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ORIGINAL RESEARCH

Pathogen Diagnosis Value of Nanopore Sequencing in Severe Hospital-Acquired Pneumonia Patients

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Background: Next-generation sequencing of the metagenome (mNGS) is increasingly used in pathogen diagnosis for infectious diseases due to its short detection time. The time for Oxford Nanopore Technologies (ONT) sequencing-based etiology detection is further shortened compared with that of mNGS, but only a few studies have verified the time advantage and accuracy of ONT sequencing for etiology diagnosis. In 2022, a study confirmed that there was no significant difference in sensitivity and specificity between ONT and mNGS in suspected community-acquired pneumonia patients, which there was no clinical study verified in patients with SHAP.

Methods: From October 24 to November 20, 2022, 10 patients with severe hospital-acquired pneumonia (SHAP) in the Nanfang Hospital intensive care unit (ICU) were prospectively enrolled. Bronchoalveolar lavage fluid (BALF) was collected for ONT sequencing, mNGS, and traditional culture. The differences in pathogen detection time and diagnostic agreement among ONT sequencing, mNGS, traditional culture method, and clinical composite diagnosis were compared.

Results: Compared with mNGS and the traditional culture method, ONT sequencing had a significant advantage in pathogen detection time (9.6±0.7 h versus 24.7±2.7 h versus 132±58 h, P <0.05). The agreement rate between ONT sequencing and the clinical composite diagnosis was 73.3% (kappa value=0.737, P <0.05).

Conclusion: ONT sequencing has a potential advantage for rapidly identifying pathogens.

Keywords: Oxford Nanopore Technologies, Nanopore sequencing, metagenomic next-generation sequencing, severe hospital-acquired pneumonia, pathogen

Introduction

Severe hospital-acquired pneumonia (SHAP) is the most common acquired infection in the intensive care unit (ICU) and one of the main causes of increased mortality in ICU patients.[1,2] Potential multidrug-resistant pathogens are very common in ICU-acquired pneumonia, including *Pseudomonas aeruginosa, Acinetobacter* spp., methicillin-resistant *Staphylococcus aureus* (MRSA), and ultrabroad spectrum β-lactamase (ESBL)-producing and carbapenem-resistant Enterobacteriaceae (CRE).[3] Timely and appropriate antibiotic treatment is effective in reducing patient mortality, but it often takes up to 48 hours from respiratory sampling to the acquisition of definitive microbiological test results, which leads to untimely antibiotic therapy and increased mortality.[3] Therefore, early identification of the pathogen and timely use of targeted antibiotics is particularly important to improve prognosis.

The traditional culture of bronchoscopy specimens remains the gold standard of pathogenic detection. However, the turnaround time of bacterial/fungal cultures is long (3–7 days) in conventional microbiological testing.[4] The low positive

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rates cannot meet the need for early pathogen diagnosis.[5] mNGS, known as a powerful, low-cost, shorter turn round-time method, has been used in pathogen diagnosis in recent years. The turnaround time of mNGS is usually 24–36 hours, and the positive rate is 15% higher than that of culture.[6] Several studies have shown that the diagnostic accuracy of mNGS was not inferior to traditional culture (61.7% vs 76.7%, P=0.11), and the 28-day mortality of the mNGS group was significantly lower than culture group (47.32% vs 62.2%, P=0.043) due to its shorter pathogen detection time.[7,8] Nevertheless, each step of mNGS requires sequence restriction to short 200–500 bp read lengths, and these shorter read lengths make genome, transcriptome, and metagenome assembly more challenging.[9]

Third-generation sequencing, also known as Nanopore sequencing, uses a long-read length assay to compensate for the shortcomings of mNGS.[10] The turnaround time of Nanopore sequencing is only 6–9 hours, which is much shorter than that of mNGS. However, it is not widely used in the clinic because of its high error rate, which is close to 30%.[11] In recent years, research has shown that removing human background gene interference significantly improves the pathogenic detection accuracy of Nanopore sequencing, which exhibits striking agreement with culture and mNGS.[12] The study has shown that there was no significant difference in the consistency between ONT sequencing and mNGS with clinical diagnosis (59.38% vs 57.81%, P>0.05) in suspected community-acquired pneumonia patients, and the detection time of ONT sequencing was significantly shorter than mNGS.[13] However, fewer clinical studies have yet validated the consistency between clinical composite diagnosis with Nanopore sequencing with human background genes removed for pathogen detection in bronchoalveolar lavage fluid (BALF) obtained from patients with SHAP. In this study, we proposed to evaluate the value of pathogen diagnosis based on BALF Nanopore sequencing application in adults with SHAP.

