Clinical diagnostic application of metagenomic next-generation sequencing in children with severe pneumonia

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Background: Pneumonia is one of the most important causes of morbidity and mortality in children. Identification and characterization of pathogens that cause infections are crucial for accurate treatment and accelerated recovery of the patients. However, in most cases the causative agent cannot be identified partly due to the limited spectrum covered by current diagnostics based on nucleic acid amplification. Therefore, in this study we explored the application of metagenomic next-generation sequencing (mNGS) for the diagnosis of children with severe pneumonia. Methods: From April to July 2017, 32 children were hospitalized with severe pneumonia in Shenzhen Children’s Hospital. Blood tests were conducted immediately after hospitalization to assess infection, oropharyngeal swabs were collected to identify common pathogens. After bronchoscopy, bronchoalveolar lavage fluids (BALFs) were collected for further pathogen identification using standardized laboratory and mNGS. Results: Blood tests were normal in 3 of the 32 children. In oropharyngeal swabs from 5 patients Mycoplasma pneumoniae by qPCR, 27 cases showed negative results for common pathogens. In BALFs we detected 6 cases with Mycoplasma pneumoniae with qPCR, 9 patients with adenovirus by using a Direct Immunofluorescence Assay (DFA) and 4 patients with bacterial infections, as determined by culture, In 3 of the cases a co-infection was detected. In 15 cases no common pathogens were found in BALF samples, using the current diagnostics, while in all the 32 BALFS pathogens were identified using mNGS, including adenovirus, Mycoplasma pneumoniae, Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, cytomegalovirus and bocavirus. Conclusions: mNGS can increase the sensitivity of detection of the causative pathogens in children with severe pneumonia. In addition, mNGS will give more strain specific information, will help to identify new pathogens and could potentially help to trace and control outbreaks. In this study we have shown that it is feasible to have the
results within 24 hours, making the application of mNGS feasible for clinical diagnostics.

Background

Pneumonia is one of the most important causes of morbidity and mortality in children [1]. A multitude of pathogens have been identified as potential causes making it challenging to determine the microbial etiology of pneumonia. Identification and characterization of micro-organisms that cause infections are crucial for targeted treatment to enable fast recovery of the patients. Culture-based techniques, nucleic acid amplification tests (NAATs) and immunological assays target only a fraction of the currently known pathogens [2]. In addition, culture-based tests require two days and even longer to identify the causative pathogen while immunological assays lack sensitivity and are therefore generally prone to false negative results. This often leads to empirical treatment solely based on clinical examination potentially resulting in misuse or overuse of antibiotics.

Metagenomic next-generation sequencing (mNGS) technologies have previously shown to be a promising for the identification of the causative in a given sample [3]. The advent of rapid and low-cost mNGS technologies has improved their applications from laboratory research to clinical diagnostics of pathogens [4-7]. mNGS has been mainly used to diagnose emerging pathogens and rare infectious diseases[8, 9]. In this study, we simultaneously used mNGS to detect pathogens in bronchoalveolar lavage fluid (BALF) and clinical examination to compare the differences in diagnostic outcome, to finally adjust the treatment option and observe the recovery of the patients.
Methods

Samples collection and information

From April to July 2017, the Department of Respiratory Diseases in Shenzhen Children’s Hospital received a cluster of pediatric patients with persistent fever, wheezing and coughing for at least 7 days. All patients were diagnosed with standardized radiography to measure abnormalities associated with pneumonia. Blood tests were conducted immediately after hospitalization to assess the total numbers of leukocytes, neutrophils and lymphocytes counts and to measure the concentration of C-reactive protein (CRP) and procalcitonin (PCT). Meanwhile, oropharyngeal swabs (155C, COPAN, Murrieta, California, USA) were collected to identify the common pathogens, as mentioned here below. Bronchoscopy was required for the disease condition of these patients, allowing the collection of BALF for further pathogen identification by mNGS analysis and for standardized assays to facilitate the clinical decision for treatment, as described here below. This study was approved by the Ethical Committee of Shenzhen Children’s Hospital with registration number 2016013. All parents and caregivers of included children provided informed consents.

BALF clinical diagnosis

D3 Ultra Direct Immunofluorescence Assay (DFA) Respiratory Virus Screening & ID Kit (Diagnostic hybirds, Inc, Athens, Ohio, USA) was employed to detect respiratory syncytial virus, adenovirus, influenza virus and parainfluenza virus. Rapid antigen detection was applied to identify H1N1 virus. *Mycoplasma pneumoniae* was diagnosed through Diagnostic Kit for *M. pneumoniae* DNA (PCR Fluoresence Probing) (DaAnGene, Guangzhou, China). Bacterial culturing was conducted to detect *Acinetobacter baumannii, Haemophilus influenzae, Haemophilus parainfluenzae, Moraxella catarrhalis, Staphylococcus aureus, Staphylococcus haemolyticus* and *Streptococcus pneumoniae*. 
Sequencing and data analysis

In mNGS analysis, DNA was extracted from BALF using the TIANamp Micro DNA Kit (DP316, Tiangen Biotech) in accordance with the manufacturer's standard protocols. Agilent 2100 was used for quality control of the DNA libraries, which were sequenced on BGISEQ-100 platform [10]. Processed by removing low-quality and short (length < 35 bp) reads, sequencing data then were aligned with the human reference genome (hg19) to remove human-derived sequences using Burrows-Wheeler Alignment. The remaining data were classified by simultaneously aligning to four Microbial Genome Databases, consisting of 2,686 viruses, 1,492 bacteria, 60 species of fungi, and 33 parasites[8]. If there were more than 50 unique reads found it was regarded as likely causative pathogen and further verified by Sanger sequencing.

