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ORIGINAL RESEARCH

Diagnostic Value of Metagenomic Next-Generation Sequencing for Multi-Pathogenic Pneumonia in HIV-Infected Patients

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Background: To evaluate the value and challenges of real-world clinical application of metagenomic next-generation sequencing (mNGS) for bronchoalveolar lavage fluid (BALF) in HIV-infected patients with suspected multi-pathogenic pneumonia.

Methods: Fifty-seven HIV-infected patients with suspected mixed pneumonia who were agreed to undergo the bronchoscopy were recruited and retrospectively reviewed the results of mNGS and conventional microbiological tests (CMTs) of BALF from July 2020 to June 2022.

Results: 54 patients were diagnosed with pneumonia including 49 patients with definite pathogens and five patients with probable pathogens. mNGS exhibited a higher diagnostic accuracy for fungal detection than CMTs in HIV-infected patients with suspected pulmonary infection. The sensitivity of mNGS in diagnosis of pneumonia in HIV-infected patients was much higher than that of CMTs (79.6% vs 61.1%; $P < 0.05$). Patients with mixed infection had lower CD4 T-cell count and higher symptom duration before admitting to the hospital than those with single infection. The detection rate of mNGS for mixed infection was significantly higher than that of CMTs and more co-pathogens could be identified by mNGS. The most common pattern of mixed infection observed was fungi-virus (11/29, 37.9%), followed by fungi-virus-bacteria (6/29, 20.7%) coinfection in HIV-infected patients with multi-pathogenic pneumonia.

Conclusion: mNGS improved the pathogens detection rate and exhibited advantages in identifying multi-pathogenic pneumonia in HIV-infected patients. Early performance of bronchoscopy and mNGS are recommended in HIV-infected patients with low CD4 T cell counts and long duration of symptoms. The most common pattern of mixed infection observed was fungi-virus, followed by fungi-virus-bacteria coinfection in HIV infected patients with multi-pathogenic pneumonia.

Keywords: HIV, multi-pathogenic pneumonia, metagenomic next-generation sequencing, conventional microbiological tests, bronchoalveolar lavage fluid

Introduction

Pulmonary infection is the most common cause of morbidity and mortality in immunocompromised patients.[1] The pathogens spectrum of pneumonia that can affect patients with human immunodeficiency virus (HIV) is wide, such as bacterial, fungal, viral, parasitic organisms, and so on.[2–4] Compared to patients with monomicrobial pulmonary infection, the clinical manifestations of patients with multi-pathogenic pneumonia can be severe and fatal, and are difficult to diagnose, particularly among immunocompromised individuals.[5–7] The risk of pulmonary infection is high in HIV-infected patients, especially those who with CD4 count of less than 200 cells/μL.[1] Multi-pathogenic pneumonia which is

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difficult to diagnose using conventional procedures were report among people living with human immunodeficiency virus (PLWH).[8,9]

Although various current conventional assays exist, rapid and accurate diagnosis of causative mixed pathogens is still difficult in HIV-infected individuals. More importantly, the diagnosis of multi-pathogenic pneumonia must be as accurate and fast as possible, because of severe clinical manifestation in HIV-infected individuals and potential side effects of combined treatment. Several current conventional tests, such as smear, culture, and molecular assays for one or a few pathogens are available now, however, the fast identification of the precise etiology of pulmonary infection is still challenging. As conventional tests have limitations in terms of speed, sensitivity, spectrum for pathogen detection, simultaneous multi-pathogen detection, and lack of diagnostic tests for some rare pathogens, sometimes we still cannot draw a correct conclusion according to these tests, especially in HIV-infected patients with suspected mixed pulmonary infection.[10,11] What's more, the requirement for invasive procedures bronchoalveolar lavage, further impedes its repeat many times to make the diagnosis in HIV-infected patients with suspected multi-pathogenic pneumonia.

Metagenomic next-generation sequencing (mNGS) is a highly sensitive, culture-independent, and unbiased method to identify the precise etiology of infection.[12,13] mNGS which could provide valuable information for identifying a comprehensive spectrum of pathogens by a single assay, has shown its efficacy in the diagnosis of pulmonary infectious in non-HIV-infected immunocompromised hosts.[14–16] Previously, we report an HIV-positive case who was diagnosed multi-pathogenic pneumonia by mNGS of bronchoalveolar lavage fluid (BALF) following negative results in multiple conventional diagnostic tests.[17] However, there is still a lack of studies on the value of mNGS in HIV-infected patients with suspected multi-pathogenic pneumonia. Here, we aimed to explore the value and challenges of real-world clinical application of mNGS for diagnosis of mixed pulmonary infection in HIV-infected patients.

