Bone & Joint Research

Supplementary Material

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The mNGS method and interpretation of mNGS results were summarized as follows, based on previous papers:

mNGS

The metagenomic next-generation sequencing (mNGS) procedure was conducted as follows. 1) Nucleic acid extraction: 500 μl of liquid or homogenized tissue was taken and total DNA was extracted using TIANamp Micro DNA Kit (DP316; Tiangen, China), according to the instructions provided with the reagents. 2) Library construction and sequencing. DNA were randomly fragmented into 200 to 300 bp fragments, and the concentration of DNA libraries was detected using the dsDNA HS Assay Kit (Thermo Fisher Scientific, USA). After cyclization, the libraries were replicated by rolling cycles to generate DNA nanospheres. The prepared DNA nanospheres were loaded onto sequencing chips and sequenced using the BGISEQ-500 platform (UW Genetics, China). 3) Bioinformatics analysis: low-quality data and data smaller than 35 bp were removed. The remaining data were processed using BWA (Burrows-Wheeler alignment, http://bio-bwa. sourceforge.net) to remove the human reference genome sequence (Hg19). The resulting data were compared with the microbial database and classified as viruses, fungi, bacteria, parasites, etc. The microbial reference whole genome data included 1,494 bacteria, 2,700 viruses, 73 fungi, 40 mycoplasma or chlamydia, and 48 parasites, all from the National Center for Biotechnology Information (https://ncbi.nlm.nih.gov/genome).

Interpretation of mNGS results

The standardized ratio was calculated to eliminate differences in reading between samples. First, the original total number of reads was divided by 20,000,000 to obtain the normalized ratio. Then, the number of total microbial reads (TMR), the number of reads stringently mapped to the pathogen in genus-level (SMRNG), and the number of reads stringently mapped to the pathogen in species-level (SMRNS) were multiplied by the standardized ratio to obtain standardized TMR (SDTMR), standardized SMRNG (SDSMRNG), and

standardized SMRNS (SDSMRNS), respectively. The genome coverage rate (CR) of a pathogen was calculated as the length of the detected pathogen sequence divided by the total length of the reference genome on the alignment. Relative abundance in genus level (RAG) was calculated as the proportion of microbial genera in the same broad category (bacteria, fungi, viruses, parasites) of all detected pathogens.

The original results of microbial species comparison using mNGS included a large number of background microorganisms in addition to pathogenic ones. Therefore, appropriate thresholds needed to be set to improve the detection rate of true pathogenic bacteria and reduce the probability of misclassifying background bacteria as pathogens. Our interpretation of mNGS results was based on previous literature and our preliminary study. The following detection thresholds were established: 1) *Burkholderia*, *Ralstonia*, *Delftia*, *Sphingobium*, *Alternaria*, *Sodaria*, *Aspergillus*, *Albugo*, and other genera were the most common background bacteria, measured within other sample species in our laboratory. The pathogenic bacteria were identified when their relative abundance at the genus level was ≥ 80%. 2) SDSMRNG < 3 was considered to be meaningless for detection. 3) The highest CR and SDSMRNS among pathogenic genera was considered to be pathogenic. 4) The optimal threshold for bacterial identification was determined to be ≥ 15% relative abundance at the level of non-human genera and ≥ 30% relative abundance at the level of fungal genera threshold. 5) Due to the extremely low amount of nucleic acids, mycobacterium tuberculosis complex (MTCP) was identified as pathogenic after normalization to the number of reads SDSMRNG ≥ 1 that strictly mapped to pathogens at the genus level.

References

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Typical cases of BJIs caused by rare organisms accurately diagnosed by mNGS

Table i. Representative rare cases precisely diagnosed by metagenomic next-generation sequencing.

Case	Sex	Age, yrs	mNGS	Microbial culture	Diagnosis
1	F	50	*Mycolicibacterium houstonense*	*Mycolicibacterium houstonense*	Implant-related infection
2	F	65	*Parvimonas micra*	Negative	PJI
3	F	63	*Mycobacterium tuberculosis*	Negative	PJI
4	F	31	*Mycobacterium colombiense*	Negative	PJI
5	M	87	*Staphylococcus aureus Mycoplasma hominis*	*Staphylococcus aureus*	PJI

PJI, periprosthetic joint infection.

Case 1: *Mycobacterium fortuitum* (*Mycobacterium houstonense*)

A 60-year-old female patient with no underlying disease history suffered from an open fracture of the left tibia and fibula because of a fall from a height. The fracture was treated with debridement and internal fixation using steel plates. Due to poor healing, an autologous iliac bone graft was performed three months after surgery. Two months after bone grafting, the patient developed fever, local swelling, pain, pus discharge, and sinus formation. Several debridements and antibiotic treatments were performed at the local hospital, but the bacterial culture was negative. After referral to our hospital, both preoperative and intraoperative tissue cultures were negative, and empirical anti-infection therapy with vancomycin and meropenem was administered postoperatively. Intraoperative tissue mNGS indicated the presence of *Mycobacterium houstonense*, and antibiotics were adjusted to clarithromycin and levofloxacin based on the mNGS results. The strain grew in the mycobacteria testing BACTEC MGIT system and was identified as *Mycobacterium houstonense*. During the follow-up of five months, the inflammatory indicators were normalized, and iliac bone implantation and internal fixation were performed in the second stage. The original anti-*Mycobacterium houstonense* treatment was continued, and the fracture healed without sinus tract or pus discharge.

