■ HIP

Can next generation sequencing play a role in detecting pathogens in synovial fluid?

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Aims

The diagnosis of periprosthetic joint infection can be difficult due to the high rate of culture-negative infections. The aim of this study was to assess the use of next-generation sequencing for detecting organisms in synovial fluid.

Materials and Methods

In this prospective, single-blinded study, 86 anonymized samples of synovial fluid were obtained from patients undergoing aspiration of the hip or knee as part of the investigation of a periprosthetic infection. A panel of synovial fluid tests, including levels of C-reactive protein, human neutrophil elastase, total neutrophil count, alpha-defensin, and culture were performed prior to next-generation sequencing.

Results

Of these 86 samples, 30 were alpha-defensin-positive and culture-positive (Group I), 24 were alpha-defensin-positive and culture-negative (Group II) and 32 were alpha-defensin-negative and culture-negative (Group III). Next-generation sequencing was concordant with 25 results for Group I. In four of these, it detected antibiotic resistant bacteria whereas culture did not. In another four samples with relatively low levels of inflammatory biomarkers, culture was positive but next-generation sequencing was negative.

A total of ten samples had a positive next-generation sequencing result and a negative culture. In five of these, alpha-defensin was positive and the levels of inflammatory markers were high. In the other five, alpha-defensin was negative and the levels of inflammatory markers were low. While next-generation sequencing detected several organisms in each sample, in most samples with a higher probability of infection, there was a predominant organism present, while in those presumed not to be infected, many organisms were identified with no predominant organism.

Conclusion

Pathogens causing periprosthetic infection in both culture-positive and culture-negative samples of synovial fluid could be identified by next-generation sequencing.

Cite this article: *Bone Joint J* 2018;100-B:??–??.

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10.1302/0301-620X.100B2.BJJ-2017-0531.R2 $2.00

Bone Joint J 2018;100-B:–.
Received; Accepted after revision

Periprosthetic joint infection may be a devastating complication of arthroplasty, resulting in significant morbidity and mortality.[1-6] The diagnosis of periprosthetic joint infection continues to be difficult, with no single test providing absolute accuracy.[7] Several biomarkers have been introduced in recent years, such as leukocyte esterase and alpha-defensin, which provide a binary result indicating the presence or absence of infection.[8,9] While this information is critical, it allows only partial insight into the diagnosis and subsequent treatment. The isolation of the infecting organism remains fundamental in the treatment of any infection.

Culture-negative infections continue to challenge clinicians in several fields of medicine including orthopaedics. Several measures have been introduced to reduce the burden of culture-negative periprosthetic infections, including withholding antibiotics prior to the collection of samples for culture, culturing synovial fluid in blood culture bottles and extending the incubation period for culture samples.[10-12] Nevertheless, the rate of culture-negative infections continues to be high, between 27% and 55%.[13-17] In an era when molecular diagnostics play a greater role, there is an urgent need to address this problem. Sev-

eral molecular techniques have been previously explored, but none have proved to be accurate enough to supplant culture as the benchmark.[16,18,19,20,21] Species-specific polymerase chain reaction (PCR) has a high degree of sensitivity. This technology, however, only detects the presence of one organism.[18,19] Although later iterations of PCR techniques, such as multiplex PCR and broad-range PCR,[16,20-22] have included attempts to broaden the range of organisms which are detected, these efforts have had limited success. Multiplex PCR, which tests for a panel of commonly implicated organisms, showed acceptable sensitivity, but also detected an organism in almost 90% of patients with aseptic failure, which creates confusion in patients with an equivocal clinical picture.[22] Broad-range PCR, which amplifies a highly conserved region of the bacterial genome, theoretically should identify any organism. This technique, however, has low sensitivities, between 67.1% and 73.3%, and thus is not significantly more helpful than culture.[16,20,21] Furthermore, an organism is only detected if it forms more than 70% of the amplicon and the technique only detects single organisms.[23] This has been partially mitigated by sequencing many clones from the PCR amplification but may also result in the increased detection of contaminants.[23]

Next-generation sequencing may provide a solution, as it allows the sequencing and identification of all amplicons in a sample, thus avoiding the problems of the aforementioned assays.[24] While this technology was previously prohibitively expensive, the cost has declined in recent years and it is now commercially available. The clinical application of next-generation sequencing has shown promising results, from the detection of *Abiotrophia defectiva* in culture-negative endocarditis to neuroleptospirosis in culture-negative meningitis.[25,26]

While tissue cultures have been shown to provide a higher yield than synovial fluid, the latter has advantages[16] as it can be aspirated from the joint preoperatively, providing additional information before surgery. Tissue, on the other hand, can only be taken intraoperatively and, given the time taken for bacteriological results to be available, does not provide data in real-time.