Patients and Methods

Patients

This was a retrospective cohort study in which HIV-infected people with clinical suspected pulmonary infection admitted to the First Affiliated Hospital of Zhejiang University were included. The First Affiliated Hospital of Zhejiang University is the general hospital and is also a provincial tertiary referral hospital for complicated or difficult-to-treat cases of HIV infection in eastern China. HIV-infected patients with suspected pneumonia were recruited when meet following criteria: (1) new-onset of symptoms, such as fever, cough, expectoration, and dyspnea; (2) new-onset of abnormal chest imaging manifestations. The inclusion criteria were as follows: (1) HIV-infected patients had suspected pneumonia and agreed to undergo the bronchoscopy; (2) BALF samples passed quality control for mNGS; (3) medical data of the patients were recorded completely. Total 57 hospitalized HIV-infected patients with suspected pulmonary infections were recruited from July 2020 to June 2022.

Microbiological Tests

All patients underwent the bronchoalveolar lavage operation following a standard safety protocol.[8] The BALF and brush specimens were sent for the conventional microbiological tests (CMTs) including the smear and culture of the bacteria, Mycobacterium tuberculosis, and fungi; the detection of *Cryptococcus* capsular polysaccharide antigen (CrAg) and galactomannan antigen; and X-pert MTB/RIF specific for Mycobacterium Tuberculosis (MTB). Other conventional diagnostic testing included real-time polymerase-chain reaction (PCR) for cytomegalovirus (CMV) and Epstein–Barr virus (EBV), T-SPOT test for Mycobacterium tuberculosis. The BALF specimens were subjected to mNGS testing by the Molecular diagnostic department of the First Affiliated Hospital of Zhejiang University. Briefly, the mNGS procedure included sample collection, library construction, sequencing, and bioinformatic analyses. According to the manufacturer's protocols, the DNA and RNA were extracted, then the DNA/RNA concentration was measured using a Qubit Fluorometer, and the molecular size was estimated by agarose gel electrophoresis. Then, the pooled libraries were sequenced on an Illumina NextSeq 550 system using a 75 bp, single-end sequencing kit (Illumina, San Diego, CA). For quality control and bioinformatic analysis, raw reads of sequencing were removed the low-quality reads or short lengths (<35 bp) by using fastp and adapter contamination to produce clean reads. Then, the clean reads were mapped to the human-source database (National Center for Biotechnology

Information GRCh38 assembly) to filter the human-source sequence. Finally, the remaining reads were aligned to the reference database (NCBI database and GenBank, ftp://ftp.ncbi.nlm.nih.gov/genomes/) to identify microbial species.[18] The turnaround time for mNGS process was 24h to 48h.

Data Collection

The following data were recorded: age, sex, duration of ART and clinical manifestations, chest computed tomography (CT), results of laboratory examination, and patient outcomes. Specimens were subjected to mNGS testing and pairwise regular clinical microbiological assay. The final diagnosis was determined by three experienced senior clinicians. Meanwhile, clinical data of all enrolled patients, including complete blood count, neutrophil count, red blood cells count, platelets count, ALB (albumin), LDH (lactate dehydrogenase), CD4 T cell count, CD8 T cell count, C-reactive protein (CRP), procalcitonin (PCT), interleukin (IL)-2, IL-4, IL-6, tumor necrosis factor-α (TNF-α), IL-10, IL-17A and interferon-γ (INF-γ) were collected.

Criteria for mNGS Result

SMRN was the number of sequences strictly mapped at the species level. Microorganisms detected by mNGS were identified when suspected pathogens meet all the following criteria: (1) the suspected pathogens were the definite pathogens of pulmonary infection which exclude the normal flora of the oral cavity, respiratory tract, or the skin (via literature search); (2) the thresholds for different types of microorganisms were set as follows: bacterial/virus/fungus/mycoplasma/chlamydia: SMRN > 3; Mycobacterium tuberculosis: SMRN > 1; Parasite: SMRN > 100; (3) the suspected pathogens meet clinical judgment by three experienced senior clinicians or meet targeted treatment response.[19,20]

Statistical Analyses

Continuous variables showed as the mean± standard deviation (SD) or the median (25th, 75th percentiles) depending on whether they were normally distributed or non-normal distribution. Categorical variables were presented as numbers (percentages). Determination of microbiological etiology and clinical composite diagnosis was used as the reference standard. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated by VassarStats (http://vassarstats.net/clin1.html). The results are presented with 95% confidence intervals (CIs). The comparisons between groups were conducted through the Chi-square test, Independent-Samples *t*-test, and Mann–Whitney *U*-test using SPSS 26.0 software. P values < 0.05 were considered significant and all tests were 2-tailed.

Results

Patient Characteristics

Between 1 July 2020 and 30 June 2022, 57 eligible HIV-infected patients whose BALF samples were sent for mNGS and CMTs to identify the infectious pathogens were enrolled in this study. The detailed clinical characteristics of all patients including demographic characteristics and laboratory findings are listed in Table 1. Among them, 91.2% (52/57) of patients were male, and the median age was 44.3 (range 24–73) years. Thirty-three (58.49%) patients were naïve to ART, and 11 (20.75%) patients had received ART for less than 6 months. The patients were at an advanced stage of immunosuppression with a median CD4 T-cell count of 54 (IQR:142) cells/mL, CD4/CD8 was 0.12 (IQR: 0.23). According to the retrospective review of medical records, including laboratory findings, 54 patients were diagnosed with pneumonia which include 49 patients with definite pathogens and five patients with probable pathogens. All patients with pneumonia had consequent favorable clinical outcomes. Another three patients were considered non-infection diseases including lung cancer (n=1), lymphoma with lung involvement (n=1), and drug-related interstitial lung disease (n=1).