Results

Clinical diagnosis and treatment

The 32 cases were diagnosed as severe pneumonia based on diffused lung consolidation, as well as atelectasis or pleural effusion. Of these patients, aged from 5 months to 8 years and 7 months, the median age was 21.5 months, 18 were males and 14 were females. Total leukocyte counts ranged from 2.34 - 21.9×10⁹/L, while total number lymphocyte and neutrophil counted were 0.58 - 10.6 and 1.17 - 22.1×10⁹/L, respectively. Normal values for total leukocytes, lymphocytes and neutrophils were found in 18, 20 and 23 cases, respectively (Table 1). The concentration of serum CRP was higher than 10.0mg/L in 18 cases, and for PCT higher than 0.5µg/L in 22 cases (Table 1). Empirical antibiotic treatment was applied in all cases to and bronchoscopy was used for diagnostic purposes. Steroid therapy was applied in 20 cases, based on patients' conditions.

Table 1. Results of laboratory tests on peripheral blood samples derived from 32 pediatric
pneumonia patients.

Pathogen detection using oropharyngeal swab and BALF samples

Routine laboratory tests showed negative results for common pathogens in oropharyngeal (OP) swabs in 27 hospitalized children with severe pneumonia, while 5 cases were positive for *Mycoplasma pneumoniae* by PCR. Routine laboratory tests on BALF found identical pathogens, of which in 6 cases *Mycoplasma pneumoniae* by PCR, of which 5 cases were also positive based on the OP samples, however, the load of *M. pneumoniae* was 100 times more than measured in OP samples. In 9 cases adenovirus was detected by DFA and in 5 cases bacterial pathogens were cultivated (2 cases with *S. pneumoniae*, 2 cases with *H. influenzae* and 1 with *M. catarrhalis*). In 3 cases co-infection was found, including 2 cases with adenovirus and *M. pneumoniae*, 1 case with adenovirus and *H. influenzae*. In 15 cases no detection of common pathogens was found in BALF samples (Table 2).

Table 2. Pathogens were detected in BALF in 32 cases by clinical diagnosis and mNGS.

mNGS results in BALF of children with severe pneumonia

Within 24 hours after collection of the BALF samples, we received the mNGS results, which indicated infection of adenovirus in 25 cases, *Mycoplasma pneumoniae* in 5 cases, *S. pneumoniae* in 2 cases, *H. influenzae*, *M. catarrhalis*, cytomegalovirus (CMV) or bocavirus each in 1 case. In 4 cases we found a co-infection including 3 cases co-infected with adenovirus and *M. pneumoniae*, 1 case with bocavirus and *H. influenzae* (Table 2 and 3). In particular, after receiving the patient result of CMV, we confirmed that the patient was indeed infected by CMV using real time PCR. Bocavirus has not be confirmed by qPCR.

Unique reads of mNGS used in distinguishing co-infection

In patients co-infected with multiple pathogens, we can distinguish the main pathogens according to the number of unique reads by mNGS, then provided reference for treatment. In this study, we identified 2 patients co-infected with adenovirus and *M. pneumoniae*
(adenovirus/M. pneumoniae unique reads 17653/146, 25230/65, respectively) and two other co-infection cases (adenovirus/M. pneumoniae unique reads 7339/7337, bocavirus/M. catarrhalis unireads 1716/810) (Table 3).

Table 3. Pathogen detection based on >50 unique reads of mNGS in 4 co-infection cases. 

Depth: the ratio of the total amount of the microbial base detected to the detected length of the microbial genome sequence, one of the indicators for evaluating the amount of sequencing.

Discussion

In this study, we present a proof of principle study using clinical samples obtained from pediatric patients with severe pneumonia.

Limitations of the current diagnostic practice is the delay in results to be able to take the right medical decision [11]. In addition, there is high proportion of pneumonia cases with unknown microbial etiology [12], leading to an increase in mortality and to empirical treatment with antibiotics. Using mNGS directly on clinical samples broadens the range of pathogen detection. In this study, we have shown the feasibility of a turnaround of 24 hours, which enables re-direction of the initial treatment, potentially accelerating the recovery and improving the outcome of disease.