The aims of this study were to assess the ability of next-generation sequencing to detect pathogens in synovial fluid and to examine its correspondence with conventional culture.

Materials and Methods

This prospective, blinded, single-centre study included analysis of 86 anonymous samples of synovial fluid from aspirations of the hip or knee. All samples were obtained from patients undergoing aspiration as part of the routine evaluation of prosthetic infection, when the C-reactive protein (CRP), total neutrophil count, human neutrophil elastase and alpha-defensin were measured, and culture undertaken. All samples were obtained between August 2016 and November 2016 and were given an identifying number and shipped overnight at ambient temperature to MicrogenDx Laboratories (Lubbock, Texas) for performance of next generation sequencing. On arrival at the laboratory, each sample is transferred to a 2 mL screw cap tube for the extraction of DNA. It is centrifuged for ten minutes and the supernatant is taken for the extraction process which is performed using the Roche High Pure PCR Template Preparation kit (Hoffman La Roche, Basel, Switzerland) in accordance with the manufacturer's protocol. This technique is slightly modified by including a beading step for disruption of the cells before extraction of the DNA, with the addition of 5 mm steel beads (Qiagen, Hilden, Germany) and 0.5 mm zirconium oxide beads (Next Advance, Averill Park, New York), as well as the Qiagen Tissuelyser II instrument (Qiagen, Hilden, Germany).

Following extraction of the DNA, a real-time PCR panel is performed on the sample, using the LightCycler 480 (Hoffman La Roche, Basel, Switzerland). The panel consists of a range of commonly implicated organisms. As an orthopaedic panel has not been designed, a chronic wound panel is used including: *Enterococcus faecalis*, *Klebsiella pneumonia*, *Enterococcus faecium*, *Streptococcus pyogenes*, *Streptococcus agalacticae*, *Candida albicans*, *Pseudomonas aeruginosa*, *Staphylococcus (S.) aureus*, and *Serratia marcescens*. It also includes an antibiotic resistance gene panel for the following antibiotics: methicillin, vancomycin, beta-lactam, carbapenem, macrolide, aminoglycoside and tetracycline. The real-time PCR assay detects these bacteria and their concentrations in the sample can be determined. This is made possible by exploiting the 5' nuclease activity of DNA polymerase to cleave a TaqMan probe during PCR extension. The TaqMan probe has a reporter dye, which increases fluorescence, at its 5' end and a quencher dye, which decreases fluorescence, at its 3' end. The separation of the two dyes because of the DNA polymerase cleaving the TaqMan probe causes an increase in fluorescence. Thus, the accumulation of PCR products is detected by an increase in fluorescence, due to increasing amounts of reporter dye being separated from quencher dye. The respective organisms on the panel are then identified based on a standard curve for each organism in the panel.

Next-generation sequencing. A conventional PCR reaction is performed to amplify the microbial DNA. Forward and reverse primers homologous to the regions flanking the 16S rRNA gene and the internal transcribed spacer gene are used to identify bacteria and fungi. These two regions are highly conserved regions of the bacterial and fungal genomes, enabling their accurate identification.[27,28] The amplified DNA is given unique tags, in order to differentiate them when being run on the sequencer. The amplified DNA is then pooled based on the strength of the amplification. Sample DNA is loaded onto beads for the emulsion PCR which generates high enough levels of the DNA in the sample for next-generation sequencing. This is necessary for each base to be sequenced. The sample is then

Table I. Comparison between the three subgroups based on inflammatory markers in the synovial fluid presented as mean and standard deviation