Concordance Between mNGS and CMTs

In this study, mNGS and CMTs (including culture) were both positive for pathogens detection in 47 (47/57, 82.46%) cases. A total of 7 cases (7/57, 12.28%) were positive for pathogens detection by mNGS only, while there were only 3 cases (3/57, 5.26%)

Table 1 Clinical Characteristics Data Summary

Characteristics	Value
Age, (years), mean±SD	44.3±12.68
Gender male/female	52/5
Onset of symptoms/signs	
Fever, n (%)	29 (50.88%)
Dry cough, n (%)	33 (57.89%)
Expectoration, n (%)	17 (29.82%)
Hypoxemia, n (%)	21 (36.84%)
Chest pain, n (%)	2 (3.51%)
Duration of symptoms	
<1 week, n (%)	2 (3.51%)
1–2 weeks, n (%)	11 (19.3%)
2 weeks–3 months, n (%)	30 (52.63%)
>3 months, n (%)	14 (24.56%)
Duration of ART	
ART naive, n (%)	33 (58.49%)
<6 months, n (%)	11 (20.75%)
>6 months, n (%)	11 (20.75%)
Laboratory test	
CD4 T-cell count (cells/mm^3) (median, 25th, 75th percentiles)	54 (17, 158.5)
CD8 T-cell count (cells/mm^3) (median, 25th, 75th percentiles)	344 (245.75, 523)
CD4 /CD8 ratio (median, IQR)	0.12 (0.23)
White blood cells (×10^9/l), mean±SD	5.05±2.95
Neutrophils (×10^9/l), mean±SD	3.68±2.61
Hemoglobin (g/l), mean±SD	120.30±25.37
Platelet (×10^9/l), mean±SD	227.16±99.62
CRP (mg/l), mean±SD	33.16±38.2
PCT (ng/mL), mean±SD	0.14±0.13
Albumin (g/L), mean±SD	37.59±11.11
LDH (U/L), mean±SD	268.63±155.26
IL-2(pg/mL), mean±SD	1.22±1.99
IL-4 (pg/mL), mean±SD	1.63±3.18
IL-6 (pg/mL), mean±SD	51.15±78.80
IL-10 (pg/mL), mean±SD	7.85±19.1
IL-17 (pg/mL), mean±SD	13.79±31.03
TNF-α (pg/mL), mean±SD	3.4±6.53
INF-γ(pg/mL), mean±SD	14.04±25.19

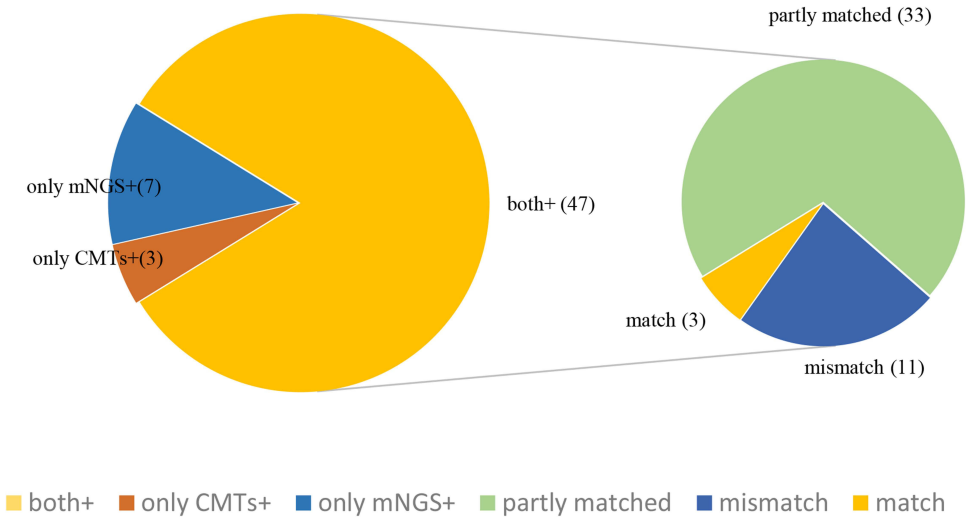

Figure 1 Concordance between CMTs and mNGS.
Notes: Both+, results of mNGS and CMTs were both positive; Both-, results of mNGS and CMTs were both negative; only mNGS+, only the mNGS result was positive; only CMTs+, only the CMTs was positive.
Abbreviations: mNGS, metagenomic next-generation sequencing; CMTs, conventional microbiological tests.

positive by CMTs only. For the 47 both-positive cases, results of mNGS and CMTs were matched in 3 cases (3/47, 6.38%) and were completely mismatched in 11 cases (11/47, 23.40%). A total of 33 cases (33/47, 70.21%) were found to be partly matched which means at least one detected pathogen was overlapped between CMTs and mNGS (Figure 1).

