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Identification of *Cutibacterium modestum* in Spondylitis by Metagenomics Analysis

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Abstract. *Background/Aim: Identifying pathogens with culture-negative pyogenic spondylitis is difficult. Shotgun metagenomic sequencing is an unbiased and culture-free approach in the diagnosis of infectious diseases. There are, however, a variety of contaminating factors that can confound the precision of metagenomic sequencing. Case Report: In a 65-year-old man suffering from culture-negative L3-5 spondylitis, metagenomics was applied to facilitate the diagnosis. The patient underwent percutaneous endoscopic lumbar discectomy. We applied metagenomic sequencing with a robust contamination-free protocol to the bone biopsy. By comparing the abundance for each taxon between the replicates and negative controls, we reliably identified Cutibacterium modestum as having a statistically higher abundance in all replicates. The patient's antibiotic therapy was switched to penicillin and doxycycline based upon the resistome analysis; the patient fully recovered. Conclusion: This application of next-generation sequencing provides a new perspective in the clinical approach to spinal osteomyelitis and illustrates the potential of this technique in rapid etiological diagnosis.*

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Key Words: Cutibacterium modestum, culture-negative pyogenic spondylitis, resistome, antibiotic-resistant genes, shotgun metagenomic sequencing.

Pyogenic spondylitis, characterized by bacterial infection of the spine or paraspinal structures and subsequent epidural and paraspinal involvement, can lead to neurological deficits in approximately one-third of cases. Early therapy is essential for a favorable outcome (1). However, the microbial etiology of pyogenic spondylitis is highly diverse. When bacterial cultures do not show the causative microorganism, the initial treatment regimen can only be based on previous records and the experiences of the physicians (2). In patients with pyogenic spondylitis, more than a third do not have culture-positive tissues or blood, and are considered to have culture-negative pyogenic spondylitis (3). A lower bacterial burden can result in a milder clinical course. The erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels of these patients are often lower, further complicating the diagnostic process (3).

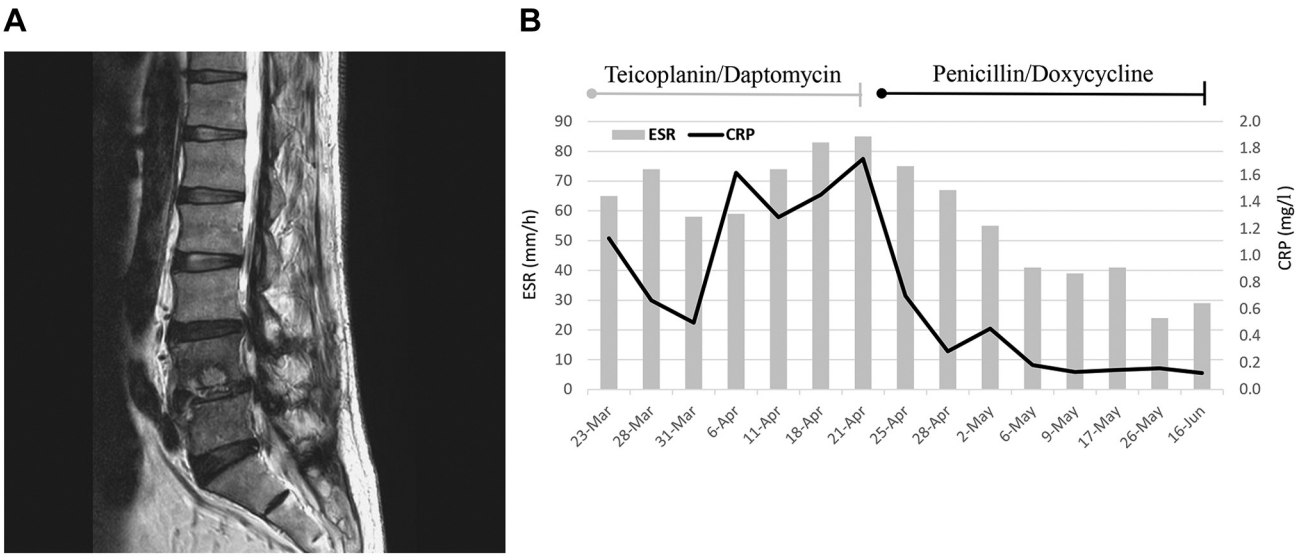

Figure 1. *Clinical presentation of the 65-year-old patient with spondylitis. A: Magnetic resonance imaging showing spondylitis in the lumbar spine (L3-5). B: Clinical course of the patient laboratory values and antimicrobial therapy during the patient's hospitalization.*