	Group I (n = 30)	Group II (n = 24)	Group III (n = 32)	Significance*
HNE (units)	7.34 (0.95)	5.90 (2.24)	0.20 (0.25)	$1.36*10^{-28}$
TNC (cells/μL)	40 235.31 (33 888.67)	23 889.25 (19 836.91)	855.72 (746.72)	$1.32*10^{-8}$
CRP (mg/L)	38.68 (39.41)	27.97 (35.94)	1.76 (3.41)	0.000021

*P-values represent between group differences based on ANOVA. In a post-hoc analysis, (Bonferroni) there were significant differences between all three groups for total neutrophil count (TNC) and human neutrophil count (HNE), while CRP was only significantly lower in Group III (negative culture, negative alpha-defensin) compared with Group I and Group II, between which there was no significant difference. (p = 0.59)

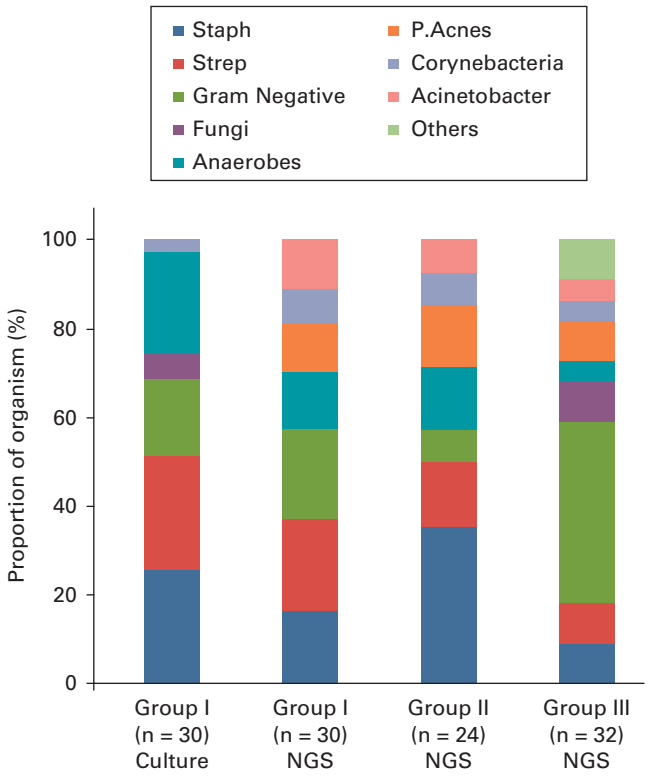

Fig. 1

Graph showing the proportion of organisms detected by next-generation sequencing and culture in the different subgroups. Group I, positive culture, positive alpha-defensin; Group II, negative culture, positive alpha-defensin; Group III, negative culture, negative alpha-defensin. NGS, next-generation sequencing.

sequenced on the Ion Torrent PGM sequencing platform (ThermoFisher Scientific, Waltham, Massachusetts), which 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 corresponds to the number of nucleotides incorporated into the sequence which is detected by the sequencer. The final step before analysis of the data consists of denoising, to remove short sequences that may interfere with the interpretation of the data, as well as the elimination of chimeric sequences. The sequences are then compared against a curated NIH/Genbank database using USearch7,[29] and an agreement of at least 90% between the sequences and the database is necessary. A report is then published providing quantitative details of the bacteria and fungi in the sample.

Statistical analysis. A power analysis was conducted to determine the sample size. Using prior institutional data on molecular techniques[22] and aiming for a 30% difference in sensitivity between next-generation sequencing and culture, a power of 80%, and an alpha error of 0.05, a sample size of 72 patients was determined. Descriptive statistics were calculated, including means and standard deviations for continuous variables and frequencies of categorical variables. Student's *t*-test was used to calculate the differences in continuous variables between groups, while chi-squared analysis was used to measure differences in categorical

Table II. Correspondence between culture growth and the predominant organism detected by next-generation sequencing

Organism detected	Group I	
	Culture (n = 30)	NGS (n = 26)
Actinomyces neuii	1	0
Bacteroides fragilis	1	0
Candida	2	0
Corynebacterium striatum	1	1
Enterococcus Fecalis	1	1
Escherichia coli	2	1
Klebsiella Oxytoca	1	1
Klebsiella Pneumonia	1	1
Pseudomonas aeruginosa	1	1
Ralstonia detusculanense	0	1
Serratia marcescens	3	3
Staphylococcus	10	10
Staphylococcus (resistant)	0	4
Streptococcus	6	6