Pathogen Detection by mNGS Relative to CMTs

The comparison between the CMTs and mNGS for different classes of pathogens detection was shown in Figures 2 and 3. The percentage of mNGS-positive patients was significantly higher than that of CMTs–positive patients regarding fungal

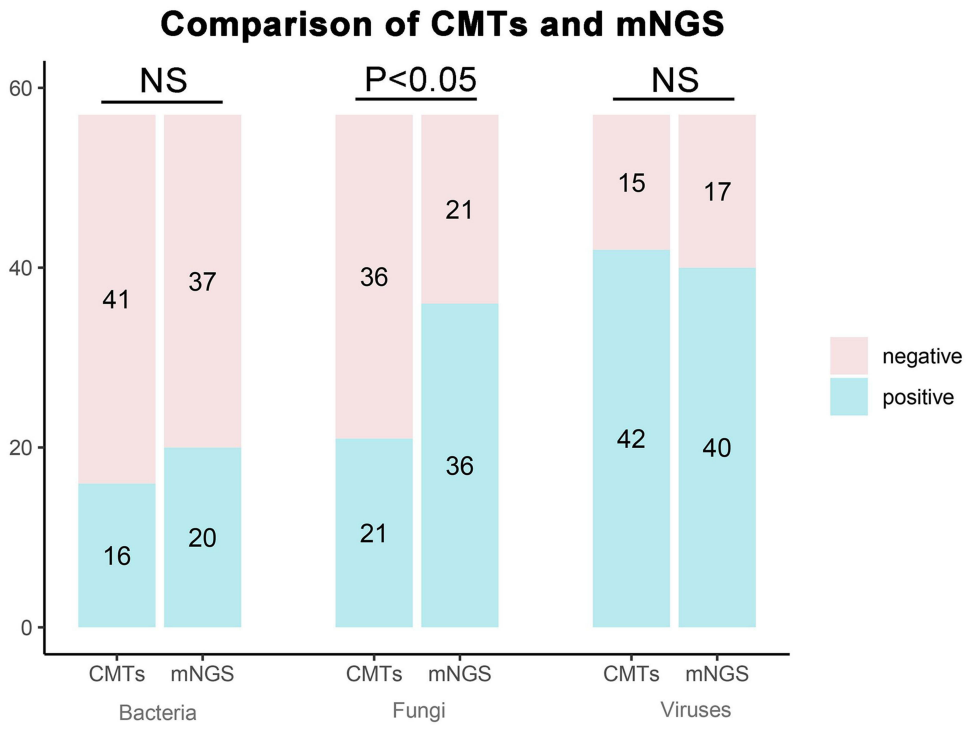

Figure 2 Comparison of different classes of pathogens detected by the CMTs and mNGS in HIV-infected patients.
Notes: The number of positive samples (y-axis) for pairwise mNGS and CMTs is plotted against the bacteria, fungi, and virus groups (x-axis).