Shotgun metagenomic sequencing is an unbiased and ultra-sensitive approach for diagnosing infectious diseases, in particular when conventional culture-based methods have reached their limit. However, the accuracy of metagenomic sequencing is often hindered by contamination during sample collection (*e.g.*, needle biopsy), by environmental pathogens (*e.g.*, airborne viruses), and from DNA extraction and sequencing reagents (the so-called 'kitome') (4). Moreover, many of these contaminating microbes are known human pathogens (*e.g.*, *Candida spp.*). During the sequencing process, these contaminants may lead to inconsistent results among laboratories and to incorrect diagnoses. A number of studies have demonstrated success in rapidly identifying infections without the need for culture (5-7). However, others have argued the concordance rate between metagenomic sequencing and culture validation is still inadequate (8). Therefore, contamination needs to be addressed in order to provide an accurate diagnosis, particularly when using samples with a low biomass.

This work presents the use of a robust, contamination-free protocol for clinical metagenomic sequencing by including replicates and differential abundance analysis. The protocol was applied to a patient with infectious spondylitis in whom the causative pathogen was identified, leading to switching of antibiotic therapy and successful treatment.

Case Report

A 65-year-old man presented to the hospital after suffering with low back pain for several weeks. Findings on magnetic resonance imaging were consistent with spondylitis in the lumbar spine (L3-5), as shown in Figure 1A. At the time of admission to the hospital, the patient had elevated levels of inflammatory markers (leukocytes: 11×10^9/l, high-sensitivity CRP: 1.2 mg/l, ESR: 65 mm/h). Teicoplanin was initiated but a lack of clinical improvement was seen, with persisting elevation of ESR and CRP. The blood culture showed no positive growth. The patient underwent percutaneous endoscopic lumbar discectomy. Next-generation sequencing of the tissue specimen and plasma were performed, which identified the presence of *Cutibacterium modestum*. Antibiotic therapy was changed to penicillin and doxycycline. Clinical improvement was identified during the following 2 weeks and he was discharged with amoxicillin. After 6 weeks of antimicrobial therapy, he was symptom-free with normal CRP and ESR, as shown in Figure 1B.

This study was approved by the Institutional Review Board of Taichung Veterans General Hospital (CF22022A) and written-informed consent was obtained from the patient for publication of this article and the accompanying images.

Sample preparation and sequencing. A 0.25 g bone biopsy sample extracted by percutaneous endoscopic lumbar discectomy was mixed with 1 g of 0.5 mm-diameter glass beads and then placed on a vortex mixer for 30 min at 3,000 rpm. A DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) was used for DNA extraction in 300 μl of the sample following the manufacturer's instructions. An enzymatic method was used to fragment DNA into sizes in the range of 150-200 bp. Qubit DNA Assay (Thermo Fisher, Waltham, MA, USA) was used to determine DNA concentrations and an Agilent BioAnalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) was used to assess DNA quality. The DNA library was built