†NGS, next generation sequencing

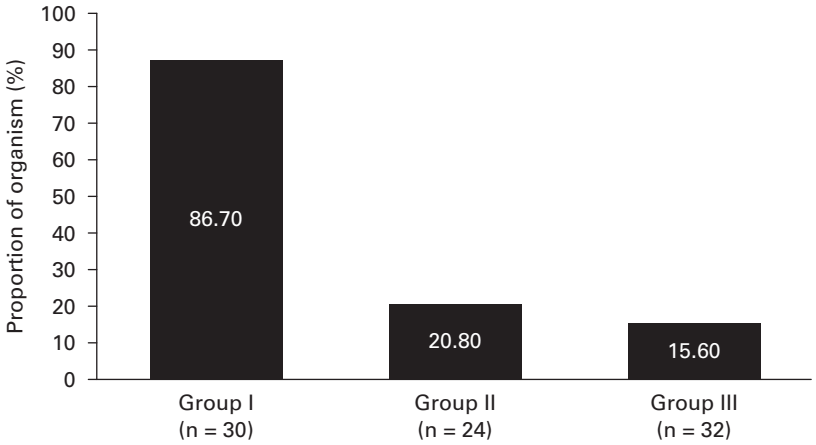

Fig. 2

Graph showing the percentage of samples in which next-generation sequencing detected at least one pathogen in each group. Culture-positive (Group I)*versus* culture-negative (Groups II and III).

variables. Since next-generation sequencing detects all organisms in a sample, many species were detected in several samples. Correspondence between next-generation sequencing and culture was defined only when both were positive. If the predominant organism on next-generation sequencing was identical to that grown in culture, they were considered concordant. If they identified different bacteria, they were considered discordant.

Results

The 86 samples were subdivided into three groups: positive alpha-defensin and positive culture (Group I) (30 samples), positive alpha-defensin and negative culture (Group II) (24 samples) and negative alpha-defensin and negative culture (Group III) (32 samples). Analysis of the subgroups based on the level of inflammatory markers and proportion of pathogens is shown in Table I and Figure 1.

Group I. Next-generation sequencing detected an organism in 26 samples; in 25 (96.1%), there was concordance between the bacteria detected in culture and the predominant organism detected on next-generation sequencing (Table II). In most of these samples, the percentage of organism detected by next-generation sequencing was > 90% (Figs 2 and 3). In three samples, while the species detected were similar, next-generation sequencing detected the mecA gene and classified the organisms as methicillin resistant *S. aureus* while culture was positive for methicillin sensitive *S. aureus*. In one, next-generation sequencing also detected *Staphylococcus lugdunensis*. There was discordance in bacterial resistance in another sample as cultures detected both *E. coli* and *S. epidermidis* while next-generation sequencing detected methicillin-resistant *S. epidermidis*. In another discordant sample, *Actinomyces neuii* was isolated by culture while next-generation sequencing detected a mixture of organisms, predominantly *Ralstonia detusculanense*.

[*Group I, positive culture, positive alpha-defensin group

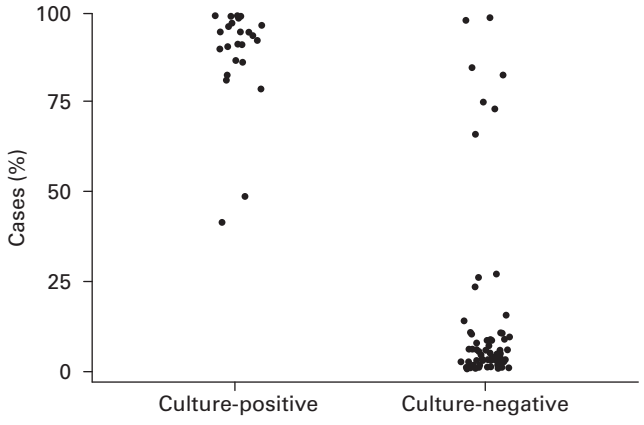

Fig. 3

Scatter plot showing next-generation sequencing results of organisms that grew on culture*versus* organisms that did not grow on culture. Group I, positive culture, positive alpha-defensin; Group II, negative culture, positive alpha-defensin; Group III, negative culture, negative alpha-defensin.