Materials and Methods

Study Participants and Study Design

A prospective cohort study was conducted on patients with severe hospital-acquired pneumonia admitted to the intensive care unit of Nanfang Hospital from October 24, 2022, to November 20, 2022. Both Oxford Nanopore Technologies (ONT) sequencing and mNGS were used to detect pathogens in BALF. Hospital-acquired pneumonia (HAP) was defined as pneumonia that manifested 48 h after hospital admission and encompasses two entities: ventilator-associated pneumonia (VAP) and severe pneumonia that developed in the hospital.[14] SHAP was defined for patients who met one major or at least three minor Infectious Diseases Society of America (IDSA)/American Thoracic Society (ATS) criteria based on HAP.[15] Patients were excluded if they left the ICU within 48 hours or if bronchoalveolar lavage was contraindicated. This study was approved by the Medical Ethics Committee of Nanfang Hospital (NFEC-2022-399), and informed consent was obtained from each patient or guardian. The study was performed in line with the Declaration Helsinki.

In our study, we compared the mNGS, ONT sequencing results for BALF from patients who had a matched BALF culture result for bacteria and fungi. Due to the absence of an available conventional test, we did not compare the numbers of sequences in the mNGS and ONT sequencing results for BALF with respiratory viral tests for viruses.

DNA Extraction

Five milliliters of BALF were collected, 600 μL was added to 250 μL of 0.5 mm glass beads for physical degradation, followed by 7.2 μL of lyticase lysis enzyme (RT410-TA, Tiangen Biotech, Beijing, China) for the enzyme degradation reaction, mixed and shaken, and centrifuge at 8000 g for 5 min. For mNGS, 300 μL of the sample was extracted according to the TIANamp Micro DNA Kit (DP316, Tiangen Biotech, Beijing, China) to extract DNA. As for ONT sequencing, we applied a human DNA removal step in BALF samples to improve the correct rate, as recently described.[16] And we extracted the genomic DNA with MagNA Pure compact (Roche Diagnostics GmbH, Germany).

Library Preparation and Sequencing

Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific Inc.) was used to construct mNGS libraries and the MGISEQ-2000 platform (BGI-Tianjin, Tianjin, China) was used for sequencing. The Rapid Barcoding kit SQK-RBK004 (Oxford

Nanopore) was used to construct ONT sequencing libraries and MinION flowcells (Oxford Nanopore) were used for sequencing.

Bioinformatics Analyses

High-quality data were obtained by removing low-quality data and short (length < 35bp) reads. The high-quality data were filtered by BWA (BWA: http://bio-bwa.sourceforge.net/) to match the human reference genome to exclude human sequences data.[17] The remaining data were compared with the BGI-PMDB pathogen database (including 6350 bacteria, 1064 fungi, 4945 viruses, and 234 parasites) after removing low-complexity reads to obtain the number of sequences that could match a certain pathogen.

Identification of Pathogens

Culture-Identified Pathogens

(i) Bacteria: A bacterium concentration more e than 10^4 CFU/mL was regarded as a positive criterion. (ii) Fungi and parasites: Positive BALF culture and smear of fungi and parasites were used to determine positivity. (iii) Tuberculosis: Tuberculosis (TB) pneumonia was diagnosed by sputum smear exams for Mycobacterium tuberculosis and/or TB culture.