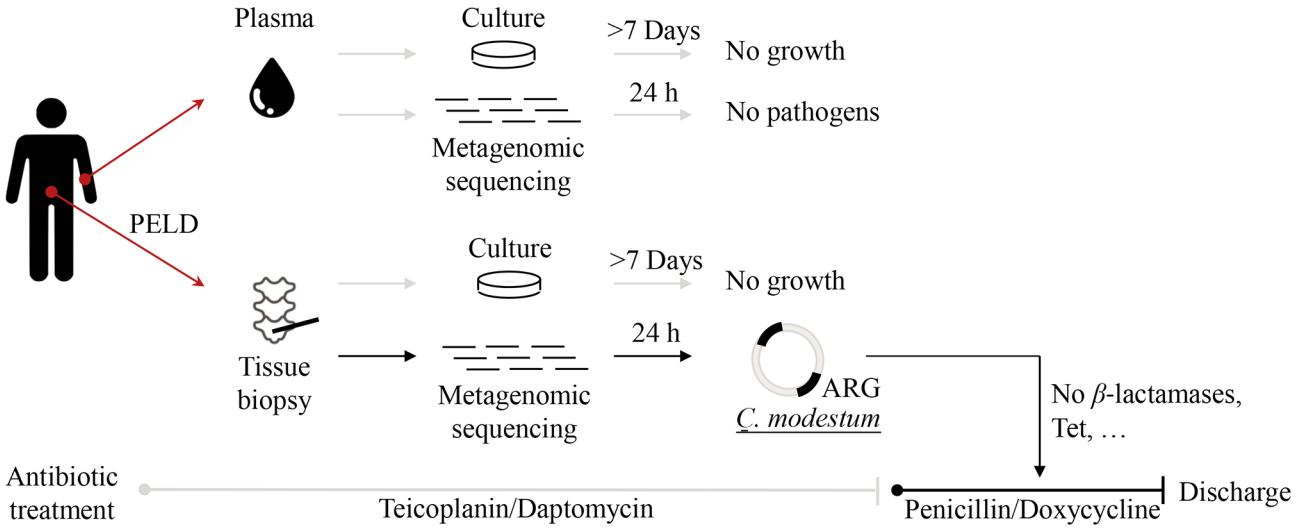

Figure 2. *Samples from plasma and tissue biopsy extracted by percutaneous endoscopic lumbar discectomy (PELD) were cultured and sequenced by a metagenomic approach. Both were culture-negative after 7 days. The metagenomic sequencing of plasma also found no pathogens. Only the tissue biopsy sequencing revealed the presence of Cutibacterium modestum within 24 hours. The high genomic coverage detected antibiotic-resistance genes (ARGs), which indicated no resistance to frontline antibiotics (i.e., penicillin and tetracycline). The treatment was switched from teicoplanin/daptomycin to penicillin/doxycycline 24 hours after sequencing. The patient recovered and was discharged. Diagnosis of culture-negative infectious spondylitis by metagenomic sequencing. A robust contamination-free protocol was used for clinical metagenomic sequencing. Each sample (e.g., plasma and tissue) and the negative control was sequenced using two replicates. The sequencing reads were mapped to the microbial genome database for calculating the taxonomic abundance of each species. The taxa of significantly higher abundance between each replicate and control were identified. The protocol only outputs the taxa concordantly detected in the two replicates.*

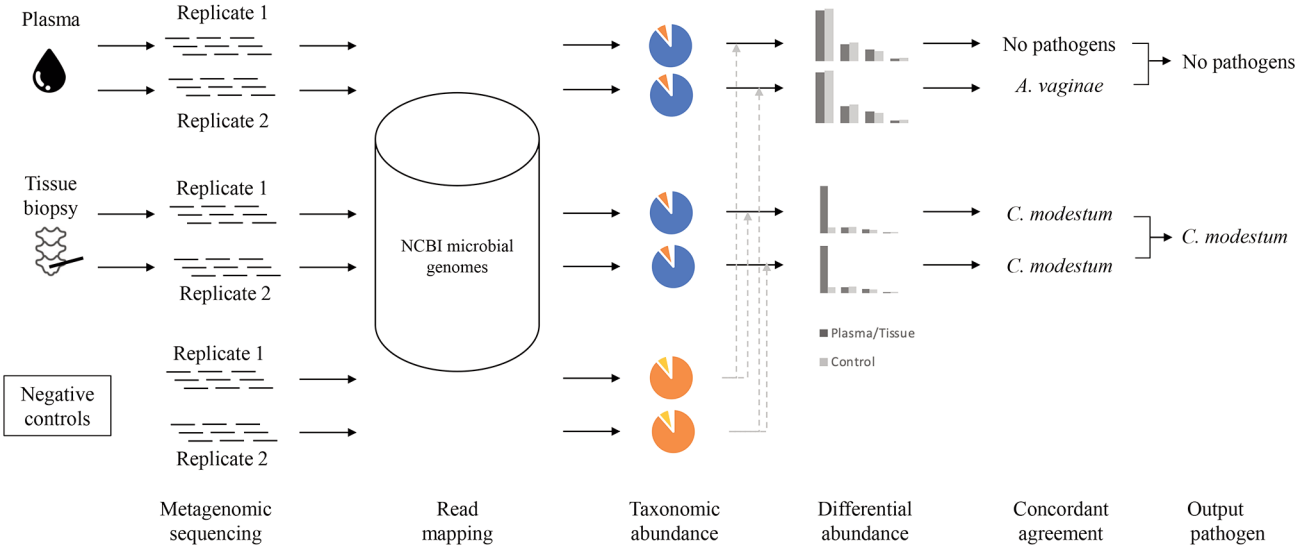

Figure 3. *Plasma and tissue biopsy (extracted by percutaneous endoscopic lumbar discectomy) samples were cultured and sequenced by a robust, contamination-free protocol. Both samples were culture-negative after 7 days. The metagenomic sequencing of plasma also found no pathogens. Only the tissue biopsy sequencing revealed the presence of Cutibacterium modestum within 24 hours. The high genomic coverage detected antibiotic-resistance genes but indicated no known resistance genes.*

