Peer Review File

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Reviewer A

Comment 1: The generalizability of the results is problematic as mNGS is not compared with the current state-of-art diagnostic tools (PCR, panels, etc). Therefore, the higher yield of mNGS does not come as a surprise.

Reply 1: There is no routine panels and virus culture for testing, so it cannot be compared, which is the deficiency of our article. However, PCR detection has been conducted, and the previous article description is not accurate enough and has been added. So we believe that the higher yield of mNGS still makes sense.(see line 136-139)

Changes in the text: After treatment, the 3-5 ml BALF was extracted and put into a sterile sputum container, and then sent to the microbiology laboratory for mNGS test and CMT test, including microbial isolation and culture, smear microscopy, antibody and antigen detection, and PCR.

Comment 2: The paper heavily reports bacterial data, these should be included formally (and the title adapted) – it is not clear why also for bacteria, the yield was so much in favor of mNGS as compared with routine diagnostics.

Reply 2: We did not consider the results carefully, although most of the children after surgery for congenital heart disease have bacterial pneumonia, and the high detection rate of mNGS has important guiding significance for the adjustment of postoperative antibiotic regimen. However, as an article focusing on viral pneumonia, our description was not specific enough, so we appropriately deleted the description about bacteria to highlight the influence of mNGS examination results on viral infection. We did not consider the results carefully, although most of the children after surgery for congenital heart disease have bacterial pneumonia, and the high detection rate of mNGS has important guiding significance for the adjustment of postoperative antibiotic regimen. However, as an article focusing on viral pneumonia, our description was not specific enough, so we appropriately deleted the description about bacteria to highlight the influence of mNGS examination results on viral infection.

Changes in the text: A description of the bacterial fraction was removed appropriately removed.

Comment 3: Line 60: The claim that mNGS has a fast turnover time needs to be substantiated, in regards to which other method?

Reply 3: Compared with conventional virus culture, immunology technology and

PCR techniques, relevant descriptions have been added in the text.(see line 48-70)

Changes in the text: At present, the detection methods of RVI include virus culture, immunological techniques, polymerase chain reaction (PCR) techniques, and so on. Virus culture is the "gold standard." However, this method is not suitable for clinical laboratory application due to its complicated operation steps, time-consuming and labor-intensive requirements and high technical equipment requirements. The immunological technique is relatively simple, but IgM antibody can not be produced until at least 6 days after viral infection, and antibodies produced by previous infections in patients can cause false positives in the detection, and the sensitivity and specificity of the detection still need to be improved. PCR method is sensitive and specific for virus detection, but in general, conventional PCR can only detect 1~3 viruses in one reaction, and the throughput is low.8,9 In the clinical treatment of congenital heart disease complicated with respiratory virus infection, due to the great difference in medical level, the diagnosis of respiratory pathogens is not clear and not timely, clinicians often use empirical treatment, which is easy to lead to the unscientific use of drugs and the delay of treatment. Therefore, it is particularly important to find an accurate, rapid, high throughput, high sensitivity and strong specificity method for respiratory virus detection.

As a standard diagnostic procedure for pulmonary diseases, bronchoalveolar lavage fluid (BALF) has been widely used to diagnose pneumonia. At present, traditional etiological detection methods, such as culture, immunological detection, and PCR, are widely used in the clinical diagnosis of pneumonia. However, its application has been limited due to poor timeliness, low pathogen coverage, and insufficient positive detection rate.

Comment 4: Line 93: Please explain: mNGS quality control: One will always have >99% human sequences?

Reply 4: A key disadvantage inherent to mNGS, given its shotgun sequencing approach, is that microbial nucleic acids from most patients' samples are dominated by human host background. The vast majority of reads, generally >99%, derive from the human host, thus limiting the overall analytical sensitivity of the approach for pathogen detection, given the relative scarcity of microbial nonhuman reads that are sequenced.

Changes in the text: /

Comment 5: Lines 96, 97: Why would patients not agree to mNGS? Where they asked before bronchoscopy?

