Dear Editors and Reviewers:

Thanks very much for your letter and for the reviewers' comments concerning our manuscript entitled "Pulmonary nocardiosis with bloodstream infection diagnosed by metagenomic next-generation sequencing in a kidney transplant recipient: A case report" (Manuscript NO.: 82021). These comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to this report. We have studied comments carefully and have made some corrections. Revised portion are marked in yellow highlight in the manuscript. The responds to the reviewer's comments are below:

Reviewer #1:

This case was very interesting and the metagenomic NGS may be a solution in the future for difficult diagnosis as in the case. The problem is that this method in very expensive at the moment and some laboratories will have difficulties to implement this new methodology in clinical practice. One problem is the high interlaboratory variability found in both identifying microbes and distinguishing true pathogens emphasizes the urgent need for improving the accuracy and comparability of the results generated across different mNGS laboratories, especially in the detection of low-microbial-biomass samples (DOI: 10.1016/j.jare.2021.09.011).

Response: As reviewer mentioned that the high cost of mNGS limits its application in clinical practice. Therefore, mNGS is currently mostly used in difficult, acute and (or) severe cases. At the same time, it also pointed out the shortcoming of mNGS - the difficulty in distinguishing pathogenic and background microorganisms, which makes it a great challenge to interpret results. We have to combine the detection parameters, clinical information and more clues for consideration before reporting the pathogens.

Taking this case report as an example, mNGS detected *Nocardia farcinica* from the patient's samples, and its specific sequence and relative abundance were more prominent. Meanwhile, *Nocardia farcinica* was not detected in negative control and

the same batch of samples. In addition, we found that *Nocardia* was not common background microorganisms in the historical samples from this medical center. Thus, contamination from experiment and sampling process were initially excluded. Finally, considering infection characteristics of *Nocardia* and immunosuppression of the patient, *Nocardia farcinica* was highly suspected as true pathogen. However, following culture and acid-fast staining for verification were all negative. With some regrets in this report, verification of Q-PCR and first-generation sequencing were not performed because of the patient's acute condition and cost reason.

In a word, to distinguish true pathogens and background microorganisms, especially in low-microbial-biomass samples, the multifaceted information needs to be integrated. And if necessary, re-examination and (or) related detection for verification should be performed to obtain more accurate diagnosis.

Reviewer #2:

Dear Authors i found your manuscript interesting. New techniques for rapid infection diagnosis, such as mNGS for Nocardia in immunocompromised patients would mean a huge help to get early diagnosis and save the patient's life, as in your case.

(1) Some minor language polishing would be required.

Response: As Reviewer suggested that the manuscript has been polished by a professional English language center. This agency is recommended by the *World Journal of Clinical Cases* and we got a Grade A (priority publishing; no language polishing required) after editing.

(2) All abbreviations should be explained upon first mentioned.

Response: **The abbreviation of "T-SPOT" has changed to "T cells spot test of tuberculosis infection".** No other unexplained abbreviations were found after careful reading.

(3) Furthermore, Please describe the used NGS method/platform.

Response: **The method of mNGS is added to the manuscript as Supplementary Material.** The contents are below:

The methods of mNGS in Guangxi KingMed Diagnostics were same as that described in a previous published mNGS article which was conducted in Guangzhou KingMed Diagnostics [1].

① Sample Processing:

Pleural effusion was collected and transported with dry ice. Blood was stored in EDTA tubes, from which plasma was separated by centrifuging at 1600 g for 10 min at 4℃.

② Sequencing and Quality Control:

In this study, nucleic acid (DNA and RNA) extraction and library preparation were performed on samples through the lab's self-built process. Both nucleic acid extraction and library preparation were conducted in parallel with quality control samples. Qubit was used to measure the concentration of the library. Single-end 75bp sequencing was carried out using Illumina nextseq 550 system with 75 cycles Reagent Kit. Then, the low-quality sequencing data were filtered out. After the removal of the sequences mapped to human reference genome, the remaining data were aligned to the microbial genome database.

③ Bioinformatic Analysis:

The adapter sequences, low-quality data, and polyG tails were remove to generate the clean data using fastp v0.20.0 [2]. Then sequences that can be mapped to human reference genome were filtered using bwa v0.7.10-r789 [3]. The alignment of the remaining microbial data was carried out using bwa v0.7.10-r789 [3] and Sequence-Based Ultra-Rapid Pathogen Identification (SURPI v1.0.18) pipeline (UCSF), a previously published research pipeline for pathogen identification [4, 5]. For clinical usage, a self-built microorganisms database “MetagenomicX” was used to aligned the sequencing data. The database contains 36497 microorganisms’ genomes, which covers most of the microorganisms which have been sequenced. 8704 of the total microorganisms in the first-grade database have integral sequence of whole genome and detailed clinical analysis, which covers most of the known pathogenic bacteria, viruses, fungi, and parasites. This first-grade database with high-quality genomes is used as first choice.

References:

[1] Shi Y, Chen J, Shi X, Hu J, Li H, Li X, et al. A case of chlamydia psittaci caused severe pneumonia and meningitis diagnosed by metagenome next-generation sequencing and clinical analysis: a case report and literature review. BMC Infect Dis. 2021;21(1):621. doi:10.1186/s12879-021-06205-5.

[2] Chen S, Zhou Y, Chen Y, Gu J. fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics. 2018;34(17):i884-i90. doi:10.1093/bioinformatics/bty560.

[3] Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25(14):1754-60. doi:10.1093/bioinformatics/btp324.

[4] Miller S, Naccache SN, Samayoa E, Messacar K, Arevalo S, Federman S, et al. Laboratory validation of a clinical metagenomic sequencing assay for pathogen detection in cerebrospinal fluid. Genome Res. 2019;29(5):831-42. doi:10.1101/gr.238170.118.

[5] Naccache SN, Federman S, Veeraraghavan N, Zaharia M, Lee D, Samayoa E, et al. A cloud-compatible bioinformatics pipeline for ultrarapid pathogen identification from next-generation sequencing of clinical samples. Genome Res. 2014;24(7):1180-92. doi:10.1101/gr.171934.113.

(4) Were there any additional studies performed on patient's material afterwards the mNGS diagnosis? I mean how did you try to prove your diagnosis with some other methods?

Response: As reviewer mentioned that proving the accuracy of mNGS is an important step. In this case, we combined the detection parameters, clinical information and more clues for reporting the pathogens and diagnosis. First, mNGS detected *Nocardia farcinica* from the patient’s samples, and its specific sequence and relative abundance were more prominent. Meanwhile, *Nocardia farcinica* was not detected in negative control and the same batch of samples. In addition, we found that *Nocardia* was not common background microorganisms in the historical samples from this medical center. Thus, contamination from experiment and sampling process were initially excluded. Finally, considering infection characteristics of *Nocardia* and immunosuppression of the patient, *Nocardia farcinica* was highly suspected as true pathogen. However, following culture and acid-fast staining for verification were all negative. With some regrets in this report, verification of Q-PCR and first-generation sequencing were not performed because of the patient’s acute condition and cost reason. In fact, to prove the true pathogens and diagnosis, we realized that the multifaceted information needs to be integrated. And if necessary, re-examination and (or) related detection for verification should be performed to obtain more accurate diagnosis.

We tried our best to improve the manuscript and made some changes in the revised manuscript. These changes will not influence the framework of the paper. We appreciate for Editors/Reviewers' warm work earnestly, and hope that the correction will meet with approval.

Once again, thanks very much for your comments and suggestions.