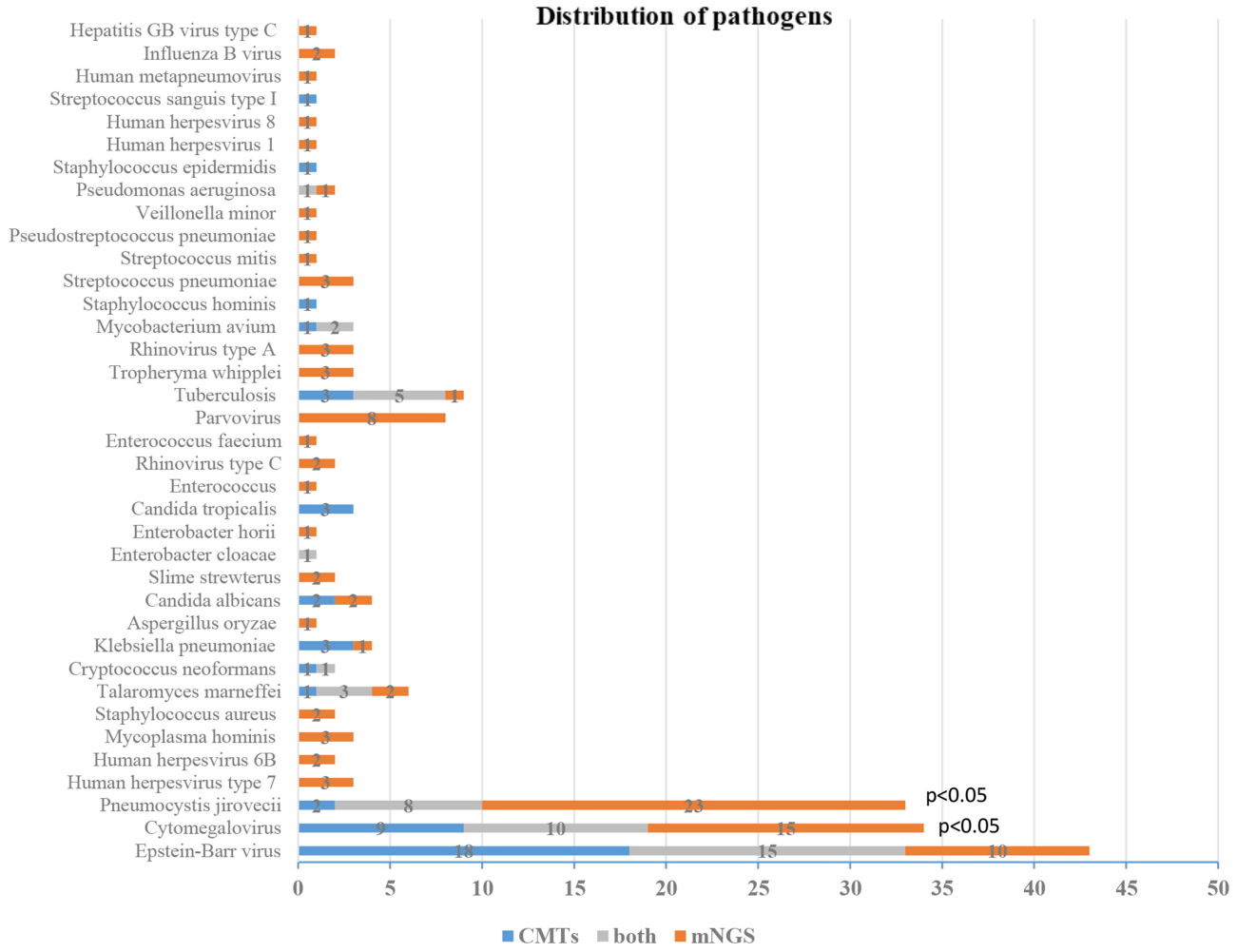

Figure 3 Distribution of pathogens identified in HIV-infected patients using CMTs versus mNGS.

detection (P < 0.05), but no significant differences were found regarding bacterial and virus detection (Figure 2). The distribution of pathogens identified by CMTs and mNGS for all 57 patients is shown in Figure 3. The detected pathogens isolated by mNGS test including 31 *Pneumocystis jirovecii*, 25 Cytomegalovirus, 25 Epstein–Barr viruses, and so on. While for CMTs, 10 *Pneumocystis jirovecii*, 19 Cytomegalovirus, and 33 Epstein–Barr viruses were detected. The detection rate of mNGS for *Pneumocystis jirovecii* and Cytomegalovirus was significantly higher than that of CMTs (P < 0.05) (Figure 3).

Comparison of mNGS and CMTs Methods

The performance of mNGS and CMTs in the diagnosis of pulmonary infection in HIV-infected patients is shown in Table 2. The sensitivity and specificity of mNGS for diagnosing pulmonary infection were 79.25% (95% CI 66.54–88%) and 60% (95% CI 23.07–92.89%), with NPV and PPV being 95.45% and 21.43%, respectively. As for the CMTs, the sensitivity and specificity were 54.72% (95% CI 41.45–67.34%) and 75% (95% CI 30.06–98.72%), with NPV and PPV

Table 2 Comparison of the Diagnostic Value Between CMTs and mNGS

	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
mNGS	79.25% (66.54–88%)	60% (23.07–92.89%)	95.45% (84.87–99.19%)	21.43% (7.57–47.59%)
CMTs	54.72% (41.45–67.34%)	75% (30.06–98.72%)	96.67% (83.33–99.83%)	11.11% (3.85–28.06%)

Abbreviations: CI, confidence intervals, PPV positive predictive value, NPV negative predictive value.

being 96.67% and 11.11%, respectively. The sensitivity of mNGS in pulmonary infection diagnosis was much higher than that of CMTs (79.25% vs 54.72%; $P < 0.05$), while the specificity with no statistically significant difference between mNGS and CMTs (60.00% vs 75%; $P > 0.05$).

Clinical Characteristics Comparison Between Mixed and Single Pulmonary Infection

Mixed pulmonary infection was defined when two or more pathogens identified by mNGS or CMTs. As shown in Table 3, there were 29 cases of mixed pulmonary infection and 25 cases of single pulmonary infection. No difference in age was observed between mixed infection group (43.55 ± 13.08) and single infection group (42.80 ± 10.89) (P=0.821).

