Diagnosis of Periprosthetic Joint Infection: The Promise of Next Generation Sequencing

Abstract

Background: Next-generation sequencing (NGS) is a well-established technique for sequencing of DNA and has recently gained attention in many fields of medicine. Our aim was to evaluate the NGS' accuracy in identifying the causative organism(s) in patients with periprosthetic joint infection (PJI).

Methods: In this prospective study samples were collected from 65 revision arthroplasties (39 knees, 26 hips) and 17 primary arthroplasties (9 hips, 8 knees). Synovial fluid, deep tissue and swabs were obtained at the time of surgery and shipped to the laboratory for NGS analysis. Deep tissue specimens were also sent to the institutional lab for culture. Sensitivity, Specificity, positive and negative predictive values were calculated for NGS, using the Musculoskeletal infection society (MSIS) definition of PJI as the gold standard.

Results: Twenty-eight revisions were considered infected; culture was positive in 17 (60.7%, 95% confidence interval [CI] 40.6%-78.5%), while NGS was positive in 25 (89.3%, 95% CI 71.8%-97.7%), with concordance between NGS and culture in 15 cases. Among the 11 cases of culture-negative PJI, NGS was able to identify an organism in 9 cases (81.8%, 95% CI 48.2%-97.7%). NGS identified microbes in 9 of 36 (25.0%, 95% CI 12.1%-42.2%) "aseptic" revisions with negative cultures and in 6 of 17 primary TJA (35.3%, 95% CI 14.2%-61.7%). NGS detected several organisms in most positive samples. However, in the majority of patients that were infected one or two organisms were dominant.

Conclusion: NGS may be a useful adjunct in identification of causative organism(s) in culture-negative PJI. Our findings suggest that some cases of PJI may be

polymicrobial that escape detection using culture. **Further study is required to determine the significance of isolated organisms in samples from patients who are not thought to be infected.**

Level of evidence: Diagnostic level III.

Keywords: Periprosthetic join infection, Next generation sequencing, culture-negative

Introduction:

Periprosthetic joint infection (PJI) is a grave complication following total joint arthroplasty, with broad implications.(1–4) Perhaps the most challenging facet of managing PJI is reaching a prompt and definitive diagnosis, with identification of the causative organism. (5,6). In up to 50% of PJI cases, cultures fail to isolate the infecting organism.(7–11) Negative cultures pose a challenge as the lack of identity of the infecting organism leads to the use of empiric antimicrobial therapy, with the potential to miss the true infecting pathogen, **and negative cultures have been associated with a 4.5 times increased risk of reinfection following revision arthroplasty.(6,12)**

We have a long-standing interest in employing molecular techniques for the diagnosis of PJI.(13) Our initial studies using multiplex polymerase chain reaction (PCR) revealed that the molecular techniques for isolation of the infecting organism held promise.(14) **However, this technique demonstrated a false positive rate of 88%, and in other studies did not outperform traditional culture with a sensitivity of 81%.(14,15)** Other techniques such as broad-range PCR are limited in the output they can produce, as 70% of the amplicons must be of a single sequence in order to generate a meaningful result. Additionally, only one organism can be detected at a time, unless sequencing of several clones is to be performed. (16) **Broad-range PCR has also shown a limited sensitivity ranging from 67.1-73.3%, and hence does not hold a clear advantage over**

culture.(9,17,18)

Next generation sequencing (NGS), is capable of sequencing all DNA present in a given sample, giving a more complete picture of the microbial profile present.**(19) NGS has been shown to identify pathogens, in patients with neurological infections and systemic sepsis.(20,21) To our knowledge, there are no studies evaluating NGS for identifying infectious organisms in PJI.** In recent years, with the rapid decline in the cost of sequencing, we have been exploring NGS' role in diagnosing PJI. A prospective study was designed to evaluate the role of NGS in diagnosing PJI, and culture negative PJI(CN-PJI) in particular.