Reply 5: Because mNGS examination is more expensive, it is difficult for families to afford. Before the child underwent bronchoscopy, the doctor informed the family members of the necessity and related risks of bronchoscopy in detail, and the family members signed the informed consent form.（see line97-100）
Changes in the text: Because the cause of infection was unknown, the empirical anti-infection effect was not good after three days, and the mNGS test was agreed to be included in the mNGS group.

Comment 6: Line 98: What did the conventional microbiological test methods consist of? (It seems that starting lines 127 a number of tests are outlined: all were direct immunofluorescence?) -Herpes simplex virus and cytomegalovirus antibody detection is not a suitable test to look for RVI.（see line100-103）
Reply 6: conventional laboratory staining, real-time PCR test, enzyme-linked immunospot detection, GeneXpert, serum (1-3) -β-D-glucan test.
Changes in the text: Those who refused mNGS test because of the high test cost were included in the CMT, and tested using routine microbiological test methods, such as conventional microbial culture, conventional routine laboratory smear staining, real-time PCR test, enzyme-linked immunospot detection, GeneXpert, serum (1-3) -β-D-glucan test.

Comment 7: Line 102: Detection of any virus? Of a potentially pathogenic virus?
Reply 7: Not any virus (see line 114)
Changes in the text: The observed outcome measure was the detection rate of respiratory viruses causing study subjects with viral pneumonia.

Comment 8: Line 113: Abbreviations must be introduced (or is there list with accepted ones by the journal?)
Reply 8: We have added the relevant abbreviations. (see line 124)
Changes in the text: inflammatory parameters (white blood cell (WBC), C-reactive protein (CRP), Procalcitonin (PCT), pro-brain natriuretic peptide (pro-BNP) within 24 hours before specimen examination).

Comment 9: Line 185: HHV-7 has most likely no pathogenic properties and should not be listed in the relevant virus list.
Reply 9: HHV-7 is a ubiquitous virus, commonly found in human white blood cells, and the positive rate of serum HHV-7 antibody in healthy people is higher. The primary infection usually occurs in childhood, and the virus is latent or persistent in some organs and tissues, including the lungs and salivary glands. HHV-7 can be reactivated when the host immune function is reduced and can

cause infection with significant clinical symptoms. The virus can cause neuroinfectious diseases such as myelitis, encephalitis and facial neuritis. It can also be reactivated in people who have received hematopoietic stem cells or organ transplantation, radiation therapy or chemotherapy, and immunosuppressant treatment, which presents as fever, rash, hepatitis, pneumonia, and myocarditis. Children with congenital heart disease have low immunity, so HHV-7 is included in the test results.
Changes in the text: /

Comment 10: Oseltamivir has no role in treatment of parainfluenza virus – in general, all treatment information should be removed as it is not about treatment.
Reply 10: Although we tried to write this article as well as possible, we made a serious mistake. After reviewing the data, we found that patients with parainfluenza virus were not treated with oseltamivir clinically. We will be more rigorous in the future.
Changes in the text: We have removed the relevant treatment data.

Comment 11: Line 214: I do not understand what prompted the use of anti-infective therapy?
Reply 11: When the empirical resistance to infection was ineffective, the mNGS detected the relevant respiratory viruses, and it was consistent with the clinical symptoms of the child, and prompted us to conduct antiviral treatment. See line (236-238)
Changes in the text: In this study, we performed antiviral treatment only when the existing anti-infection regimen was ineffective and the symptoms coincided with the symptoms of pneumonia caused by the monitored virus.

Comment 12: Discussion: The part on bacterial pathogens is confusing: The study way about viral detection?
Reply 12: As an article focusing on viral pneumonia, our description was not targeted enough, so we appropriately deleted the description of the bacterial part, highlighting the impact of mNGS examination results on virus infection.
Changes in the text: We appropriately deleted the description of the bacterial part.