Case 2: *Micromonas parvum*

A 65-year-old female patient who underwent a left total hip arthroplasty (THA) seven years ago due to aseptic necrosis of the left femoral head presented with

persistent mild pain in the left hip. Three years ago, a mass was found in the left thigh. On admission, the ESR was 48 mm/h, CRP was 2.8 mg/l, preoperative synovial leucocyte count (SF-WBC) was 42,478 × 10^6/l, and synovial polymorphonuclear neutrophil percentage (SF-PMN) was 92.3%. Although the preoperative synovial fluid puncture culture was negative, Stage II revision was performed. After surgery, an empirical anti-infection regimen of vancomycin and ceftazidime was administered. Intraoperative mNGS of ultrasound lysis fluid showed that *Micromonas spp.* accounted for more than 90% of bacterial reads. Intraoperative synovial fluid was tested with 16S rRNA broad-range polymerase chain reaction (PCR), and the coincidence rate of *Micromonas spp.* was 99%. Vancomycin and ceftazidime were discontinued, and infection was controlled after treatment with amoxicillin.

Case 3: *Mycobacterium tuberculosis*

A 63-year-old female patient underwent total knee arthroplasty (TKA) for osteoarthritis of the left knee eight months ago. Despite the surgery, the patient presented with recurrent swelling and pain. On admission, laboratory results revealed an elevated ESR of 80 mm/h, CRP of 33.4 mg/l, preoperative SF-WBC of 9,436 × 10^6/l, and SF-PMN at 66.63%. Despite a ten-day preoperative bacterial and fungal culture in the synovial fluid puncture, results were still negative, and the patient underwent Stage II revision. An empirical anti-infection regimen of vancomycin and meropenem was administered after surgery. Intraoperative mNGS of synovial fluid revealed *Mycobacterium tuberculosis* infection, which was confirmed by tuberculosis DNA-PCR. The patient was finally diagnosed with chronic infection caused by *Mycobacterium tuberculosis* after the TKA, and was successfully treated with anti-tuberculosis therapy.

Case 4: *Mycobacterium columbiae*

A 31-year-old female with a medical history of systemic lupus erythematosus and diabetes underwent THA ten years ago due to a left femoral neck fracture. Two years ago, she developed pain in the left acetabular region, and was treated for left acetabular prosthesis loosening and underwent acetabular lateral revision with anti-infection therapy despite multiple tissue cultures being negative at other hospitals. However, ten months after the revision surgery, persistent pain, sinus tracts, and purulent discharge developed, and she was referred to our hospital. The patient was diagnosed with periprosthetic joint infection (PJI) of the left hip based on the 2018 International Consensus Meeting on Periprosthetic Joint Infection criteria, and underwent a stage II revision. Bacterial cultures of periprosthetic tissue, joint fluid, and ultrasound lysis fluid during the operation remained negative for 14 days. Despite being administered an empirical anti-infection regimen of vancomycin and meropenem, no significant reduction in inflammatory markers was observed. Intraoperative mNGS of ultrasound lysis fluid indicated the presence of *Mycobacterium columbiae*, which was confirmed by 16S rRNA broad-range

PCR in the synovial fluid. The patient was treated with clindamycin, ethambutol, and rifampicin to control the infection after the anti-infection therapy.

Case 5: Staphylococcus aureus and *Mycoplasma* co-infection

An 87-year-old male patient with a history of hypertension and diabetes mellitus underwent bilateral TKA surgery due to osteoarthritis. Three days after surgery, the patient developed chills, red swelling, and pain in the right knee and purulent discharge from the incision. The local hospital provided anti-infective treatment. Three days after a cystostomy procedure, the patient reported pain and urgency during urination, and urinary routine examination revealed elevated white blood cells. Upon admission, laboratory results revealed an elevated ESR of 120 mm/h, CRP of 90 mg/l, and preoperative SF-WBC of 26,286 × 106/l, with SF-PMN at 85.6%. Based on the patient's symptoms and in accordance with the 2018 ICE PJI diagnostic criteria, a diagnosis of right knee PJI was made and stage II revision was performed. During the operation, bacterial cultures were taken from the surrounding tissue of the prosthesis and ultrasound lysis fluid, revealing *Staphylococcus aureus*. Post-surgery, vancomycin and meropenem were administered for anti-infective treatment, but the patient's CRP and ESR remained high. The results of intraoperative tissue mNGS showed the presence of *Mycoplasma hominis*. Considering the urinary system symptoms of the patient, the possibility of *Mycoplasma* PJI caused by urinary system infection was considered. After treatment with doxycycline, the inflammatory indicators returned to normal and the infection was controlled.