Methods

Following institutional review board approval, consecutive patients undergoing revision arthroplasty between June to November 2016 by a single surgeon were prospectively enrolled in this study. All patients undergoing revision total knee and total hip arthroplasty were eligible for recruitment. In addition, a cohort of patients undergoing primary arthroplasty was also included provided there was no previous surgery in the index joint.

Preoperative assessment:

Patients undergoing revision arthroplasty were screened preoperatively according to institutional protocols, including obtaining blood for measurement of erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP).(22) Patients were aspirated at the discretion of the treating surgeon, if it was felt that a definitive diagnosis had not been reached. In these cases, synovial fluid was assessed for white blood cell count, white blood cell differential, leukocyte esterase (LE) and culture. **Preoperative antibiotics were withheld from 2 weeks prior to surgery until samples were collected for culture/NGS in all revision arthroplasty cases.**

Intraoperative sample collection:

Synovial fluid, deep tissue specimens and swabs from the intramedullary canals were obtained for all patients at the time of surgery. Synovial fluid was obtained in a sterile fashion, using an 18-gauge needle prior to arthrotomy. Deep tissue specimens were taken from the synovium and intramedullary canals. Swabs of the acetabulum and the intramedullary canal of the femur were obtained from hips, and from the intramedullary canal of the femur and tibia in knees. All samples were promptly stored in sterile

containers and shipped overnight at ambient temperature to the laboratory (MicrogenDx Laboratories, Lubbock, TX) for NGS. Deep tissue specimens were also sent to the institutional laboratory for routine culture, including aerobic and anaerobic bacterial cultures, fungal cultures and acid-fast bacillus cultures. **Samples for culture were not collected from primary arthroplasty cases.**

Next Generation Sequencing:

Upon arrival at the lab, the first step is DNA extraction and performance of a quantitative PCR to determine the bacterial burden present in the sample. This process is described in Appendix A. The second step is the NGS assay. Initially, the DNA is amplified via a PCR reaction using forward and reverse primers flanking the region of interest. For the detection of bacterial and fungal species, the two regions of interest are the 16S and internal transcribed spacer (ITS), which are highly conserved regions of the rRNA gene in bacteria and fungi, respectively.(23,24). Following the amplification process, the amplified DNA was then pooled based on amplification strength. Sample DNA was then loaded onto beads for the emulsion PCR. Emulsion PCR was then carried out to generate high levels of the sample DNA for NGS. The sample was then sequenced on the Ion Torrent PGM sequencing platform (ThermoFisher Scientific, Waltham, MA). The Ion Torrent sequencer relies on the principle that a hydrogen ion is released each time a nucleotide is incorporated into the DNA, thus generating a change in pH. This change in pH corresponds to the number of nucleotides incorporated into the growing sequence, which is then detected by the sequencer. The final step before data analysis consists of denoising, to remove short sequences that may interfere with the interpretation of the data generated.(25) The sequence reads generated are then compared against a curated

NIH/Genbank database. The comparison against the database is performed using USearch7, and an agreement of at least 90% between the sequence reads and the database is necessary.

Antimicrobial therapy:

For all patients with positive culture at the time of surgery, antimicrobial therapy was administered intravenously to cover organisms in accordance with the culture results. For culture-negative PJI patients, also intravenous antimicrobial therapy was initiated and continued. Our Infectious Disease physicians took into account the findings of the NGS and tailored the antimicrobial therapy based on the NGS findings. The outcome of all patients with regard to infection control was evaluated. .

Data and statistical analysis:

Power analysis was conducted to determine the sample size. Using prior institutional data on molecular techniques(14), we used a 30% difference in sensitivity between NGS and culture, a power of 80%, and an alpha error of 0.05 a sample size of 55 patients was determined.