Comment 13: The author should detail the validation steps taken before introduction of the method
Reply 13: We added more specific details of the mNGS detection method, hoping

to meet the modification requirements. (See 132-182)

Changes in the text: Sample processing and routine microbiological testing

After the patient's written informed consent, bedside bronchoscopy was performed by the attending physician. The doctor followed standard procedures for treatment with a fiberoptic bronchoscope and continued monitoring of ECG, blood pressure, and pulse oxygen saturation during treatment. After treatment, the 3-5 ml BALF was extracted and put into a sterile sputum container, and then sent to the microbiology laboratory for mNGS test and CMT test, including microbial isolation and culture, smear microscopy, antibody and antigen detection, and PCR. The BALF specimen should be promptly sent to the laboratory and processed immediately (within 1 h after collection). If it cannot be processed and tested in time, it should be stored in a refrigerator with -70°C or below, and transported to the laboratory under freezing conditions, but not more than 24 hours. After the elimination of background pathogens, if any of the routine etiological tests were positive and consistent with clinical characteristics, the result was considered positive.

Metagenomic next-generation sequencing methods and bioinformatics analysis

The 3-5 ml BALF samples were collected according to standard procedures. A 1.5 ml microcentrifuge tube containing 0.5 ml BALF sample and 1 g of 0.5 mm glass beads were attached to the horizontal platform of the vortex mixer. The mixture was then vigorously stirred at 3,000 rpm for approximately 30 minutes. According to the manufacturer's recommendations, 0.3 ml samples were transferred to new 1.5 ml microfuge tubes using Tianamp microtubes and DNA extraction DNA kit (DP316, Tiangen Biotechnology). DNA libraries were then constructed by DNA fragmentation, end repair, adapter ligation, and PCR amplification. Agilent 2100 was used for DNA library quality control. The BGISEQ-50 platform ranked qualified DNA libraries.

High-throughput sequencing technology was used to analyze microbial nucleic acid sequences in specimens and identify microorganisms by comparing them with nucleic acid sequences of existing microorganisms in databases. The detection process includes pretreatment, nucleic acid extraction, library construction (preparation of DNA library: including end repair, junction joint, PCR enrichment, purification, clone generation; preparation of RNA library: including removal of the ribosome, reverse transcription and hybridization, fragmentation, first / second strand synthesis, end repair, junction joint, PCR enrichment, purification, cloning, etc.), sequencing, bioinformation analysis, and result interpretation, etc. The mNGS assays were performed based on the Illumina sequencing high-throughput sequencing platform. Quality control requirements included sequencing data volume ≥ 20 M, sequencing read length >

50 bp, dehumanized sequence of more than 90%, and pathogen database > 10,000 species. This database was composed of genomes of archaea, bacteria, fungi, protozoa, viruses, and parasites from the NCBI Genome Database.16 The number of unique alignment reads was calculated and normalized to obtain the number of reads strictly localized to the pathogen species and those strictly localized to the pathogen genus. The workflow for mNGS is shown in Figure 1.

Criteria for positive mNGS: 1. Bacteria (mycobacteria excluded): ≥50 reads mapped to pathogen species and with a reads number no less than times of any other microorganism or supported by CMT results. 2. Fungus/mycoplasma/chlamydia/virus: the reads mapped to pathogen species with reads no less than five times any other fungus or supported by CMT results. 3. Mycobacterium tuberculosis (MTB): no less than one particular sequence was mapped to the reference genome of genus or species level, owing to the difficulty of nucleic acid extraction and the low likelihood of environmental contamination. 4. Nontuberculous Mycobacterium (NTM): a relative bacterial abundance ranking in the top 10 of the bacterial list in view of common environmental contamination.

Comment 14: CMT: Introduce abbreviations at first use, line 98 too late, also important for CICU, CHS

Reply 14: We added the relevant abbreviations at the first appearance. (see line24, line34, line79)

Changes in the text: the conventional microbiological test (CMT) group, cardiac intensive care unit (CICU) , congenital heart surgery (CHS)

Reviewer B

Comment 1: Introduction and sections are well written; the methodology is adapted to the research question but required to be more detailed.

