DNA EXTRACTION OF MICROBIAL DNA DIRECTLY FROM INFECTED TISSUE

An optimized protocol for use in nanopore sequencing

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Abstract:

- Traditional methods make discovering bacteria that cause tissue infections difficult, in the case of low-growing bacteria or difficult to cultivate microbes.
- We use shotgun metagenomic sequencing on bacterial DNA extracted directly from the infected tissue may reduce time to diagnosis and targeted treatment.
- Infected tissue, is made up of human DNA (hDNA), which makes bacterial identification difficult, so we present a modified version of the Ultra-Deep Microbiome Prep kit for DNA extraction, which removes additional human DNA.
- Tissue biopsies from 3 patients with orthopedic implant-related infections containing varying degrees of Staphylococcus aureus were included. Microbe and antibiotic resistance genes were identified using DNA shotgun metagenomic sequencing and Oxford Nanopore Technologies' (ONT) MinION network, as well as ONT's EPI2ME WIMP and ARMA bioinformatics workflows.
- The modified DNA extraction protocol reduced human DNA by a tenth of a percent while keeping S. aureus DNA. The presented protocol has the ability to classify the infection-causing pathogen in infected tissue within 7 hours of biopsy.
- Positive discovery of antibiotic resistance genes was not effective due to a lack of S. aureus reads.

Introduction:

As next-generation sequencing (NGS) gains acceptance as the gold standard in bacteriology, the market for efficient DNA extraction procedures increases. The ability to remove microbial DNA directly from human samples and then sequence it with shotgun metagenomic sequencing will speed up diagnosis. The development of a DNA extraction protocol capable of depleting hDNA while preserving microbial DNA is important for improving NGS microbe identification.

The Ultra-Deep Microbiome Prep kit (Molzym, Bremen, Germany) is a DNA extraction kit that extracts enriched microbial DNA from a range of sample forms, including biopsies. It can recognize and identify all types of bacteria and fungi, including those that aren't culturable. The kit has been used for shotgun metagenomic sequencing of bronchi-alveolar lavage fluid using Illumina sequencing platform. Additional examples are found in the characterization of human breast tissue biopsies, and the profiling of oral bacteria on pathologically changed heart valves using 16 S rRNA sequencing on an Ion PGM Sequencer and Sanger sequencing. However, used NGS sequencing platforms for metagenomic sequencing, such as Ion Torrent and Illumina, require extensive pre-sequencing sample preparation and require the sequencing run to be completed before analysis can begin (exception of a recently published tool) (LiveKraken).These obstacles may be overcome by using nanopore sequencing technology where pre-sequencing preparation is short (15 min–2 h) and data analysis can be done in near real-time using the web- analysis platform including for microbe identification, and ARMA for antibiotic resistance genes based on the Comprehensive Antibiotic Resistance Database .

These technological advancements may help patients with orthopedic implant-associated infections. The implications of these infections are severe and relying on empirical antibiotic therapy may cause inefficient treatment, increasing risk of mortality.

The rapid detection of these infectious agents is critical for medical care. Traditional OIAI microbiological diagnostics necessitate the cultivation of 5 biopsies from each patient for at least 5 days on several media. Another 24 hours would be required to test phenotypic antibiotic resistance.

The aim of this proof was to demonstrate the feasibility of using a modified version of the Ultra-Deep Microbiome Prep kit for DNA extraction and subsequent shotgun metagenomic sequencing with ONT's nanopore sequencing and bioinformatics platform for near real-time detection of microbes and antibiotic resistance genes directly from infected tissue.

Related work:

From Jan 2017 to December 2018, diagnostic soft tissue biopsies were taken from patients at Akershus University Hospital who had OIAI. They were collected from the areas directly adjacent to the infected implant.

Every biopsy was split into two parts, one was cultivated using standard microbiological diagnostics and the other used for sequencing, was initially frozen at 80 °C. Antibiotic sensitivity testing was carried out according to the European Committee on Antimicrobial Susceptibility Testing guidelines, with EUCAST breakpoints used to classify the isolate as susceptible (S), intermediate (I), or resistant (R). There were a total of 33 patients in the study. Based on the semi-quantification of Staphylococcus aureus development during routine diagnostic cultivation, biopsies from three patients (13 unique biopsies) were chosen. In this proof of concept analysis, patients with S. aureus infection were chosen because it is one of the most frequent causes of OIAI12.