Patients were categorized as infected or aseptic using the Musculoskeletal infection society (MSIS) criteria(26). These two groups were further subdivided based on whether culture results were positive. Student's t test was used to calculate difference in continuous variables between groups, while Chi-squared analysis was used to measure differences in categorical variables. Sensitivity and specificity were calculated and compared between NGS and culture using McNemar's test. We examined the peak percentage of organism in a NGS sample as a predictor for infection using the area under the ROC curve (AUC). Concordance between culture and NGS was also examined**, and**

all cases with at least one positive intraoperative culture were considered culture-positive. Given that NGS detects all organisms in a given sample, the detection of multiple species is not an infrequent occurrence. Complete concordance was defined as NGS and culture picking up identical organisms. **If culture detected multiple organisms that were undetected on NGS**, then any overlap in regard to organisms identified was considered to be partial concordance. If NGS/culture identified completely different bacteria, this was considered discordant.

Results

Overall, samples were obtained from 78 patients undergoing 86 procedures. Sixty-nine procedures were revision arthroplasties (39 knees, 26 hips) and 17 were primary arthroplasties (8 knees, 9 hips). Two patients were excluded due to insufficient data to allocate them as infected/aseptic. Another two patients were excluded since culture was not obtained (Figure 1). Overall, **Twenty-eight** samples were classified as infected and 37 were considered "aseptic" (Table 1). Culture was positive in 17 (60.7%, 95% confidence interval [CI]:40.6%-78.5%) of the infected cases and in one (2.7%, 95% CI:0.1%-14.2%) "aseptic" revision. NGS was positive in 25 (89.3%, 95% CI:71.8%-97.7%) infected cases and in 10 (27.0%, 95% CI 13.8%-44.1%) "aseptic" revisions (Figure 2). **6 month follow up was obtained for all patients and is displayed in Table 2.**

Culture positive infections:

The first analysis was to examine the concordance between culture and NGS in patients who were infected and had positive cultures (n=17). There was one case of E. coli positive cultures in which NGS did not detect the organism. NGS was positive in the remaining 16 cases. Of these, 15 cases showed **complete concordance** between NGS and

culture. In six cases NGS had detected several other organisms. Yet, in most cases one organism predominated, making up more than 90% of the sample (Figure 3). In three cultures multiple organisms were detected. In these cases concordance with NGS was only partial; two patients were identified as being infected with *S.aureus* by culture, whereas NGS had detected *Staphylococcus Lugdunensis*. One patient had cultures positive for *Klebsiella pneumoniae, S.epidermidis and S.aureus,* while NGS detected only *S.epidermidis.* There were 2 cases of discordance related to bacterial resistance. In one of them NGS detected the Mec-A gene and classified the organisms as methicillin resistant *S.aureus* (MRSA) while culture identified the organism as methicillin sensitive *S.aureus* (MSSA). In the other, NGS detected MSSA while culture was positive for MRSA.

Culture negative infections:

There were eleven patients (39.3%, 95% CI:21.5%-59.4%) classified as MSIS positive who had negative cultures. NGS was able to identify an organism in nine of these cases (81.8%, 95% CI:48.2%-97.7%) that included known pathogens such as *S.epidermidis, Streptococcus canis, Burkholderia cepacia,* and *Pseudomonas stutzeri*.(27). Two patients with negative NGS result were classified as infected based on the presence of sinus tracts. Notably, in both cases both NGS and culture failed to isolate an infecting organism.

***Aseptic** revisions:*

One patient who did not meet the MSIS criteria for PJI had a single positive "very light" growth of Coryneform bacteria on culture, which was assumed to be a contaminant. NGS identified *P.acnes* in the same patient.

There were 36 patients undergoing revision arthroplasty who did not meet the criteria for

PJI and had negative cultures. NGS isolated microbial DNA in 9 (25.0%, 95% CI:12.1%-42.2%) of these cases. In all cases more than 3 different bacteria were present in the sample. *P.acnes* was the most prevalent organism in this group, positive in 6 cases. There was one case positive for fungi.