Reply 1: We added more descriptions of mNGS for detecting the respiratory tract after congenital heart disease, with the hope of achieving modification requirements.(see line 40-81)

Changes in the text: Respiratory viral infection (RVI) is common in infants and young children. Respiratory syncytial virus (RSV) is the most common pathogen; approximately 95% of children under two years of age have been infected with RSV, and RSV infection is one of the leading causes of hospitalization.1,2 However, rhinovirus, influenza and parainfluenza virus, human metapneumovirus, coronavirus, and bocavirus can also be detected in patients admitted with respiratory symptoms.3,4 In children with congenital heart disease (CHD), RVI may extend the length of hospital stay, intensive care stay, and mechanical

ventilation duration and is associated with delays in elective cardiac surgery, even leading to patient death.5-7 At present, the detection methods of RVI include virus culture, immunological techniques, polymerase chain reaction (PCR) techniques, and so on. Virus culture is the “gold standard.” However, this method is not suitable for clinical laboratory application due to its complicated operation steps, time-consuming and labor-intensive requirements and high technical equipment requirements. The immunological technique is relatively simple, but IgM antibody can not be produced until at least 6 days after viral infection, and antibodies produced by previous infections in patients can cause false positives in the detection, and the sensitivity and specificity of the detection still need to be improved. PCR method is sensitive and specific for virus detection, but in general, conventional PCR can only detect 1~3 viruses in one reaction, and the throughput is low.8,9 In the clinical treatment of congenital heart disease complicated with respiratory virus infection, due to the great difference in medical level, the diagnosis of respiratory pathogens is not clear and not timely, clinicians often use empirical treatment, which is easy to lead to the unscientific use of drugs and the delay of treatment. Therefore, it is particularly important to find an accurate, rapid, high throughput, high sensitivity and strong specificity method for respiratory virus detection.

As a standard diagnostic procedure for pulmonary diseases, bronchoalveolar lavage fluid (BALF) has been widely used to diagnose pneumonia. At present, traditional etiological detection methods, such as culture, immunological detection, and PCR, are widely used in the clinical diagnosis of pneumonia. However, its application has been limited due to poor timeliness, low pathogen coverage, and insufficient positive detection rate. Metagenomics next-generation sequencing is a new technology for the rapid, efficient, and unbiased acquisition of microbial nucleic acid sequence information.10 Since mNGS was first used to diagnose Leptospirosis in 2014, its diagnostic value has been increasingly recognized.11 Due to its high sensitivity, fast turnover time, unbiased, and unrelated to culture, mNGS has been applied as an emerging and powerful diagnostic technology for infectious diseases, especially for pathogens that cannot be identified by conventional diagnostic methods.12

Although mNGS has been used for microbial identification of infectious diseases and severe pneumonia, the application value of mNGS in the post-operation infection of congenital heart surgery (CHS) is unclear. In this paper, we retrospectively analyzed and summarized the clinical data to explore the value of mNGS in diagnosing respiratory virus infection after CHS.

Comment 2: Material and Methods: The authors should mention how BAL

samples were stored.

Reply 2: We have added how BAL samples were stored.(see 133-145)

Changes in the text: After the patient's written informed consent, bedside bronchoscopy was performed by the attending physician. The doctor followed standard procedures for treatment with a fiberoptic bronchoscope and continued monitoring of ECG, blood pressure, and pulse oxygen saturation during treatment. After treatment, the 3-5 ml BALF was extracted and put into a sterile sputum container, and then sent to the microbiology laboratory for mNGS test and CMT test, including microbial isolation and culture, smear microscopy, antibody and antigen detection, and PCR. The BALF specimen should be promptly sent to the laboratory and processed immediately (within 1 h after collection). If it cannot be processed and tested in time, it should be stored in a refrigerator with -70°C or below, and transported to the laboratory under freezing conditions, but not more than 24 hours. After the elimination of background pathogens, if any of the routine etiological tests were positive and consistent with clinical characteristics, the result was considered positive.

