a clinical scenario, metagenomic results were compared to all available culture results, including intraoperative tissue and synovial fluid cultured any time prior to surgery. With these, an additional 31 PJI cases were classified as culture positive (Table 3). Metagenomics resulted in identical identifications in 121 of 146 (82.9%) culture-positive cases, with additional potential pathogens detected in 12 cases. However, 16 (11.0%) cases had at least 1 known pathogen not detected by metagenomics. In 14 of these cases, antibiotics had been administered prior to prosthesis removal. In 7 cases, there was no growth from the sonicate fluid that was being used for metagenomic analysis. Metagenomics was still able to identify new potential pathogens in 21 of 67 (31.3%) culture-negative cases.

Read Counts of Detected Microorganisms

Read counts identified as the primary pathogen or potential pathogen were calculated to estimate the depth of genome

Table 2. Performance of Metagenomic Shotgun Sequencing Versus Sonicate Fluid Culture

Case Classification	Samples, No.	Identical Findings	Organisms Not Identified by Metagenomics	New Organisms Detected by Metagenomics
Aseptic failure	195	188 (96.4)	NA	7 (3.6)
Culture-positive PJI	115	99 (86.1)	6 (5.2)	11 (9.6)
Culture-negative PJI	98	55 (56.1)	NA	43 (43.9)

Abbreviations: NA, not applicable; PJI, prosthetic joint infection.

Data are the No. (%) of samples in which identical findings or discrepant findings between sonicate fluid culture and metagenomic sequencing were observed.

Table 3. Metagenomic Sequencing Versus All Cultures From All Sources (Intraoperative Tissue, Preoperative Synovial Fluid, and Sonicate Fluid)

Case Classification	Samples, No.	Identical Findings	Organisms Not Identified by Metagenomics	New Organisms Detected by Metagenomics
Aseptic failure	195	188 (96.4)	NA	7 (3.6)
Culture-positive PJI	146	121 (82.9)	16 (11.0)	12 (8.2)
Culture-negative PJI	67	46 (68.7)	NA	21 (31.3)

Abbreviations: NA, not applicable; PJI, prosthetic joint infection.

Data are the No. (%) of samples in which identical findings or discrepant findings between sonicate fluid culture and metagenomic sequencing were observed.

coverage, which is important for gene content interpretative analyses, such as antibiotic resistance prediction. Of the 110 pathogens detected by culture, the average number of paired-end reads attributable to the known pathogen was 4 989 251, with a median of 1 978 090 reads per pathogen, and up to 32 892 968 reads (out of 37 499 849 total) in 1 sample (Table 5). Reads counts for noncultured potential pathogens were fewer, with the average number of reads from the 3 other categories ranging from 113 969 reads in the aseptic failure cases, up to 1 948 403 reads from new potential pathogens not previously identified in culture-positive PJI cases.

DISCUSSION

Metagenomic shotgun sequencing has the potential to enhance or change how many difficult-to-detect pathogens are identified. Using this approach to evaluate sonicate fluids generated from 408 resected arthroplasties, we observed that metagenomics was able to detect most pathogens identified by culture (89.0%–94.8% depending on culture positivity definition), as well as many that were not detected by culture. This was particularly true in the culture-negative PJI group in which potential pathogens were detected in 43.9% of cases with no sonicate fluid growth. Of these, 48.8% (21 of 43) were not found in cultures of other sources, that is, intraoperative tissue specimens or synovial fluid.

The species newly detected by metagenomics as potential pathogens consisted largely of common PJI pathogens (Table 4). Some notable exceptions included *M. salivarium* and *Mycobacterium bovis* bacillus Calmette-Guérin, bacteria that can go undetected with routine bacterial culture techniques. Interestingly, the individual with *M. salivarium* detected by metagenomics (sample 1116) later developed recurrent PJI and *M. salivarium* was detected using molecular techniques, as previously reported [14], as well as by specialized culture.