Table 3 Comparison of Clinical Characteristics Between Mixed Infection Group and Single Infection Group

Characteristics	Mixed Infection (n =29)	Single Infection (n = 25)	*P* value
Age, (years), mean±SD	43.55±13.08	42.8±10.89	0.821
Gender male/female	28/1	22/3	**<0.05**
Onset of symptoms/signs			
Fever, n (%)	55.2%	36.0%	0.159
Dry cough, n (%)	65.5%	52.0%	0.313
Expectoration, n (%)	12 (44.8%)	3 (12%)	**0.008**
Hypoxemia, n (%)	41.4%	32.0%	0.477
Chest pain, n (%)	0.0%	4.0%	0.94
Duration of symptoms			
<1 week, n (%)	0.0%	8.0%	0.407
1–2 weeks, n (%)	0.0%	44.0%	**<0.05**
2 weeks–3 months, n (%)	58.6%	48.0%	0.435
>3 months, n (%)	41.4%	0.0%	**<0.05**
Duration of ART			
ART naive, n (%)	75.0%	45.5%	**0.033**
<6 months, n (%)	17.9%	27.3%	0.65
>6 months, n (%)	7.1%	27.3%	0.124
Laboratory test			
CD4 T-cell count (cells/mm^3) (median, 25th, 75th percentiles)	28 (6, 52)	108.5 (30.5, 238.75)	**<0.05**
CD8 T-cell count (cells/mm^3) (median, 25th, 75th percentiles)	338 (200, 506)	405.5 (270, 749.5)	0.365
CD4 /CD8 ratio (median, IQR)	0.07 (0.1)	0.26 (0.32)	**<0.05**
White blood cells ($\times 10^9$/l), mean±SD	4.68±2.61	5.63±3.35	0.235
Neutrophils ($\times 10^9$/l), mean±SD	3.61±2.36	3.97±2.99	0.828
Hemoglobin (g/l), mean±SD	119.14±25.66	120.96±26.22	0.798
Platelet ($\times 10^9$/l), mean±SD	240.07±119.3	218.56±70.5	0.456
CRP (mg/l), mean±SD	39.29±40.73	29.26±35.61	0.180
PCT (ng/mL), mean±SD	0.14±0.12	0.14±0.14	0.628

(*Continued*)

Table 3 (Continued).

Characteristics	Mixed Infection (n =29)	Single Infection (n = 25)	*P* value
Albumin (g/L), mean±SD	35.77±11.11	38.45±10.25	0.075
LDH (U/L), mean±SD	302.93±191.17	236.52±101.9	**0.027**
IL-2(pg/mL), mean±SD	1.05±1.159	0.98±1.13	0.808
IL-4(pg/mL), mean±SD	2.076±4.41	1.13±1.08	0.917
IL-6(pg/mL), mean±SD	62.56±95.54	43.97±61.77	0.565
IL-10(pg/mL), mean±SD	11.36±26.78	4.53±4.78	**0.026**
IL-17(pg/mL), mean±SD	18.36±36.18	10.15±26.79	0.513
TNF-α (pg/mL), mean±SD	2.24±2.81	3.12±7.36	0.990
INF-γ(pg/mL), mean±SD	12.31±17.33	17.37±32.85	0.625

Note: P-value lower than 0.05 are shown in bold.

The most common symptoms were dry cough and fever, but without statistical significance between the two groups. Significant statistical differences were observed between mixed infection group (12/29, 44.8%) and single infection group (3/25, 12.0%) in terms of expectoration (P=0.008). Most patients were admitted to the hospital within 2 weeks-3 months of symptoms onset. The duration of symptoms within 1–2 weeks was significantly higher in the single infection group than in the mixed infection group (44% vs 0%). The rate of symptoms duration >3 months was significantly higher in the mixed infection cases than in the single infection cases (41.4% vs 0%). Additionally, patients with mixed infection had lower CD4 T-cell count (28 vs 108.5 cells/mL) and CD4/CD8 ratio (0.07 vs.0.26) than those with single infection ($P < 0.05$). There were no significant differences in most of the inflammatory indices between the two groups, however, the level of LDH and IL-10 were significantly higher in the mixed infection group than in single infection group ($P < 0.05$).

Comparison of mNGS and CMTs in Mixed Pulmonary Infection

A total of 29 HIV-infected patients were diagnosed with mixed infection (including 16 cases with two pathogens, 12 cases with three pathogens, and one case with more than three pathogens). The detection rate of mNGS for mixed pulmonary infection was significantly higher than that of CMTs (89.66% vs 55.17%, $P < 0.05$), and more co-pathogens could be detected by mNGS. Fungi-virus (11/29, 37.9%) and fungi-virus-bacteria (6/29, 20.7%) coinfection were the most common co-pathogens observed in HIV infected patients with multi-pathogenic pneumonia (Figure 4A). The most common pathogen in co-infection was Pneumocystis jirovecii (20/29, 69.0%) and Cytomegalovirus (20/29, 69.0%) (Figure 4B). The most common pathogens were pneumocystis jirovecii (26/54, 48.15%), cytomegalovirus (20/54, 37.04%), mycobacterium tuberculosis (11/54, 20.37%), bacterial (16/54, 29.62%), and talaromyces marneffei (6/54, 11.11%).