This study was accepted by the Regional Committee for Medical and Health Research Ethics and the local Data Protection Officer (17/024) at Akershus University Hospital. The patients gave their written. All research was performed in accordance with relevant guidelines.

Methodology:

DNA extraction according to master's thesis "Rapid molecular diagnostic tool for identification of bacteria causing orthopedic implant-related infections". The current procedure for the Ultra-Deep Microbiome Prep kit for DNA extraction might benefit from adjustment of the hDNA depletion stage. As a result, the updated methodology was put to the test, which included extra cell lysis and human DNA depletion procedures.

Before performing DNA extraction with the Ultra-Deep Microbiome Prep kit, each of the 13 included biopsies was split into two equal-sized pieces and weighed. In a type 2 microbiological safety cabinet. The manufacturer's protocol was used to extract Biopsy (P I). To extract DNA for biopsy (P II), the initial incubation with proteinase K was extended from 10 to 20 minutes, followed by lysis of human cells and extracellular DNA degradation. Then performed particle resuspension in 1 mL TSB, followed by lysis of human cells degradation of extracellular DNA step was repeated. In all methods, the centrifugations were done at 14000 g.

Assessment of optimization steps two qPCRs for the detection of human DNA (human -globin gene HBB) and S. aureus DNA (nuc-gene) were performed to test the effect of the optimization stages.

HBB was detected using a previously published method. The nuc qPCR consisted of a 20 µl reaction containing primers and probe sequences and concentrations from Tunsjø and co-workers, and TaqMan FAST Universal PCR Mastermix 2X.Both qPCR protocols were performed on a 7900HT Fast Real-time PCR instrument. All samples were run in parallel and DNA concentrations were estimated using 10-fold dilution standard curves for both qPCR assays. Samples with Ct-values higher than 40 were characterized as negative and, in calculation of the mean Ct-values, calculated as 40.

Library preparation and MinION sequencing Library preparation was performed using Rapid PCR Barcoding Kit (SQK-RPB004, Oxford Nanopore Technologies) following manufacturers guidelines (RPB_9059_v1_revD_ 08 Mar2 018). The input volume of all samples was 3 µl and to best preserve all DNA, the pooling of samples was performed without quantification after the AMPure XP beads step. 1 µl of RAP (Rapid Adapter) was added to 10 µl of the pooled eluate and the library was kept on ice until loaded onto the flow cell. One library was prepared for each patient, including five P I samples and five P II samples, indexed and multiplexed on one flow cell. One no template control (NTC) was included in the library preparation for patients 2 and 3. Shotgun metagenomic sequencing was carried out on a MinION sequencer (Oxford Nanopore Technologies) using R9.4.1 FLO-MIN 106 flow cells. The operating software MinKNOW was used for local base calling (Patient 1: MinKNOW v. 1.15.4, Patients 2 and 3: MinKNOW v. 3.3.2, Guppy 3.0.3). Demultiplexing and identification of both pathogen and antibiotic resistance genes were performed using the cloud-based bioinformatics platform, EPI2ME (Patient 1: EPI2ME v. 2.57.1769546, Patients 2 and 3: EPI2ME v 2.59.1896509). The QC and Barcoding, WIMP, and ARMA workflows were employed using default Q-score ≥7. The MinION was run for up to 48 hours.

The results files from the 3 workflows were combined in order to extract the run data for each read. The protein homolog model of antibiotic resistance genes with average alignment accuracy of ≥90% were reported.

Results

Patient 1. Patient 1 was chosen based on the dense growth of S. aureus by standard cultivation of the biopsies .The human β-globin gene qPCR showed an increase in mean Ct-values from biopsies using manufacturer's

protocol, P I, 28.6 [25.7–31.3] to 32.1 [29.9–35.1] (3.5 Ct-values) using modified protocol, P II, corresponding to approximately a 10-fold reduction of human DNA using the modified protocol (P II). For the related nuc qPCR, Ct-values stayed virtually unchanged resulting in mean Ct-values of 28.5 [26.7–31.9] for P I, and 28.7 [26.4–30.8] for P II.

