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Reviewer A

This study addresses relevant points of timely detection of the etiological agent in bloodstream infections. Work surrounding the utility of sequencing based methods towards the same is increasingly gaining focus. However, there remain some major points in this manuscript that the authors need to clarify:

1. Concept of study needs to be backed by adequate blinding between different testing methods

Reply 1: Culture, mNGS, and tNGS were conducted by different technicians not aware of the results of each other, which ensured adequate blinding between different testing methods.

Changes in the text: We have modified our text as advised: Page 5, Line 143-145.

2. Baseline criteria is clinical evaluation at death/discharge which would follow the testing and thus further introduce bias as evaluations would be done retrospectively (Line 186-187)

Reply 2: None of the known etiological diagnostic tests can avoid false negativity. Thus, it is inappropriate to set culture or mNGS or tNGS as the gold standard in this study. A clinical diagnosis of BSI or noninfectious fever was made at discharge/death by a clinician team consisting of at least two senior clinicians/professors and four attending doctors, according to the patient's medical history, signs and symptoms, laboratory and imaging examinations, and response to antibiotic treatment. Compared to a single diagnostic test of culture or mNGS or tNGS, the clinical diagnosis at discharge/death by the clinician team is much more reliable and appropriate to set as the gold standard because the consideration of the clinicians is more comprehensive and discreet. Bias exists inevitably but limited because the clinician team made the final diagnosis based on many factors, not only a single diagnostic test.

Changes in the text: We have modified our text as advised: Page 14, Line 453-455.

3. Need to include criteria to address for how NGS-based pathogen identification was done - Methods (Line 122-124) suggest that the majority pathogen was categorized however no mention is made for how polymicrobial assessments were made

Reply 3: If more than one potential pathogen was identified by mNGS or tNGS with approximate read number (within one-fold) and all the identified potential pathogens were consistent with the clinical course of the patient, the mNGS or tNGS results were recognized as polymicrobial infection.

Changes in the text: We have modified our text as advised: Page 5, Line 153-156.

Additional comments

1. Line 134-136: Methods of extraction for blood samples is mentioned but not for other sample types

Reply 1: Sputum was liquefied by 0.1% DTT (dithiothreitol) for 30 minutes at room temperature. Pus or body fluids could go directly to the next operation.

Changes in the text: We have modified our text as advised: Page 6, Line 167-168.

2. Line 141-142: Mention which library kit was used

Reply 2: DNA libraries were constructed using MetaCAP Pathogen Capture Metagenomic Assay Kit (KingCreate China).

Changes in the text: We have modified our text as advised: Page 6, Line 175-176.

3. Line 161-162: The pathogen targets included are not clarified

Reply 3: The enrichment panel targeted >3,000 pathogens, including viruses, bacteria, fungi, and parasites. The pathogen target was screened according to the following rules: 1. The target had at least one completed genome sequence; 2. The target had at least one article related to human infection; 3. The target had research reports. Finally, this capture range was 1060 bacteria, 1402 viruses, 388 fungi and 210 parasites. Probe design selected the conserved regions such as ribosomal RNA gene (16S rRNA, 18S rRNA or internal transcribed spacer), house-keeping genes which allowed for pathogen identification down to the genus or species level.

Changes in the text: We have modified our text as advised: Page 7, Line 196-203.

4. How was confidence of calling pathogens maintained using single-end 50 bp for MGI (Line 145-146) and 100 bp for Illumina (Line 165-166)?

Reply 4: Compared with tNGS, mNGS has much more data burdens and the data interpretation process is more complex. The data size is approximately 100-120M per sample for mNGS. 50bp is recommended by BGI to balance the diagnosis performance and clinical turnaround time. If 50bp increases to 100bp, time spent on sequencing will double, consuming almost 30 hours on the sequencing platform. TAT of 100bp in mNGS cannot meet clinical needs. Howerver, in tNGS, target capture and enrichment can reduce data burdens. The data size is approximately 1M per sample for tNGS and sequencing needs only 6 hours on Illumina Miniseq platfrom. 100bp is more suitable for tNGS because specific target for pathogen identification requires certain length. These are the inherent differences between mNGS and tNGS. The aim of this study is to evaluate diagnostic performance of blood culture, mNGS, and tNGS.