Primary arthroplasty:

Patients undergoing primary knee (n=8) and hip (n=9) arthroplasty were also examined. NGS identified an organism in six cases (35.3%, 95% CI:14.2%-61.7%). All positive samples originated from tissue, while swabs and fluid were all negative. Many of these were organisms originating from phyla shown to be part of the microbiome (28); in three cases the predominant organism originated from the Proteobacteria phylum, representing 98, 66 and 50 percent of the sample. In other samples, organism from the Fusobacteria and Actinobacteria phylum were detected in high percentage.

Performance of NGS and correlation to cultures:

In patients that were clearly infected (with more than two positive cultures), one or two organisms were predominant in the majority of cases. On the other hand, in patients presumed to be not infected, NGS detected a large number of organisms, with no predominant species. There was a significant difference in the mean number of pathogens detected by NGS between the infected and non-infected groups (4.7 vs. 8.9, $P<0.001$). The sensitivity, specificity, PPV and NPV of NGS were compared to culture (Tables 3-5). NGS was more sensitive (89.3% vs. 60.7%; 28.6% difference, 95% CI:9.1%-48%,P=0.01) but less specific than culture (73.0% vs. 97.3%;24.3% difference, 95% CI:4.9%-43.8%,P=0.003) in detecting any presence of bacteria in the sample. Setting a threshold of 59.5% of bacteria present in the NGS sample showed the highest AUC value

(0.85). Although this improved specificity (94.6%, 95% CI:81.81%-99.34%) it decreased sensitivity (71.4%, 95% CI:51.33%-86.78%).

Discussion

This study investigating the utility of NGS in diagnosing PJI reveals several findings. First, NGS was found to be capable of identifying an organism in almost 90% of patients with PJI (as determined by the MSIS criteria) compared to culture that had a sensitivity of 60.7%. Second, and perhaps more importantly, NGS detected a potential pathogen in 80% of CN-PJI. Third, NGS had 88.2% concordance with culture at our institution. **On the other hand, NGS was positive in 35% of primary arthroplasties and 25% of revision of arthroplasties, presumed to be non-infected.** Given that the incidence of CN-PJI is 27-55%, our results indicate that **NGS may be useful as an adjunct in the diagnosis** of CN-PJI(7–11). **Given the high rate of positive NGS results in both primary and aseptic revision arthroplasty, pre-test probability determined by the clinical picture and other laboratory investigations should be closely examined when interpreting the results of NGS.**

Infections associated with implants, are known to exist as biofilm, which interferes with the isolation of the infecting organism using culture.(29) **In the last several years, there has been a drive to identify a biomarker for PJI with alpha-defensin and leukocyte esterase being two examples. While these biomarkers may provide crucial diagnostic information, they simply indicate the presence or absence of infection with no identification of the causative organism.(30)** Several investigators have evaluated different techniques in order to remedy this problem. Xu et al. (31) examined specimens from 25 joint aspiration or revision TJA for suspected PJI. They used broad

range PCR aimed at16S rRNA gene and then cloning of the amplicon and sequencing a limited number of clones. In that study an organism was detected in only 11 patients, five of them had negative cultures. We previously reported a high detection rate of culture negative PJI with the use of PCR-based electron spray ionization time-of-flight mass spectrometry(14). That technique was able to identify an organism in all infected cases including four out of five CN-PJI. However, it had also detected an organism in many revisions presumed to be aseptic (50/57). In the present study NGS was able to detect a pathogen in more than 90% of cases and in 81.8% of culture negative cases. It also showed improved specificity. In all CN-PJI cases, NGS detected multiple organisms (3 or more). Given the quantitative results the test can provide, better insight can be obtained into these supposedly polymicrobial infections. Treatment of polymicrobial infection has been shown to have lower success rates compared to monomicrobial infection,(32) and a better understanding of these infections is needed to determine if they are truly polymicrobial in nature, or rather an infection with a dominant organism with other organisms acting in concert.