Discussion

Although researchers have evaluated the performance of mNGS in immunocompromised hosts with suspected pulmonary infection,[14–16] there is still a lack of studies on the value of mNGS of BALF in HIV-infected patients with suspected pneumonia. The sensitivity of BALF mNGS in identifying pathogens was significantly higher than CMTs (79.25% vs 54.72%; $P < 0.05$), while the specificity was comparable between mNGS and CMTs (60.00% vs 75%; $P > 0.05$) in HIV-infected patients. Consistent with our data, it was reported that mNGS increased the sensitivity rate for diagnosis of infectious disease when compared with that of culture (50.7% vs 35.2%; $P < 0.01$), while the specificity rate of mNGS was comparable with that of culture (89.1% vs 85.7%; P=0.39) in general population.[21] BALF mNGS exhibited a higher detection rate compared to conventional tests regarding fungal detection, while there were no significant differences regarding bacterial and virus detection in HIV-infected patients with suspected pneumonia. This was consistent with

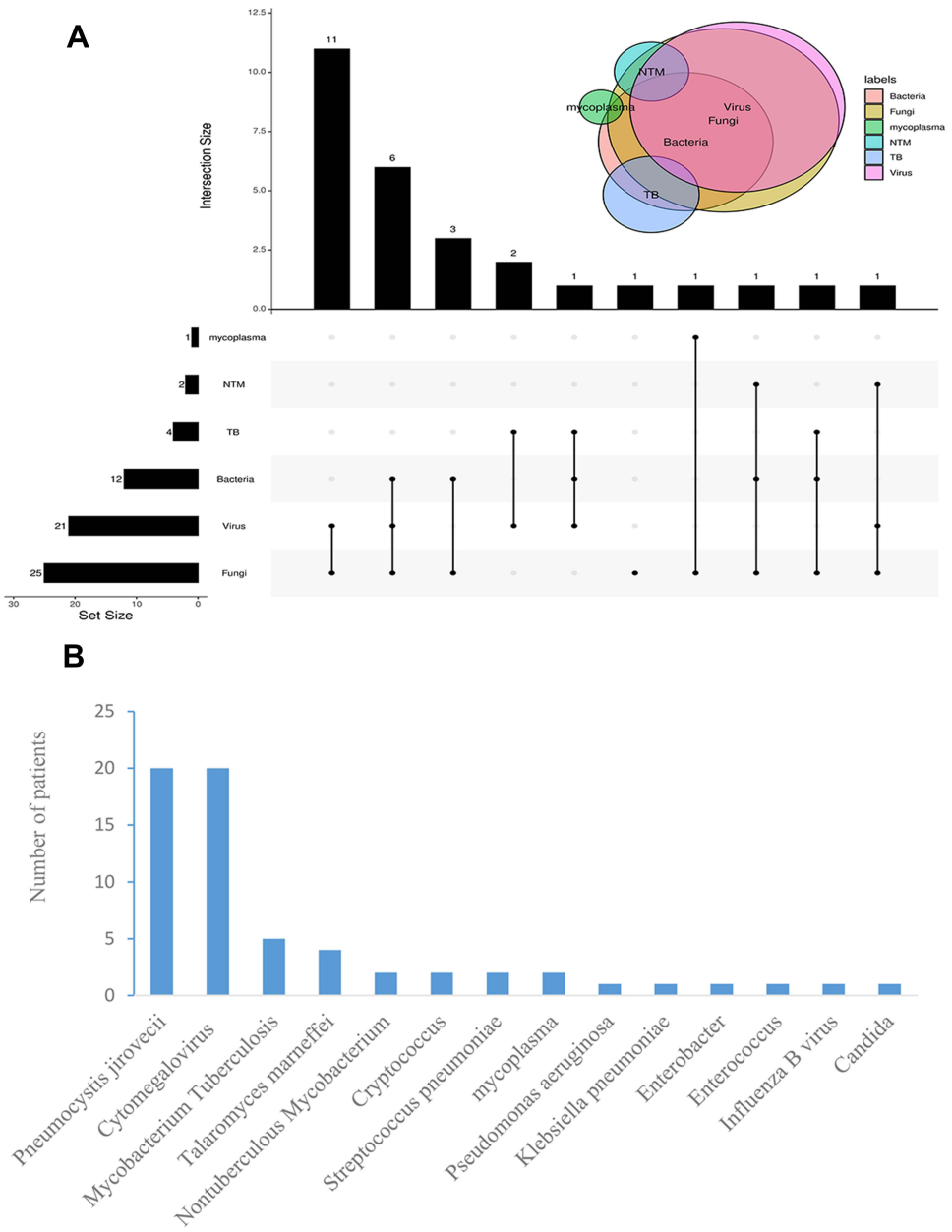

Figure 4 Distribution of pathogens identified in HIV-infected patients with co-infection.
Notes: (**A**) Percentage of patients with co-infection for various pathogens. (**B**) Pathogen spectrum of HIV-infected patients with co-infection.

previous findings that BALF mNGS has superior feasibility in detecting fungi than that of culture in immunocompromised patients.[16,21] However, some studies found that culture can identify most bacterium-associated pneumonia compared with sequencing.[22] Furthermore, it reported that there was no significant difference between CMTs and mNGS in pulmonary fungal detection, while mNGS displayed higher detecting rate for bacteria.[9] Unlike the aforementioned studies, it showed similar diagnostic performance of BALF mNGS when compared with CMTs for all types of pathogens in 60 immunocompromised patients.[23] The factors such as different diseases, different sample types, and different test conditions of CMTs and mNGS may be the possible explanations for the divergence in these studies.