Positive mNGS/ONT Criteria

Due to the lack of criteria for the interpretation of mNGS results, we used the developed criteria from previous study.[18] The infectious bacteria (excluding mycobacteria), fungi, and parasites were considered positive if they met any of the following standards of the mNGS/ONT sequencing: (i) if mNGS/ONT detected the same pathogen as culture with the number of unique reads from a single species exceeded 50; (ii) if the unique reads of pathogen less than 50, the diagnosis of infection can still be made based on the clinical manifestations; (iii) mycobacterium tuberculosis: at least one read was identified to a species, and TB was considered positive; (iv) if mNGS/ONT detected the pathogen with the number of unique reads exceeded 50 while culture missed this pathogen, it was considered potential pathogenic microbe.

Furthermore, mNGS/ONT sequencing results could not be used to determine if microorganisms were infected, colonized, or contaminated. After receiving the mNGS/ONT sequencing results, two physicians analyzed the clinical features to reach a consensus.

Clinical Data Collection

Data were obtained from hospital records, including demographic, laboratory data, APACHE II scores, and SOFA scores. Data on pathogen species and unique reads were collected from reports from the sequencing company.

Statistical Analysis

The one-way analysis of variance (ANOVA) was used to compare the turnaround time of three pathogen-testing methods. The kappa value measured the agreement between the results of mNGS and ONT tests. According to the research of Landis and Koch[19] the importance of consistency was considered as follows: a kappa value of 0.8–1 denoted close to a perfect consistency; 0.6–0.8 denoted significant consistency; 0.4–0.6 denoted moderate consistency, and less than 0.4 denoted low consistency. SPSS 26.0 software was utilized for data analysis. *P* values less than 0.05 were deemed significant.

Results

Sample and Patient Characteristics

10 patients diagnosed with critical hospital-acquired pneumonia with a median age of 63 years were included in this study with the majority of patients being male (n=7, 70%). Laboratory findings and disease severity are shown in Table 1. Among the underlying diseases, hypertension (n=3, 30%), cerebral infarction (n=3, 30%), and cardiovascular disease (n=2, 20%) were the most prevalent (Table 2).

Table 1 Clinical and Laboratory Characteristics of 10 Patients with SHAP

Characteristics	Patients
Male, n (%)	7 (70)
Age (yr)	63 (52, 81)
Vital signs	
HR (time/min)	105 (69, 116)
SBP (mmHg)	119±4.83
DBP (mmHg)	64.6±4.04
MAP (mmHg)	81.3±4.22
RR (time/min)	15 (13, 19)
T (°C)	37.34±0.34
OI (mmHg)	214.5±31.41
Laboratory findings	
WBC (×10^9/L)	16.93±3.08
HGB (g/L)	78.4±8.09
PLT (×10^9/L)	109.9±30.52
CRP (mg/L)	51.18 (36.36, 86.59)
PCT (ug/L)	4.93 (1, 17.53)
IL-6 (pg/mL)	157.95 (29.41, 286.85)
ALT (U/L)	65 (6.5, 344.75)
AST (U/L)	155.5 (24, 335.5)
ALB (g/L)	32.48±0.73
TBIL (μmol/L)	22.95 (12.85, 107.73)
DBIL (μmol/L)	18.05 (7.73, 76.8)
CR (μmol/L)	154.3±31.95
APTT (s)	38.02±3.91
PT (s)	17.21±1.95
TT (s)	18.45±0.82
INR	1.37 (1.08, 1.78)
Fbg (g/L)	2.14 (1.80, 4.46)
LAC (mmol/L)	2.76±0.66
Disease severity	
APACHE II	27 (21, 31)
Modified mortality (%)	57.36±6.97
SOFA	15 (11, 17)

Note: Data are the number (%) of patients, median (interquartile range), or mean ± standard deviation.

Abbreviations: HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; RR, respiratory rate; WBC, white blood cell count; HGB, hemoglobin; PLT, platelets; CRP, C-reactive protein; PCT, procalcitonin; IL-6, interleukin 6; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALB, albumin; TBIL, total bilirubin; DBIL, direct bilirubin; CR, creatinine; APTT, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time; INR, international normalized ratio; Fbg, plasma fibrinogen; LAC, lactic acid; APACHE, Acute Physiology And Chronic Health Evaluation scoring system; SOFA, Sequential Organ Failure Assessment; OI, oxygenation index.