There were four culture-positive samples in which next-generation sequencing did not detect genetic material for any organisms. The mean synovial CRP, human neutrophil elastase and total neutrophil counts for these samples were relatively low at 6.5 mg/L, 6.6 units and 16 894 cells/μL, respectively. The infectious organisms grown on culture were fungi in two samples; *Candida albicans* and *Candida parapsilosis* and bacterial in the other two; *Staphylococcus warneri* and *Bacteroides fragilis*.

Group II. Next-generation sequencing detected an organism in five samples (20.8%) (Fig. 2), in which the mean levels of inflammatory markers were relatively high, with the mean synovial CRP, human neutrophil elastase and total neutrophil count being 31.6 mg/L, 8.2 units and 43 241 cells/μL, respectively. The DNA material sequenced in the samples corresponded to *S. epidermidis* in two and *Staphylooccus piscifermentans* and *Streptococcus dysgalactiae* in one each.[30] In the fifth sample, several organisms were identified, predominantly *Veillonella parvula*.

Group III. Next-generation sequencing detected organisms in five samples (15.6%). One was positive for *Aureobasidium pullulans* and *Malassezia restricta*, both of which are fungi. In two samples, the predominant organism was *Pseudomonas aeruginosa*, representing 74% and 84% of the samples, respectively. *Acinetobacter baumannii* and *Propionibacterium acnes* were the main bacteria in the remaining two samples.

Discussion

In this study, we sought to evaluate the role of next-generation sequencing in detecting pathogens in synovial fluid. It reliably detected organisms in synovial fluid with a high degree (96.1%) of concordance with traditional culture and was also able to detect organisms in patients with negative cultures who had a high likelihood of infection, based on the levels of inflammatory markers in the synovial fluid. Though possessing a higher sensitivity than culture, next-generation sequencing did not appear to have excessive sensitivity, a problem that has plagued most molecular techniques which have been evaluated for the diagnosis of periprosthetic joint infection.[22]

An organism was identified in four samples by traditional culture when next-generation sequencing was negative. Although it is possible that next-generation sequencing missed a pathogen in these samples, a probable explanation is that the organisms isolated by culture were contaminants, as the levels of inflammatory markers were relatively low in these patients. Furthermore, the optimal volume of synovial fluid for next-generation sequencing is currently 2 ml and the limited volume of the samples may have played a role in the failure of next-generation sequencing to identify these organisms. Next-generation sequencing did, however, detect genetic material of potential pathogens in five of 32 samples in Group III, raising the issue that this sequencing may be associated with false positive findings in some samples. If the samples in this group were not truly infected, next-generation sequencing had a false positive rate of approximately 15%, and this is certainly encouraging when compared with earlier studies. These false positives may be because of subclinical infection, or organisms that are part of the microbiome. Perhaps the greatest indictment of many PCR-based technologies has been a high rate of false positives and it's encouraging that we noted a low rate of false positives associated with next-generation sequencing compared with these earlier techniques. Our previous experience with multiplex PCR is a good example of one such technology, with an organism being identified in > 80% of samples thought to be aseptic.[22] In two of the five false positive samples in the present study, *Aureobasidium pullulans* and *Malassezia restricta* were isolated and these are probably contaminants. This finding indicates that the results of next-generation sequencing need to be interpreted considering relevant clinical and laboratory data to avoid over-treating patients who may not be infected.

Another important finding of this study was that next-generation sequencing detected the mecA gene, in some samples that had been designated methicillin-sensitive, on culture. Any information on antibiotic resistance that can be provided expeditiously during treatment is critical to administering appropriate antimicrobial agents. In fact, the initial results of next-generation sequencing, provided within 24 hours, give this technology an immense advantage over techniques that may take two or three weeks when the infection is caused by slow-growing organisms. Another attribute of next-generation sequencing may relate to cost-effectiveness. Its cost is currently < $ 200 and likely to be reduced further in the future. This contrasts with

between $60 and $150 per sample for culture with between three and five samples being needed.