Compared with immunocompetent patients, more polymicrobial infections were found in immunocompromised patients.[24] Multi-pathogenic pneumonia is common in HIV-infected patients with low CD4 count. In this study, the patients with mixed infection had lower CD4 T-cell count (28 vs 108.5 cells/mL) and CD4/CD8 ratio (0.07 vs.0.26) than those with single infection. Polymicrobial infections can hardly be identified by CMTs because of its limitations. On the

other hand, mNGS has more advantages in the diagnosis of co-infection because it is a unbiased detection technology and can detect multiple pathogens at the same time.[25,26] It has shown that mNGS is better in coinfections identification than conventional tests like serological antibody, PCR, or culture in prior studies.[27–29] In this study, mNGS showed broader spectrum for pathogen detection and a higher detection rate than that of CMTs in pulmonary co-infection. So, mNGS might benefit HIV-infected patients who developed pneumonia with suspected multiple pathogens. Interestingly, more patients with multi-pathogenic pneumonia were naïve to ART. Moreover, HIV-infected patients with established multi-pathogenic pneumonia had significantly lower CD4 T cell counts than patients with a single infection. Furthermore, patients with established multi-pathogenic pneumonia had a long duration of symptoms than single infection individuals. These suggested that HIV-infected patients with advanced immunosuppression and long duration of symptoms are prone to mixed pulmonary infection, accordingly, mNGS may be best suited to patients with such conditions. Thus, mNGS may be a more favorable method that can improve clinical guidance in the diagnosis and treatment of multi-pathogenic pneumonia in HIV-infected patients with advanced immunosuppression.

Consistent with previous findings in immunocompromised patients, this study found that the most common combinations were fungi-virus coinfection in HIV-infected individuals.[25] Unlike our result, Wu et al reported that bacterial–viral coinfection and bacterial–fungal coinfection were the most frequent combinations in immunocompromised patients.[30] The inconsistency between these studies might result from the different kinds and different proportions of immunocompromised patients. In this study, we focused on HIV-infected patients with advanced immunosuppression and found that mNGS identified much more co-pathogens when compared with CMTs. Furthermore, Pneumocystis jirovecii and CMV were found to be the most common pathogen of pulmonary co-infection in HIV-infected patients with advanced immunosuppression, which was similar to the previous study in patients without HIV infection.[30] It indicated advantage of mNGS for identifying pathogens in HIV-infected patients with suspected mixed pulmonary infection.

However, it is still challenging for mNGS to identify *Cryptococcus* or mycobacterium because of the difficulty of DNA extraction.[31] In addition to these challenges, there are still some barriers to the widespread application of mNGS as a diagnostic tool for pulmonary infection among PLWH. Except for HIV, the mNGS sequencing found many DNA/RNA viruses in BALF, it is difficult to distinguish whether the pathogens identified by mNGS are pathogenic or colonization or whether they are the consequence of cell lysis. Therefore, the interpretation of mNGS findings should be done in combination with other clinical data, preferably by experienced clinicians in a multidisciplinary manner.[32]

This retrospective study contains certain limitations. Firstly, the study only recruited the participants who had complete available data, so the number of participants was relatively small and there was inevitable selection or recall bias in the retrospective study. In addition, some patients with severe condition are unable to tolerate invasive procedures bronchoalveolar lavage. What's more, the virus detected by mNGS was interpreted depending on the three experienced senior clinician's subjective judgment rather than diagnostically confirmed, so subjective bias is unavoidable. Furthermore, it was hard to distinguish pathogens from infection to colonization as respiratory samples are not sterile and the unbiased detection of mNGS. So, future studies should focus on developing a unified standard of distinguishing pathogens from colonization to infection, developing databases containing the pathogens involved in the disease for more accuracy in clinical interpretation of the mNGS results, and improving the mNGS protocols which include sample collection, nucleic acid extraction, the human genome and/or biofilm depletion, and microbial enrichment in library preparation protocol.

Conclusions

Pulmonary infection in HIV-infected patients presents a diagnostic dilemma. It is necessary to consider multiple etiologies in HIV-infected patients with low CD4 T cell counts and long duration of symptoms, and early performance of bronchoscopy and mNGS are recommended. In this study, mNGS exhibited better performance in identifying multi-pathogenic pneumonia, and the most common combinations we found were fungi-virus coinfection and fungi-virus-bacteria coinfection in HIV-infected patients with advanced immunosuppression. However, there are still challenges in the widespread use of mNGS in the accurate pathogen diagnosis of suspected multi-pathogenic pneumonia in HIV-infected patients and a lot of work to be done in interpreting the mNGS reports.

Ethics Approval and Informed Consent

This study conforms to the ethical norms of the 1975 Helsinki Declaration. Due to the retrospective nature of the study, written informed consent from the patients was not required. The research protocol was approved by the Institutional Review Committee of the First Affiliated Hospital of Zhejiang University. All the data used for analysis were anonymized.

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Disclosure

The authors declare no conflicts of interest.

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