Identification of Pathogens by Traditional Culture Methods, mNGS, and ONT Sequencing

In terms of the type of pathogens detected, the ONT sequencing and clinical composite diagnosis displayed consistency in 9 (90%) cases, including 7 diagnostically positive cases versus 2 diagnostically negative cases. The ONT sequencing and mNGS results were consistent in 8 (80%) cases, including 6 mNGS-positive and 2 mNGS-negative cases. And a total of 50 bacteria, 16 fungi, and 2 parasites were identified in 30 samples that 36 bacteria, 5 fungi, and 2 parasites were the true-positive pathogenic microbes (Figure 1A). 11 pathogens were identified by culture method, and 8 pathogens were considered the true positive. The most frequently detected bacteria were *Pseudomonas aeruginosa* (two cases), *Acinetobacter baumannii* (two cases) (Figure 1B). A total of 18 pathogens

Table 2 Characteristics and Detection Results of Patients

Patient ID	Sex	Age (Yr)	Underlying Disease(s)	Detection Results for:		
				Culture Detection	mNGS Test	ONT Test
P1	Male	57	Acute coronary syndrome, brain atrophy	Pae	Pae/Spn/Sma/CV/HHV-7	Pae/Aba/Sma/Kpn
P2	Male	53	Cerebral infarction, Hypertension	Ctr	Ctr/Cal	Ctr
P3	Female	50	Diabetes, Pemphigus	Aba/Smear: Parasite	Aba/Sst/HHV-1/HCMV	Aba/Lpn/Sau/Pmi/HHV-1
P4	Male	81	Cerebral infarction	Negative	Negative	San/Cal/Efa/Sau/Sma
P5	Male	67	Hypertension	Negative	Smi	Pji/Lpn/EBV
P6	Male	81	Alzheimer's, Cerebral infarction, Hypertension	Pae/Kpn/CRE	Kpn/Pae	Kpn/Kva/Eho/Ecl/Ave/Pae/Sau/Lad/Aba/Pmi/HHV-4
P7	Female	77	Coronary arteriosclerosis	Negative		Sor
P8	Male	46	None	Negative	Afu/HHV-1	Afu/HHV-1
P9	Male	89	None	Aba/Cal/Sce	Aba/Lrh/Efa/Cal	Aba/Efa/Aca/Kpn/Pae/Sau/Cal
P10	Female	59	Glaucoma	Cgl	Cgl/Cal/Efa/EBV/HCMV	Cgl/Cal/Efa/HHV-4/HHV-1/HHV-5

Abbreviations: Pae, Pseudomonas aeruginosa; Spn, Streptococcus pneumoniae; Sma, Stenotrophomonas maltophilia; Aba, Acinetobacter baumannii; Kpn, Klebsiella pneumoniae; Lpn, Legionella pneumophila; Sau, Staphylococcus aureus; Pmi, proteus mirabilis; San, Streptococcus anginosus; Efa, Enterococcus faecalium; Smi, Streptococcus mitis; Pji, Pneumocystis jirovecii; Kva, Klebsiella variicola; Eho, Enterobacter hormaechei; Ecl, Enterobacter cloacae; Ave Aeromonas veronii; Ahy, Aeromonas hydrophila; Lad, leclercia adecarboxylata; Sor, Streptococcus oralis; Lrh, Lactobacillus rhamnosus; Aca, Aeromonas caviae; Sst, Strongyloides steroralis; Ctr, Candida tropicalis; Cal, Candida albicans; Afu, Aspergillus fumigatus; Pku, Pichia kudriavzevii; Cgl, Candida glabrata; Sce, Saccharomyces cerevisiae; HCMV, Human cytomegalovirus; CV, Circovirus; HHV, human herpesvir.