An interesting finding was the frequency with which next-generation sequencing identified several organisms in a sample, with 14 of 30 culture-positive samples (46.7%) suspected of being infected, returning more than one organism on next-generation sequencing. Polymicrobial infections are poorly understood, and the question remains whether these are the result of a single dominant organism or several pathogens. Culture may provide a preferential medium for certain organisms, and this may cause an inaccurate identification of the causative organism, or could cause a polymicrobial infection to be missed. This becomes of particular concern in view of the fact that most patients, in whom treatment for infection fails, are infected with a different organism.[31,32] It may be that the growth of certain bacteria is suppressed in culture, leading to the selective growth of a non-infecting organism. This technology may provide a better understanding of polymicrobial infections. We believe it is plausible that some, if not most, cases of periprosthetic infection are caused by several organisms that escape detection using traditional culture that tends to isolate the most dominant organism that grows at the expense of other pathogens.

While a predominant organism was identified in most samples that were presumed to be infected, those that were less likely to be infected had a greater mixture of organisms. One possible explanation for this is the presence of subclinical infection. This is thought to be caused by low-virulence organisms, which do not promote a florid immune response, hence the laboratory investigations for infection are normal.[33] In a previous study evaluating multiplex PCR at our institution, approximately 12% of patients undergoing revision total knee arthroplasty for aseptic failure, and a positive result on multiplex PCR, subsequently failed due to infection.[34] The surgeon should always be aware of this possibility. Another possible explanation is that these organisms may be part of the resident microbiology of the joint. While the natural micro-organisms of the hip and knee have not been studied, next-generation sequencing is currently being used to study this in several sites in the human body and this level of sequencing is likely to detect what may be commensals in the joint.

This study has limitations. A limitation may be the lack of clinical information about these patients as the samples were retrieved from an anonymized reservoir and we were therefore not able to determine whether the patients were infected or not. Thus, categorization of these cases was performed using synovial fluid markers known to be accurate for the diagnosis of periprosthetic infection.[35] Our intention was to determine whether next-generation sequencing can detect organisms in synovial fluid and its concordance with culture. While a more detailed knowledge of the samples' clinical information may have improved our interpretation of the culture results, it would not have altered these results themselves and by extension, the concordance between next-generation sequencing and culture.

Another shortcoming of the study is that we did not evaluate another molecular technique in parallel with next-generation sequencing. However, given that culture remains the most accepted method for isolating organisms, we could not justify the selection of any culture-independent technology over another. Finally, we were only able to test synovial fluid, and hence a direct comparison to tissue or other samples cannot be made.

Conventional culture, despite its flaws, has been the mainstay of the detection of pathogens for almost 150 years. With the limited yield of synovial fluid, the difficulties associated with the current paradigm of detecting organisms have become more apparent. Typically, if the results of the culture of synovial fluid are negative, intraoperative cultures will be taken in the hope of identifying an organism. Next-generation sequencing of synovial fluid may be a useful alternative for the detection of causative organisms in prosthetic infection. This is important given the lack of yield attributed to synovial fluid both as a sample for culture and other molecular techniques.[16]

Our findings suggest that next-generation sequencing holds great promise for the detection of potential pathogens from the synovial fluid of patients with a periprosthetic infection. As further data are generated the role of this unique and promising technology for the diagnosis of periprosthetic infection may be refined.

Take home message:

-Culture-negative periprosthetic joint infection poses a significant burden and can generate diagnostic and therapeutic uncertainty.

- Previous molecular techniques have failed to displace culture as the benchmark for pathogen identification in periprosthetic joint infection.

- Next-generation sequencing may be a useful adjunct for identifying the causative organism in culture-negative periprosthetic joint infection.

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M. Tarabichi: Designing the study, Collecting and analyzing the data, Writing the manuscript.
N. Shohat: Analyzing the data, Writing the manuscript.
K. Goswami: Analyzing the data, Editing the manuscript.
J. Parvizi: Designing and supervising the study, Writing and editing the manuscript.

Funding statement:

The author or one or more of the authors have received or will receive benefits for personal or professional use from a commercial party related directly or indirectly to the subject of this article.

This article was primary edited by J. Scott.