With standard molecular diagnostics, M. pneumoniae was detected in BALFs samples of 6 patients by qPCR. This was confirmed in 5/6 samples by mNGS. Thus, sensitivity of the mNGS approach was at least similar to the sensitivity of the diagnostic PCR in this set of samples. The sensitivity of mNGS was higher than that of DFA used for the detection of adenovirus as only 9 were detected with DFA while 25 were found with mNGS. Furthermore, culture and mNGS were equally sensitive, as only one case of H. influenzae was missed by mNGS. In addition, other pathogens were detected that were not found by
standard diagnostic tests, such as CMV and bocavirus. Most striking were the co-infections found in 4 of the 32 patients, in which the added value of mNGS became most clear as by using only one method will simplify and standardize the interpretation of results.

The costs of mNGS might still be an obstacle for most hospitals to implement mNGS for routine diagnosis. In additions, efforts and investments are needed to further standardize and validate this method especially with respect to sample preparation and data processing. Nevertheless, implementation of mNGS in clinical diagnostics is promising and should be embraced by clinicians and training physicians as it enables fast identification of pathogens in severe pneumonia cases and strongly supports the improvement of clinical intervention.

Conclusions

mNGS can increase the sensitivity of detection of the causative pathogens in children with severe pneumonia. In addition, mNGS will give more strain specific information, will help to identify new pathogens and could potentially help to trace and control outbreaks.

List Of Abbreviations

mNGS : metagenomic next-generation sequencing
DFA: direct Immunofluorescence Assay
BALF: bronchoalveolar lavage fluid
CRP: C-reactive protein
PCT: procalcitonin

Declarations

Acknowledgements

We thank Zhengfang Yue from BGI to provide the support of sequencing platform.

Ethics approval and consent to participate
This study was approved by the Ethical Committee of Shenzhen Children’s Hospital with registration number 2016013. All parents and caregivers of included children provided informed consents.

Consent for publication
Not applicable.

Availability of data and material
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

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Authors’ contributions
HP-W: study design, completion of lab protocols, analysis of results, manuscript preparation. ZW-L and YM-B: recruitment of participants, critical review of the manuscript. YH-Y and RG: critical review of the manuscript. WK-D: data analysis and critical review of the manuscript. MIJ and YJ-Z: study design, analysis of results, manuscript preparation. All authors read and approved the final manuscript.

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Tables

|                             | Normal (5.0 - 12.0 × 10⁹/L) | High (>12.0 × 10⁹/L) | Low (<5.0 × 10⁹/L) |
|-----------------------------|-----------------------------|----------------------|---------------------|
| Total leukocyte count       |                             |                      |                     |
| Lymphocytes                 | 18                          | 7                    | 7                   |
| Neutrophils                 | 23                          | 5                    | 4                   |
|                             | Moderate (<10 mg/L)         |                      | No                  |
| Neutrophils                 |                             |                      |                     |
| CRP                         | 14                          | 18                   | 0                   |
|                             | Moderate (<0,5μg/L)         |                      | No                  |
| CRP                         | 10                          | 22                   | 0                   |

Table 1. Results of laboratory tests on peripheral blood samples derived from 32 pediatric pneumonia patients.

|                                    | DFA | PCR | Culture | mNGS |
|------------------------------------|-----|-----|---------|------|
| Adenovirus                         | 9   | n.d.| n.d.    | 25   |
| *Mycoplasma pneumoniae*            | n.d.| 6   | n.d.    | 5    |
| *Streptococcus pneumoniae*         | n.d.| n.d.| 2       | 2    |
| *Haemophilus influenzae*           | n.d.| n.d.| 2       | 1    |
| *Moraxella catarrhalis*            | n.d.| n.d.| 1       | 1    |
| Cytomegalovirus                    | n.d.| n.d.| n.d.    | 1    |
| Bocavirus                          | n.d.| n.d.| n.d.    | 1    |
| Co-infection                       | 3   | 2   | 1       | 4    |

Table 2. Pathogens were detected in BALF in 32 cases by clinical diagnosis and mNGS.
| Patient ID | Depth  | Pathogens                   | Unique reads |
|------------|--------|-----------------------------|--------------|
|            | 69.87  | Human adenovirus B          | 17653        |
|            | 1      | *Mycoplasma pneumoniae*    | 146          |
|            | 14.97  | Human adenovirus E          | 105          |
| Case 1     | 105.44 | Human adenovirus B          | 25230        |
|            | 20.61  | Human adenovirus E          | 152          |
|            | 1      | *Mycoplasma pneumoniae*    | 65           |
| Case 2     | 27     | Human adenovirus B          | 7339         |
|            | 1.9    | *Mycoplasma pneumoniae*    | 7337         |
| Case 3     | 43.54  | Human bocavirus             | 1716         |
|            | 1      | *Moraxella catarrhalis*    | 810          |

**Table 3.** Pathogen detection based on >50 unique reads of mNGS in 4 co-infection cases.

Depth: the ratio of the total amount of the microbial base detected to the detected length of the microbial genome sequence, one of the indicators for evaluating the amount of sequencing.