were identified by mNGS in 10 samples, while 12 pathogens were true-positive microbes. The most commonly detected bacteria were *Acinetobacter baumannii* (two cases), *Pseudomonas aeruginosa* (two cases), and *Enterococcus faecalium* (two cases) (Figure 1C). The samples were also sequenced by ONT. A total of 39 pathogens were identified while 16 strains (41%) were false-positive pathogenic microbes. And the most frequently bacteria were *Acinetobacter baumannii* (three cases), *Pseudomonas aeruginosa* (three cases), and *Klebsiella pneumonia* (three cases) (Figure 1D). Notably, *Strongyloides steroralis* was detected in 1 case by mNGS and traditional culture, but it was not discovered by ONT sequencing. Overall, ONT sequencing identified more potential kinds of pathogens than mNGS. The detailed pathogen results of ONT sequencing, mNGS, and culture for each patient are shown in Supplementary Materials.

Detection Time

Among the 10 cases in this study, the overall average time for the ONT sequencing assay was 9.6±0.7 h, which was significantly shorter than that for mNGS (24.7±2.7 h, $P<0.05$) and traditional culture (132±58 h, $P<0.05$).

Diagnostic Performance

The diagnostic positivity rates for ONT sequencing, mNGS, and traditional culture were 80%, 60%, and 60%, respectively. Take clinical composite diagnosis as the reference standard, consistent with previous studies, mNGS had good agreement with the final diagnosis (Kappa value=0.783, $p<0.05$). ONT sequencing also had good agreement with the final diagnosis (Kappa value=0.737, $p<0.05$). However, the agreement rate between traditional culture and the final diagnosis was notably only 34.8% in the 10 samples, which was not statistically significant (Kappa value=0.348, $P>0.05$) (Table 3).

"False Positive" and "False Negative" of ONT Sequencing and mNGS

"False Positive" of ONT Sequencing

In the 7 clinical composite diagnosed positive cases, there were no "False negative" cases for ONT sequencing which identified all pathogenic microbes and were consistent with the final diagnosis. For the "ONT false positive" cases, possible reasons included colonization (2/4), and potential causes of infection (2/4). In 12 pathogens that did not meet positive criteria, only 2 microbes were considered potential causes of infection, which was shown in Table 4.