through an end-repaired adapter and polymerase chain reaction amplification using the MGIEasy FS DNA Library Prep Kit (MGI Tech, Shenzhen, Guangdong, PR China). The circularized single-stranded DNA library underwent a transformation process wherein it was converted into DNA nanoballs. Subsequently, sequencing was performed using a benchtop genetic sequencer DNBSeq-G50, which generated sequence reads with an average length of 50 base pairs.

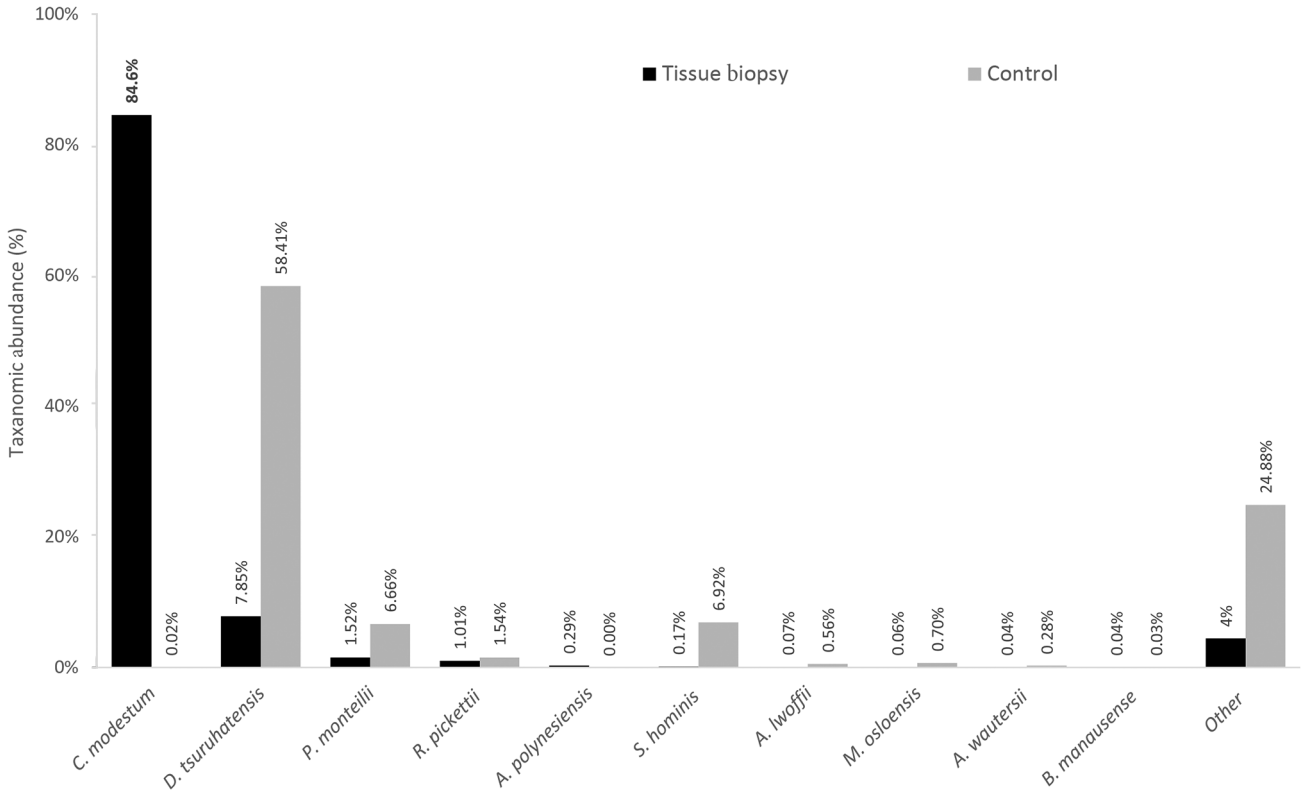

Figure 4. *Abundance of Cutibacterium modestum and other microorganisms in the clinical specimen and control.*