MinION sequencing stopped after 17 hours and showed a reduction of total number of hDNA reads in the 5 biopsies from 60063 using PI to 1755 reads using PII. S. aureus reads increased from 613 reads (PI) to 3831 reads (PII). This corresponds to roughly a 34-fold reduction of human DNA reads, and a 6-fold increase in S. aureus reads. The number of reads identified by WIMP as microbes other than S. aureus increased from 142 reads to 271 reads from PI to PII, respectively. In P I, these reads consisted mainly of S. aureus subspecies (N = 34) and Malassezia globosa (N = 12), whereas using P II the majority consisted of S. aureus subspecies reads (N = 188), staphylococcus phages (N = 20) or staphylococcus viruses (N = 17). Unfortunately, no controls were included in this run. All biopsies were positive for S. aureus during the first hour of sequencing (Supplement File 1), so identification of the infection-causing pathogen would have been possible within 7 hours after biopsy. Phenotypic antibiotic resistance testing showed resistance to ciprofloxacin. Using the ARMA bioinformatic tool for identification of antibiotic resistance genes, 4 alignments of tetC (average accuracy 91.0%), 3 alignments of arlS (average accuracy 91.3%) and 1 alignment of sav1866 (average accuracy 90.0%) were identified in the biopsies extracted by the original protocol. Using the modified DNA extraction protocol, 8 alignments of arlS (average accuracy 91.9%) and 7 alignments of sav1866 (average accuracy 91.3%) were identified.

Patient 2. Cultures from patient 2 produced intermediate growth of S. aureus in all 5 biopsies .The qPCR showed a reduction of human β-globin in all P II biopsies compared to P I biopsies with a mean Ct-value of 34.6 [27.1–40.0] in P I biopsies and a mean Ct-value of 39.5 [37.6–40.0] in P II biopsies (4.8 Ct-values). This corresponds to more than a 50-fold reduction in hDNA. nuc was only detected in Biopsy 2 and 5 following manufacturer's protocol, whereas all biopsies extracted according to modified protocol were negative for nuc. MinION sequencing stopped at 48 hours. The total human sequencing reads in biopsies from Patient 2 were reduced from 856 275 reads (P I) to 1181 (P II), whereas the total number of S. aureus reads were reduced from 637 to 155, respectively. The reduction of S. aureus reads was mainly due to biopsy 3 that showed a significant reduction of reads from PI to PII (565 reads to 26 reads in total, Supplement 1).

Reads identified as other microbes by WIMP, were reduced from 2376 reads (P I) to 48 reads (P II). The majority of the other microbes reads were identified as Malassezia globosa (N = 689 reads) and Opisthokonta (N = 512) in P I biopsies and S. aureus subspecies (N = 27) in P II biopsies. The NTC had a total of 21 reads of which 20 were human and 1 was Malassezia globosa. Due to few S. aureus reads, at least 4 hours of sequencing would have been required to positively identify the causative agent, extending the total time from biopsy to pathogen ID to 10 hours.

Phenotypic antibiotic resistance testing showed resistance to ciprofloxacin and penicillin. Biopsies extracted with manufacturer's protocol showed 1 alignment with norA (average alignment accuracy 93.0%) using ARMA, whereas biopsies extracted with modified protocol showed 1 alignment with mepA (average alignment accuracy 94.0%).

Patient 3. Patient 3 cultures had growth of S. aureus in 2 of 4 biopsies only after pre-cultivation in broth. Of these 4 biopsies, 3 were available for DNA extraction . The mean Ct-values for β-globin increased from 33.7 [31.0–38.8] in P I to 36.9 [34.0–40.0] in P II samples, whereas the mean Ct-values for nuc were stable with 36.8 [35.1–40.0] for P I and 36.0 [33.8–40.0] for P II, respectively. This corresponds to a ~10-fold decrease of human DNA.

MinION sequencing stopped after 48 hours and the total number of human DNA reads were reduced from 3 697 756 in P I biopsies to 8280 in P II biopsies. Total S. aureus DNA reads increased from 24 to 215 reads. The total number of reads for other microbes were reduced from 6058 (P I) to 112 reads (PII). The majority of other microbes in the P I biopsies consisted of Malassezia globosa (N = 4067 reads), and S.aureus subspecies (N = 88 reads) in P II biopsies. Using P II, S. aureus was identified in the first hour of sequencing, whereas using P I, S. aureus was identified during the fourth hour of sequencing. In P II, only 1 S. aureus read was identified in biopsy 1 (positive after pre-cultivating in broth), whereas the 2 culture-negative biopsies showed S. aureus reads from the first hour of sequencing.

Phenotypic antibiotic resistance testing showed resistance to penicillin. No resistance genes were detected in the biopsies extracted with the manufacturer's protocol and 2 alignments of mepA (average alignment accuracy 90.0%) were identified in the biopsies extracted with the modified protocol and using the ARMA pipeline. The NTC displayed no alignments.