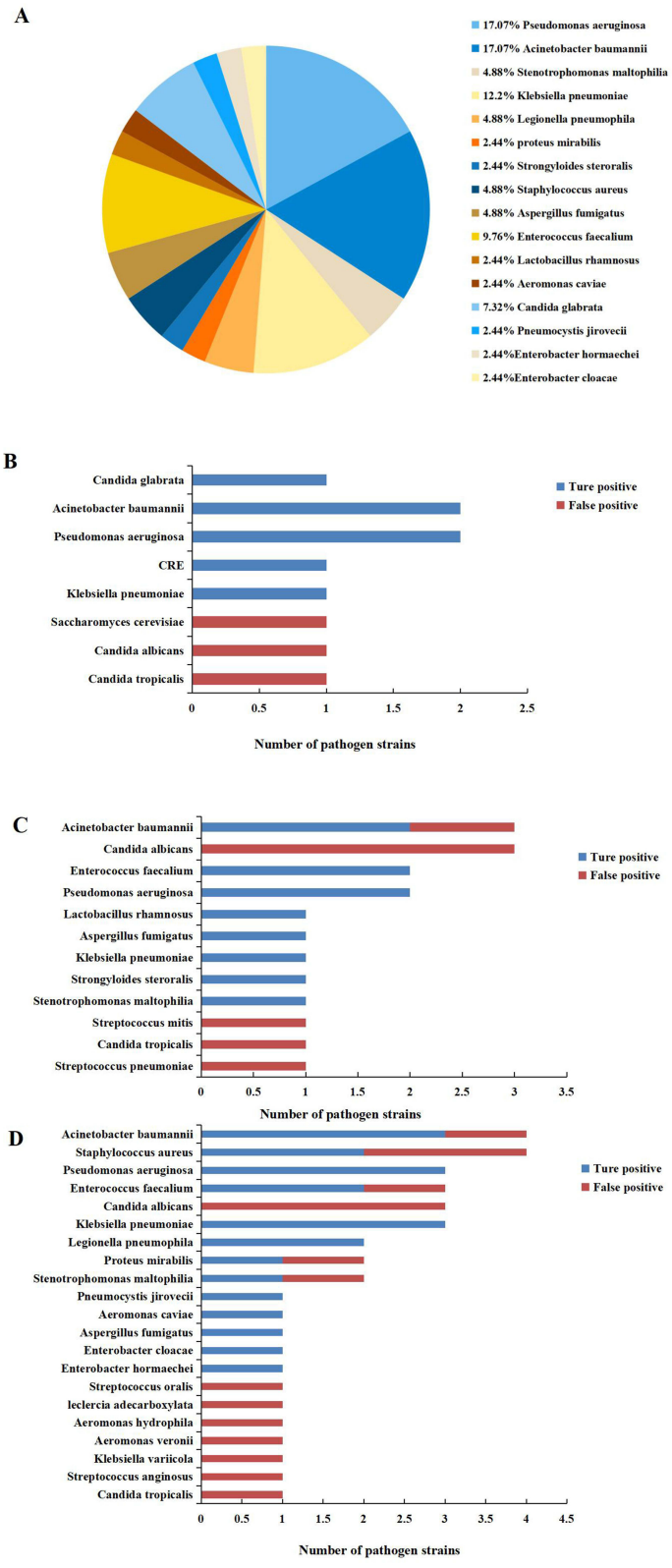

Figure 1 Identification of pathogenic microbes by traditional culture, mNGS, and ONT sequencing. (**A**) The pathogen distribution of 43 true-positive microbes. (**B**) Histogram of traditional culture method to detect pathogenic microbes. (**C**) Histogram of mNGS to detect pathogenic microbes. (**D**) Histogram of ONT sequencing detection for pathogenic microbes.

Table 3 Diagnostic Performance of ONT Sequencing, mNGS, and Traditional Culture

ONT	Clinical Composite Diagnosis		Total
	+	–	
+	7	1	8
–	0	2	2
Total	7	3	10
Kappa value=0.737, $P<0.05$			
mNGS	**Clinical Composite Diagnosis**		**Total**
	+	–	
+	6	0	6
–	1	3	4
Total	7	3	10
Kappa value=0.783, $P<0.05$			
Culture	**Clinical Composite Diagnosis**		**Total**
	+	–	
+	5	1	6
–	2	2	4
Total	7	3	10
Kappa value=0.348, $P>0.05$			

Notes: a kappa value of 0.8–1 denoted close to a perfect consistency; 0.6–0.8 denoted significant consistency; 0.4–0.6 denoted moderate consistency, and less than 0.4 denoted low consistency.

"False Positive" and "False Negative" of mNGS

In the clinical composite diagnosed positive cases, up to 11 pathogens were missed by mNGS, and were unidentifiable by mNGS. As for the "false positive" cases of mNGS, the most commonly reasons was potential causes of infection (2/3) (Table 5).

Table 4 Analysis of "False Positive"[a] Results of ONT Sequencing

Pathogens Detected Only by ONT (Unique Reads>50)				
Patient ID	Diagnosis	ONT Results	Unique Reads	Possible Explanation
P4	AMI	Streptococcus anginosus	99	Likely colonization
		Candida albicans	207	Likely colonization
P6	Septic shock	Klebsiella variicola	560	Potential cause of infection
P10	DLBCL	Candida albicans	632	Potential cause of infection
Pathogens Detected Only by ONT (Unique Reads<50)				
Patient ID	Diagnosis	ONT Results	Unique Reads	Possible Explanation
P2	STMI	Candida tropicalis	3	Likely colonization
P3	Septic shock	Staphylococcus aureus	2	Potential cause of infection
P4	AMI	Enterococcus faecalium	3	Likely colonization
		Staphylococcus aureus	1	Likely colonization

(*Continued*)

Table 4 (Continued).