Changes in the text: We have modified our text as advised: Page 13, Line 429-438.

5. Line 176-179: Which reference genomes were used?

Reply 5: The scope of the database contains all targeted pathogens and other species in their genus. In addition, it also includes species outside the genus with genome average nucleotide identity >80%. Database contains 11,958 bacteria, 7,373 viruses, 1,714 fungi and 343 parasites. The species genomes were collected and downloaded from the

NCBI genome (ftp://ftp.ncbi.nlm.nih.gov/genomes/). Genome assemble level at complete or scaffold was selected and shielded contamination introduced by assembly, such as host sequences.

Changes in the text: We have modified our text as advised: Page 7, Line 220-227.

6. Line 201: How were nosocomial infections categorised?

Reply 6: Nosocomial infections referred to infections acquired during hospitalization. Infections occurred after discharge but acquired during hospitalization still belonged to nosocomial infections.

Changes in the text: We have modified our text as advised: Page 8, Line 257-259.

7. Line 297-299: Therapy was changed in 22 cases based on the results of tNGS but no information is provided in cases where therapy was not changed or a reason for why therapy was not changed.

Reply 7: For the other 47 patients with BSI, tNGS results were concordant with the clinician's initial evaluation of the patient and the initial anti-infectious therapy continued.

Changes in the text: We have modified our text as advised: Page 11, Line 363-365.

8. Table S2: Was tNGS only performed in blood? M. tuberculosis was detected in P50 and it is an unlikely pathogen to be detected in blood.

Reply 8: tNGS was only performed in blood. For patients with disseminated or severe tuberculosis infection, it is likely for *M. tuberculosis* to be detected in blood. Here are the blood mNGS reports detecting *M. tuberculosis* of two patients in our medical group (read number of *M. tuberculosis* was 9 and 4 respectively on the genus level).

Patient A: A 19-year-old male with *M. tuberculosis* infection in eye, lung, and abdomen. The infection was so severe that his left eyeball was necrotic and had to be removed. He recovered after 18 months of anti-tuberculosis treatment.

检测信息	
样本类型：血	样本编号：22P20001656
检测日期：2022.06.29	报告日期：2022.07.01

检测结论

本次检出序列

✓ 结核分枝杆菌复合群 9条

具体请结合临床。

Patient B: A 26-year-old male with *M. tuberculosis* infection in lung and abdomen. His body weight was only 35 kg on admission (BMI 12.1). During hospitalization, intestinal obstruction and bleeding almost killed him. Finally, he survived and received 14 months of anti-tuberculosis treatment. Sorry that the mNGS report was saved in image format with no English version available.

2022-06-09 血mNGS（06-07送检）：检出结核分枝杆菌核酸序列4条

属名	属相对丰度（%）	属严格序列数	种名	种相对丰度（%）	种序列数	种严格序列数
结核分枝杆菌复合群	2.64	4	牛分枝杆菌	0.64	1	0
	2.64	4	田鼠分枝杆菌	0.67	1	0
	2.64	4	獭分枝杆菌	0.67	1	0
	2.64	4	结核分枝杆菌	0.65	1	0

9. How were S. mitis and H. parainfluenzae detected by mNGS categorized as contaminants.

Reply 9: These two patients were respectively diagnosed with Takayasu arteritis and polyarteritis nodosa. After transferred to the Rheumatology Department, they received glucocorticoid and immunosuppressant treatment and clinically improved. The clinical course did not support the diagnosis of BSI. *S. mitis* and *H. parainfluenzae* are oral normal flora. When sputum and other kind of samples such as blood are tested in the same batch, they often cause contaminantation.