Earlier studies have shown bacteria to be present in presumed “aseptic” revisions in up to 77% of cases (33). Some of these may be subclinical infections (34,35) while other organisms may reflect part of the microbiome and are unlikely to cause an infection. NGS permits the generation of thousands of individual sequences from a single broad-range-PCR. This provides comprehensive information on the organisms occupying the joint and thus a better understanding of the joint microbiome. In approximately one third of supposedly non-infected revision cases in this study, NGS had detected bacteria. In many of these cases *P.acnes* was the predominant organism. Propionibacterium is known

to cause PJI, particularly in the shoulder and typically follows an indolent postoperative course (36) yet its presence in aseptic loosening is not fully understood (37).Other organisms isolated in our study, however, were mostly microbiota and the relative contribution of each organism was low. In one "aseptic" case NGS detected an organism that resulted in the subsequent failure of that patient with culture identifying the infecting organism at failure. It is plausible that with further follow-up we may witness the failure of additional cases with the same organism that was identified by NGS. These results support the current practice of an infectious workup prior to all revision cases (38). Several molecular diagnostics methods have been suggested to address the issue of diagnosing biofilm-associated infections. (8,9,13,39,40). The main issue with these methods relates to the uncertainties of whether the identified organisms are actually resident in the joint, or are contaminants, and whether they are truly "pathogens".(41) In the current study, 6 out of 17 patients undergoing primary arthroplasty an organism was identified compared to our previous study with 5 of 7 having a positive result using mass spectrometry, and this is certainly promising.(14) The isolation of an organism in a patient with arthritic joint and no prior operation should not be overlooked. Several parts of the body have been shown to have distinct microbiomes and dysbiosis of these intrinsic microbial communities **has been postulated to contribute to the pathogenesis of conditions thought to be noninfectious in nature, such as degenerative disc disease and breast cancer** (28,42,43).

The main limitation of the present study is the sample size which precludes us from making any generalizable conclusions. Nevertheless, the numbers are sufficiently high to show the utility of NGS in isolating the infecting organism in the majority of the culture

negative infections. No molecular methods were concurrently tested alongside NGS, thus we are not able to make direct comparisons to other techniques. However, culture remains the "gold standard" for isolation of the infecting organism and hence was used as a comparison. Our follow-up data on patients is limited; therefore we cannot reach any conclusion regarding some important findings such as the clinical relevance of "aseptic" revisions who were NGS positive. Finally, our interpretation of data could be affected by poor understanding of the microbiome. Thus, we considered "aseptic" patients with positive NGS as false positives, and primary arthroplasties as negative controls, which may prove to be an erroneous assumption. A better understanding of the native organism profile in the joint could help further interpret our findings. Future studies should focus on this group of patients in the long term. It is plausible that the majority of PJIs may be polymicrobial in nature and this may lead to the design different treatment strategies for these patients in the future.

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435 **Appendix A**

436 *DNA extraction and real-time PCR:*

437 Upon arrival at the NGS lab, tissue samples were transferred to 2 mL screw cap tubes,

438 while fluid samples are centrifuged for a period of 10 minutes. This was followed by

439 DNA extraction. DNA extraction was performed using the Roche High Pure PCR

440 Template Preparation Kit (Roche Diagnostics, Basel, Switzerland). The extraction

441 process was modified by the inclusion of a beading step for tissue and cell disruption

442 using 5mm steel beads, 5mm Zirconium Oxide beads and the use of the Qiagen

tissuelyser II instrument (Qiagen, Hilden, Germany). The lysate generated from this step was then prepared using the Roche High Pure PCR Template Preparation Kit. The initial step in the performance of the assay was the performance of real-time polymerase chain reaction (real-time-PCR), using the lifecycler 480 (Roche Diagnositics, Basel, Swtizerland). This assay provides a quantitative assessment of the bacterial burden of the assay, and covers a range of organisms and antibiotic resistance genes including the following: *Enterococcus faecalis, Klebsiella pneumoniae, Streptococcus agalactiae, Streptococcus pyogenes, Candida albicans, Enterococcus faecium, Pseudomonas aeruginosa, Staphylococcus aureus, Serratia marcescens,* methicillin resistance, vancomycin resistance.