		Stenotrophomonas maltophilia	1	Likely colonization
P6	Septic shock	Aeromonas veronii	24	Likely colonization
		Aeromonas hydrophila	11	Likely colonization
		Leclercia adecarboxylata	5	Potential cause of infection
		Staphylococcus aureus	5	Likely colonization
		Proteus mirabilis	4	Likely colonization
P7	Cardiogenic shock	Proteus mirabilis	4	Likely contamination
P9	Cerebral hemorrhage	Candida albicans	4	Likely colonization

Note: [a]"False positive" occurred if ONT sequencing identified clinical composite diagnosed negative microbes.
Abbreviations: STMI, ST elevation myocardial infarction; AMI, Acute myocardial infarction; DLBCL, Diffuse large B cell lymphoma.

Table 5 Analysis of "False Negative"[b] Results of mNGS

Pathogens Detected Only by mNGS				
Patient ID	Diagnosis	mNGS Results	Unique Reads	Possible Explanation
P10	DLBCL	Candida albicans	5543	Potential cause of infection
P1	ALL (bone marrow transplant)	Streptococcus pneumoniae	1795	Potential cause of infection
P9	Cerebral hemorrhage	Candida albicans	54	Likely colonization
P2	STMI	Candida tropicalis	47	Likely colonization
		Candida albicans	25	Likely colonization
P5	Respiratory failure	Streptococcus mitis	15	Potential cause of infection
Clinical composite diagnosed positive microbes missed by mNGS				
Patient ID	Diagnosis	Missed microbes		
P1	ALL (bone marrow transplant)	Staphylococcus aureus/Klebsiella pneumoniae		
P3	Septic shock	Legionella pneumophila/Proteus mirabilis		
P5	Respiratory failure	Pneumocystis jirovecii/ Legionella pneumophila		
P6	Septic shock	CRE/Staphylococcus aureus		
P9	Cerebral hemorrhage	Klebsiella pneumoniae/ Staphylococcus aureus/ Pseudomonas aeruginosa		

Note: [b]"False negative" occurred if clinical composite diagnosed positive microbes missed by mNGS.
Abbreviations: ALL, Acute lymphocyte leukemia; STMI, ST elevation myocardial infarction; CRE, arbapenem - resistant Enterobacteriaceae.

Discussion

Oxford Nanopore Technologies (ONT) was prospective to be a viable method out of its broad breadth, long reading sequencing, and real-time production.[20] However, the interference of human DNA limited the application of ONT sequencing in clinical practice.[16] This prospective self-controlled study explored the consistency of pathogen diagnosis in SHAP patients by ONT sequencing which depleted human DNA before sequencing. In this study, we showed that ONT sequencing may be a promising method to detect pathogens in SHAP patients. Future large-sample clinical studies can further verify the sensitivity and specificity of this method and explore whether it can guide personalized antibiotic therapy to improve prognosis.

Previous studies had already demonstrated that the mNGS can improve the prognosis by guiding targeted antibiotic treatment with high sensitivity and specificity.[7,8] However, fewer clinical studies have verified the diagnostic accuracy and detection time of ONT sequencing.[13] And there was no clinical study exploring the consistency between ONT sequencing with the clinical diagnosis in patients with SHAP.

In this study, we confirmed the superior time requirements and result agreement of ONT sequencing compared with mNGS and traditional microbiological methods in pathogen diagnosis in patients with SHAP. First, the mNGS and ONT sequencing tests had a higher positive rate than traditional methods, which was consistent with earlier researches.[21]

Charalampous, Kay, Richardson, Aydin, Baldan, Jeanes, Rae, Grundy, Turner, Wain, Leggett, Livermore and O'Grady[16] found that the sensitivity of ONT sequencing with human DNA depletion is as high as 96.6% in lower respiratory tract infections which was consistent with our study. The kappa value between ONT sequencing and clinical composite diagnosis was high in SHAP patients (kappa value=0.737, indicating substantial agreement). However, the kappa value between ONT sequencing and mNGS was low (kappa value=0.545, P >0.05), which was not statistically significant. Possible reasons for the lack of concordance are as follows. First, since the study's limited sample size, inconsistent results in individual cases may cause significant bias. In 2 cases where the results of ONT sequencing were different with mNGS, one case was the false positive of ONT which was considered a colonization pathogen. Another case was the false negative of mNGS, *Pneumocystis jirovecii*, and *Legionella pneumophila* were not detected; the second possibility is that more potential pathogens are detected by ONT sequencing.[22]