Changes in the text: We have modified our text as advised: Page 11, Line 342-344.

Minor comments:

1. Line 66-68: Please include references

Reply 1: We have included references as advised.

Changes in the text: Page 3, Line 90.

2.Line 163-164: Rephrase "Quality-qualified libraries were pooled using the Qubit dsDNA HS Assay Kit."

Reply 2: The sentence has been rephrased as “The Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to construct quality-qualified libraries”.

Changes in the text: We have modified our text as advised: Page 7, Line 205-206.

3. Line 266: Replace consistency with concordance

Reply 3: We have modified our text as advised.

Changes in the text: Page 10, Line 323.

4. Lines 238, 240, 260: tNGS identified new microbes - replace new with additional

Reply 4: We have modified our text as advised.

Changes in the text: Page 9, Line 295 and 297; Page 10, Line 317.

5. Line 262-264: Genus names and anaerobes do not need to be italicised

Reply 5: We have modified our text as advised.

Changes in the text: Page 10, Line 320-321.

6. Line 285: Replace pollutant with contaminant

Reply 6: We have modified our text as advised.

Changes in the text: Page 11, Line 342.
7. Figure 2A: Name of Streptococcus is cut off on Y axis
Reply 7: In Figure 2A, "Streptococcus" generally referred to the genus, including *Streptococcus oralis*, *Streptococcus sanguinis*, and *Streptococcus agalactiae*.

Reviewer B

In their manuscript entitled „ Etiological diagnostic performance of probe capture-based targeted next-generation sequencing in bloodstream infection the authors describe the application of probe capture-based targeted Next Generation Sequencing (tNGS) for the etiological diagnosis of bloodstream infections (BSI) - a technique that has not yet been widely explored in BSI context while popular in other fields.

The study explores the clinical implication of the evolving landscape of BSI diagnostics. By examining current challenges, recent advancements, and future directions, the authors seek to highlight the potential for these technologies to revolutionize the diagnosis and management of BSIs, ultimately improving patient care and outcomes.

The single center study nicely indicates the potential of the method with its findings, discusses the findings appropriately and is generally well written.

The following issues should be addressed:

Please give some detail on how blood cultures were gained (how many, central catheter, peripher vein puncture, SOP?) - this might influence the comparison of the effectiveness of the techniques.
Reply: Details were listed in Page 20, Line 543-547.

Moreover, it should be noted that tNGS vibes the advantage for viral diagnostics along with microbial screening.
Reply: tNGS has advantages in virus detection. However, limited by the small sample size, no patient with viremia was diagnosed in this study.
Changes in the text: We have modified our text as advised: Page 14, Line 458.

Inclusion criteria: why >14 years of age? Please provide rationale. Comment on the use in pediatrics/neonatology - probe volume..
Reply: Our hospital does not have the Pediatric Department. All the patients admitted to our hospital must be ≥ 14 years of age. This study does not cover

pediatrics/neonatology. Technically speaking, the process of tNGS testing, including volume of probe and blood samples, does not differ between adult and pediatrics/neonatology.
Changes in the text: We have modified our text as advised: Page 4, Line 118-119.

Could you be a bit more specific about the cost relation of the methods?
Reply: In Shanghai, the cost of a single mNGS testing is around 3500 RMB. If mNGS covers DNA and RNA dual processes, the price will increase to 4500-6000 RMB. The price of tNGS is much lower than that of mNGS. The specific price of tNGS has not been determined yet.
Changes in the text: We have modified our text as advised: Page 3, Line 93-94.

Please address antibiotic stewardship as a benefit of downsizing antibiotic regimens.
Reply: tNGS may help clinicians to downsize antibiotic regimens and decrease the empirical usage of broad-spectrum antibiotics, which might reduce antibiotic abuse and affect the clinical prognosis.
Changes in the text: We have modified our text as advised: Page 14, Line 447-451.