Street et al recently published similar findings using a metagenomic sequencing approach to PJI pathogen identification with a genus-level sensitivity of 93% (64/69 samples) despite differences in techniques, including microbial DNA enrichment methods (NebNext microbiome enrichment kit vs MolYsis kit), sequencing methods (MiSeq vs HiSeq 2500), and analytic tools (Kraken vs LMAT and MetaPlan2) [15]. The use of MolYsis reagents and sequencing at higher depths reported here allowed a larger proportion and number of reads from organisms

Table 4. Species From Discordant Samples

Discrepancy Between Methods	New or Missed Identifications by Metagenomics vs Sonicate Fluid Culture	New or Missed Identifications by Metagenomics vs Sonicate Fluid Culture	New or Missed Identifications by Metagenomics vs Sonicate Fluid Culture
PJI organisms not detected by metagenomics	*Bacillus* spp *Candida albicans*	*Mycobacterium abscessus* *Porphyromonas* species	*Pseudomonas aeruginosa* (2)
New organisms detected in aseptic failure	*Cutibacterium acnes* (2)	*Staphylococcus aureus* (3)	*Streptococcus sanguinis* (2)
New organisms detected in culture-positive PJI	*Anaerococcus obesiensis* *Clostridium* species *C. acnes* *Enterobacter cloacae*[a]	*Finegoldia magna* (3)[a] *Peptoniphilus harei* *Prevotella nanciensis* *S. aureus*	*Staphylococcus epidermidis* (5) *Staphylococcus lugdunensis* (3) *Varibaculum cambriense*
New organisms detected in culture-negative PJI	*Anaerococcus urinae* *C. albicans* (2)[a] *Candida parapsilosis*[a] *Clostridium perfringens* *Corynebacterium pseudogenitalium* *C. acnes* *Enterococcus faecalis* (3)[a]	*E. cloacae* (2)[a] *Facklamia languida* *Granulicatella adiacens* (2)[a] *Mycobacterium bovis* BCG[a] *Mycoplasma salivarium* *Peptoniphilus* species *Pasteurella multocida*[a]	*S. aureus* (10)[a] *S. epidermidis* (5)[a] *Staphylococcus haemolyticus* (2)[a] *S. lugdunensis* *Streptococcus agalactiae* (4) *Streptococcus dysgalactiae* (4)[a] *Streptococcus oralis*[a]

Abbreviation: PJI, prosthetic joint infection.

Listed are species identified or missed by shotgun metagenomics compared to sonicate fluid culture alone. Values in parentheses indicate detection in >1 subject; the number given is the number of subjects.

[a]Indicates organism identified in cultures other than sonicate fluid.

Table 5. Read Counts for the Top Known or Potential Pathogen Identified in Samples as Identified by Livermore Metagenomics Analysis Toolkit at the Genus Level

Statistics	Organisms Also Detected by Sonicate Fluid Culture, No.	New Potential Pathogens Identified, No.		
		Culture-Positive PJI	Culture-Negative PJI	Aseptic Failure
No. of samples	110	10	43	7
Median	1 978 090	11 786	21 762	24 059
Mean	4 989 251	1 948 403	384 630	113 969
Minimum	117	595	100	5392
Maximum	32 892 968	10 196 578	3 976 409	433 462

Abbreviation: PJI, prosthetic joint infection.

detected. More complete coverage is important when performing additional analyses such as gene content, including antibiotic resistance prediction, which requires complete coverage of genomes at sufficient depth to provide confidence in predicting that a resistance-conferring gene or mutation is not present [9]. Ruppé et al also recently published a study using metagenomics to examine a variety of orthopedic infections [20]. Of the 104 samples tested, 24 specimens from 14 individuals contained sufficient DNA to perform metagenomic sequencing. Known pathogens were detected in all (8) monomicrobial specimens, while 74.5% of isolates from polymicrobial samples were detected at the genus level. They too observed large numbers of additional species. While some of them were plausible given the source (eg, mandibular or sacral infections), the overall pattern and presence of species in negative controls suggests contaminant reagent DNA to be problematic.