Comment 3: Lines 144-148: High-throughput sequencing technology should be detailed (which Illumina machine, which protocol, which pipeline were used….).

Reply 3: We have added details about high-throughput sequencing technology.(see line 147-155), However, because of the confidentiality requirements of the laboratory, some details cannot be made public, and we are still trying.

Changes in the text: The 3-5 ml BALF samples were collected according to standard procedures. A 1.5 ml microcentrifuge tube containing 0.5 ml BALF sample and 1 g of 0.5 mm glass beads were attached to the horizontal platform of the vortex mixer. The mixture was then vigorously stirred at 3,000 rpm for approximately 30 minutes. According to the manufacturer's recommendations, 0.3 ml samples were transferred to new 1.5 ml microfuge tubes using Tianamp microtubes and DNA extraction DNA kit (DP316, Tiangen Biotechnology). DNA libraries were then constructed by DNA fragmentation, end repair, adapter ligation, and PCR amplification. Agilent 2100 was used for DNA library quality control. The BGISEQ-50 platform ranked qualified DNA libraries.

Comment 4: In addition, it is mentioned that "… cDNA synthesis, fragmentation, library construction, sequencing, …" however only DNA extraction is presented… so how cDNA has been obtained? And as respiratory viruses are mainly RNA viruses how this point was addressed in mNGS approach? These points have to be clarified.

Reply 4: We have added relevant content.(see 158-164)
Changes in the text: The detection process includes pretreatment, nucleic acid extraction, library construction (preparation of DNA library: including end repair, junction joint, PCR enrichment, purification, clone generation; preparation of RNA library: including removal of the ribosome, reverse transcription and hybridization, fragmentation, first / second strand synthesis, end repair, junction joint, PCR enrichment, purification, cloning, etc.), sequencing, bioinformation analysis, and result interpretation, etc.

Comment 5: Rules to rule out false positive or background noise are not detailed; negative and positive controls should be done and presented.
Reply 5: We have added relevant rules.(see 173-182)
Changes in the text: Criteria for positive mNGS: 1. Bacteria (mycobacteria excluded): ≥50 reads mapped to pathogen species and with a reads number no less than times of any other microorganism or supported by CMT results. 2. Fungus/mycoplasma/chlamydia/virus: the reads mapped to pathogen species with reads no less than five times any other fungus or supported by CMT results. 3. Mycobacterium tuberculosis (MTB): no less than one particular sequence was mapped to the reference genome of genus or species level, owing to the difficulty of nucleic acid extraction and the low likelihood of environmental contamination. 4. Nontuberculous Mycobacterium (NTM): a relative bacterial abundance ranking in the top 10 of the bacterial list in view of common environmental contamination.

Comment 6: Conventional method to diagnose infection should be detailed.
Reply 6: We have added relevant details. (see 136-139)
Changes in the text: After treatment, the 3-5 ml BALF was extracted and put into a sterile sputum container, and then sent to the microbiology laboratory for mNGS test and CMT test, including microbial isolation and culture, smear microscopy, antibody and antigen detection, and PCR.

Comment 7: Staphylococcus aureus should be written in italic.
Reply 7: We have written Staphylococcus in italic. (see line288-289)
Changes in the text: *Staphylococcus*

Comment 8: the authors highlighted the retrospective character of this study; this should be mentioned in the Material Method section; in addition, it is therefore difficult to understand how the mNGS data were included in the patient daily monitoring to adapt the antimicrobial therapy. This point has to be clarified

at the Material Method, Results and Discussion sections.

Reply 8: Through retrospective collection of data, excluding non-conforming data analysis, mNGS detection according to the guideline consensus and combined with the clinical actual situation, due to the unknown cause of infection after 3 days, agreed to mNGS test, we take the bronchoalveolar lavage fluid, adjust according to the results.(see 97-99)

Changes in the text: Because the cause of infection was unknown, the empirical anti-infection effect was not good after three days, and the mNGS test was agreed to be included in the mNGS group.