Second, according to the HAP/VAP treatment guidelines, the patients strongly suspected of VAP who accepted the early antibiotic treatment had lower mortality, compared with inadequate therapy (38% versus 91%).[1] A prospective cohort study also indicated that the patients who accepted inappropriate antibiotic treatment had a higher mortality rate than those who accepted correct therapy.[23] Therefore, early adequate and appropriate antibiotic therapy is extremely important in the treatment of HAP, which means that rapid pathogen diagnosis tests, such as mNGS and ONT sequencing, are helpful to guide targeted antibiotic treatment due to their high sensitivity.

In this study, consistent with a previous study,[13] ONT sequencing had a shorter turnaround time than mNGS (9.6±0.7 h versus 24.7±2.7 h, P<0.05) and traditional culture methods (9.6±0.7 h versus 132±58 h, P <0.05), which means that pathogens can be identified as early as possible to guide personalized antibiotic therapy.[24]

In addition, more types of species were detected by ONT sequencing than mNGS, especially among bacteria. ONT sequencing applied the enrichment method to improve the detection rate of pathogens, while mNGS was performed directly on extracted nucleic acids. This may be a reason for the differences in microbial detection between the two methods. In this study, the most frequent pathogens were *P. aeruginosa, A. baumannii, K. pneumonia*, and fungi, which is in agreement with former reports.[25] The most frequently detected viruses included Epstein-Barr virus (EBV), cytomegalovirus (CMV), and human herpesvirus 1, 4, 5, and 7, which were considered to have no pathogenic significance when detected in BALF in most studies.[26,27]

According to our data, ONT sequencing with the removal of human DNA is a powerful method for microbiological diagnosis. As such, ONT sequencing has potential clinical application value. Large-sample clinical research is required to validate the sensitivity and specificity of ONT sequencing in microbiological detection and determine whether it can guide targeted therapy and reduce clinical antibiotic overuse.

However, there are still some disadvantages of ONT sequencing. The fecal round nematode was identified by mNGS which was considered a pathogenic microbe, while ONT sequencing did not detect this pathogen. There have been no clinical studies investigating the accuracy of ONT sequencing for parasites. The reasons we considered are the following. (i) During the process of removing human DNA, most nucleic acids of the fecal round nematode were removed, which led to failure in the amplification and library preparation process. (ii) Alternatively, the database for analysis was not comprehensive and did not include the sequence of the fecal round nematode. On the other hand, one case showed a positive ONT sequencing result but negative culture and mNGS results (*Streptococcus pyogenes* and *C. albicans*), which may be a false-positive ONT sequencing result due to the high content of oropharyngeal pathogens.[28]

Our research also has some shortcomings. First, our study was conducted in a single location with a limited sample size, there existed selection bias. Second, as a self-controlled study, we divided the bronchoalveolar lavage fluid of one patient into three-part to send for ONT sequencing, mNGS, and culture at the same time. So we were unable to compare the difference in change of antibiotic treatment between ONT sequencing and mNGS, which was to be carried out by a large sample cohort/case-control study. Third, we did not conduct the real-time ONT sequencing analysis because our objective was to verify its turnaround time and accuracy. In addition, the antibiotic resistance gene sequencing outcomes were not analyzed due to the small sample size of this study. To investigate further, we are conducting a prospective cohort study.

Conclusion

Our results demonstrated that ONT sequencing had a substantial agreement with clinical composite diagnosis in pathogen diagnosis. With shorter turn-round times of 8–10 h, ONT sequencing may play an important role in the creation of quick and accurate SHAP pathogen diagnostic tools. Further investigations are required to determine the direct influence on antibiotic options and prognosis.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

All authors declare that they have no potential conflicts of interest in this work.

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