In every sample tested, there were reads assigned to microorganisms other than known or suspected pathogens, including in uninfected cases and negative controls. This is a major challenge in the field of metagenomics as it is a common problem and can interfere with reliable identification of pathogens [10–12]. This is further complicated by the common contaminants (eg, *Acinetobacter*, *Streptococcus*, *Cutibacterium*, and *Staphylococcus* species) also being among common causes of PJI [11, 13]. We and others have observed that these background reads are largely attributable to the reagents used, as the composition can change when different brands of kits are used during DNA preparation. When analyzing negative controls or uninfected samples, we have observed that when large numbers of background reads are observed, this is due to high coverage of a few small fragments (typically <1 kb). This observation was useful to help determine "real" vs background reads by aligning reads from a sample to reference genomes; if the standard deviation was 10-fold greater than the average depth of coverage, the organism was determined to be a likely contaminant.

It is interesting that with the various sources tested by culture and metagenomic analysis of sonicate fluid, 46 cases that appeared to be infected by clinical criteria remained without a potential pathogen identified. Of these, 27 had received 1 or more antibiotics within 4 weeks prior to surgery, which is known to increase the risk of culture-negative PJI [21]. Another likely contributor is the lack of a gold standard for PJI vs aseptic failure diagnosis. PJI classification is based on a combination of clinical, microbiological, and histological findings; although some subjects may fit 1 or more criteria for PJI classification, the joint may not have actually been infected. Limitations of reference databases can also lead to microorganisms not being detected using a metagenomics approach [22]. We observed examples of this while using 2 separate analysis tools, LMAT and MetaPhlAn2. For example, samples 637, 765, and 930 each contained >1 million reads from known pathogens, yet were not detected using MetaPhlAn2 (Supplementary Table 1). These discrepancies highlight the utility of using multiple analytic tools to help overcome deficiencies of individual methods or databases.

The role of bacteria or other microorganisms as contributors to aseptic loosening or other causes of aseptic failure continues to be an active area of research. We analyzed a large set of arthroplasties removed for reasons other than infection and, of the 195 samples tested, only 7 had sufficient microbial reads beyond that of negative controls to be considered potentially positive. While these results do not rule out a potential role of microorganisms in the other 188 cases, we were unable to find evidence of the presence of bacteria in higher proportions of samples as has been reported previously using other methods [4, 5].

There are limitations to this study and the methods used. Accurate case definition and limitations of sequence databases are mentioned above. MolYsis was used for microbial DNA enrichment, and whereas multiple studies have published the wide range of bacteria detectable by PCR after MolYsis treatment, we observed that some *P. aeruginosa* strains were susceptible to DNA degradation by MolYsis pretreatment, raising the question as to whether other pathogens may have been selected against using this method [23]. Alternative microbial DNA enrichment techniques are available but have the potential to introduce other biases [24]. Skipping microbial enrichment and sequencing at much higher depths is another possibility but greatly increases the cost per sample and would still result in too few reads to reliably obtain useful information regarding gene content in many cases [9].

The role of metagenomics in infectious disease diagnostics continues to evolve. Case reports have highlighted its value in

select situations where no pathogen is identified by conventional testing methods [14, 22]. Small series of samples have also addressed the utility in specific specimen types, such as urine and feces [16, 17, 25]. High costs and slow turnaround times are barriers to routine use; however, improving technology continues to make sequencing and subsequent analysis faster and less expensive. For these studies, the sequencing method (HiSeq 2500 rapid run mode, 2 × 250 cycles) alone takes approximately 60 hours to run, putting total process time with sample preparation and analysis at approximately 6 days. Given that metagenomics found identical results to those of culture in the large majority of aseptic failures and culture-positive PJIs, the highest-yield use of metagenomics would likely come from using it in PJI cases where no pathogen is identified with initial culture. Subsequent studies are needed to determine the utility of metagenomics vs other methods, such as 16S and 18S rRNA gene PCR/sequencing, which is becoming increasingly available; the role for metagenomics may also be after these molecular methods fail to identify a potential pathogen or if additional gene content information is needed for treatment decisions.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Disclaimer. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health (NIH).

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