Bioinformatics analysis. Sequencing reads were preprocessed by removing low-quality reads [*i.e.*, <80% Phred score Q30, duplicated, and short-length (<35 bp) reads]. The remaining high-quality reads were aligned with Burrows–Wheeler aligner (BWA) against the human genome (hg38) to remove human-derived sequences (9). The non-human reads were BWA-aligned to the National Center for Biotechnology Information RefSeq (representative genomes) database for taxonomic identification. The abundance of each taxon was computed and normalized as reads per million. Differential abundance analysis was performed by comparing the taxon reads per million between the replicate and the negative control. Taxa with an abundance statistically higher than that of the controls were identified. Only the differential-abundant taxa concordantly identified in all replicates were output. The antibiotic-resistance genes were annotated by BWA-aligned reads onto the Comprehensive Antibiotic Resistance Database (https://card.mcmaster.ca/).

Two replicates of the specimens and the negative control were sequenced and independently analyzed. The sequencing reads were mapped against the human and microbial genome database, which produced the taxonomic abundance of each taxon separately for each sample. Subsequently, we identified the taxa in the sample which had significantly higher abundance than in the negative control (namely *Atopobium vaginae* in replicate 2 of plasma, *C. modestum* in replicate 1 of bone biopsy tissue). Finally, only the differentially-abundant taxa concordantly identified from the two replicates were output. Analysis of two replicates of bone biopsy tissue reported the same microorganism, namely *C. modestum* for this case.

The sequence has been deposited in GenBank under the accession number PRJNA917499.

Discussion

Metagenomics analysis is a relatively new and rapidly developing field in the field of infectious diseases diagnosis and research. This method involves the analysis of the entire genomic content of a sample, including both host and microbial DNA, to identify the presence of specific species or pathogens (7). This approach has been increasingly used for the identification of pathogens in difficult-to-diagnosis infection (10). Advantages of clinical metagenomics includes accuracy, comprehensiveness, cost-effectiveness, and speed (8). However, metagenomics analysis is a complex and technically demanding process, requiring specialized equipment and highly trained personnel. This may limit the availability of this method in some areas and make it less accessible to patients.

We applied a contamination-robust protocol for metagenomic diagnosis using two replicates per sample, two negative controls, and differential abundance analysis. By comparing each taxon abundance between the sample and control, false detection arising from contamination from the laboratory environment or reagents can be greatly reduced. With two replicates per sample, the occasional burst of abundance due to sequencing variance or the kitome can be corrected. Figure 2 illustrates the protocol using two clinical specimens (*i.e.*, plasma and tissue) as examples.

We applied the developed protocol to biopsied material from a 65-year-old male suffering from persistent low back pain and soreness after spinal surgery. magnetic resonance imaging confirmed infectious spondylitis in L3-L5. Teicoplanin was given for weeks without improvement. Tissue biopsy *via* percutaneous endoscopic lumbar discectomy were obtained from the patient (Figure 3). Both the plasma and tissue samples were culture-negative at that time. We applied metagenomic sequencing with the proposed protocol for identifying the pathogens of significantly higher abundance than the control and concordance in replicates. The protocol detected no pathogens in the plasma sample due to discordance between the two replicates (Figure 2). In the tissue biopsy sample, *C. modestum* was concordantly identified in the two replicates within 24 hours. Notably, *C. modestum* was not only the most abundant taxa in the two tissue replicates, but its abundance was also significantly higher than for the same taxa in the negative control (84.6% *vs.* 0.02%, $p<0.01$, chi-square test) (Figure 4). In addition, because there were 52,721 reads (*i.e.* ~2.6 Mbp) covering most protein-coding genes in the *C. modestum* genome (~2.6 Mbp), we further annotated the antibiotic-resistant genes in its resistome. The results indicated no known such gene. Therefore, the antibiotic was switched to penicillin and doxycycline upon receiving the metagenomic diagnosis report. The CRP and ESR values continuously dropped after switching antibiotics. The patient recovered and was discharged. In this report, we present a case of spondylitis. Shotgun metagenomic sequencing identified *C. modestum*. The potential of this application in infectious spondylitis requires further investigation.

Conflicts of Interest

The Authors declare that they have no competing interests.

Authors' Contributions

YTH, YCF, DYL, YLK, CHT, CWL, and PYL conceived and designed the study. MYY, YCM, DYL, YLK, CHT, CWL, and PYL contributed to comprehensive research and sample collection. YTH, YCF, and PYL wrote the article. MYY and YCM participated in manuscript editing. All Authors read and approved the final article.

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