

Diagnostic Value of Metagenomic Next-Generation Sequencing of Bronchoalveolar Lavage Fluid for the Diagnosis of Suspected Pneumonia in Immunocompromised Patients

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Abstract

Background: To evaluate the diagnostic value of metagenomic next-generation sequencing (mNGS) of bronchoalveolar lavage fluid (BALF) in immunocompromised patients for the diagnosis of suspected pneumonia in comparison to that of conventional microbiological tests (CMTs).

Methods: Sixty-nine immunocompromised patients with suspected pneumonia received both CMTs and mNGS of BALF were analyzed retrospectively. The diagnostic value was compared between CMTs and mNGS, using the clinical composite diagnosis as the reference standard.

Results: Sixty patients were diagnosed of pneumonia including fifty-two patients with identified pathogens and eight patients with probable pathogens. The overall detection rate of mNGS for pathogens were higher than that of CMTs. However, a comparable diagnostic accuracy of mNGS and CMTs were found for bacterial and viral infections. mNGS exhibited a higher diagnostic accuracy for fungal detection than CMTs (78% vs. 54%, $P=0.05$), which mainly because of the high sensitivity of mNGS in patients with *Pneumocystis jirovecii* pneumonia (PJP) (100% vs. 28%, $P=0.05$). Among fifty-two patients with definite pathogens, nineteen patients (37%) were identified as pulmonary mixed infection, mNGS test showed a higher detection rate and broader spectrum for pathogen detection than that of CMTs in mixed infection. Moreover, *Pneumocystis jirovecii* was the most common pathogen in mix infection and mNGS have identified much more co-pathogens of PJP than CMTs.

Conclusions: mNGS of BALF improved the microbial detection rate of pathogens and exhibited remarkable advantages in detecting PJP and identifying mixed

infections in immunocompromised patients.

Background

Pneumonia is the most common cause of morbidity and mortality in immunocompromised patients, for which early identification of causative pathogens is necessary (1). However, the conventional microbiological tests (CMTs) methods are time consuming and represent a low rate of positive detection, which can not meet the diagnosis needs of an immunocompromised patient. Even with the combination of culture and culture-independent techniques (nucleic acid amplification testing and serological testing), the aetiological diagnosis of severe pneumonia in immunocompromised patient is usually unclear.

Metagenomic next-generation sequencing (mNGS), an unbiased pathogen detection method, has been used in infectious diseases increasingly in recent decades. It is mainly used for assessing the sterile body fluids in the clinical setting, including cerebrospinal fluid, joint effusion and blood(2–4). For non-sterile body fluids, such as bronchoalveolar lavage fluid (BALF), the applications of mNGS are greatly limited, especially for immunocompromised patients. Pan et al explored the application of mNGS of BALF in thirteen immunocompromised patients for the diagnosis of pneumonia and showed improved detection of opportunistic pathogens(5). However, peng et al reported a similar diagnostic value of mNGS of BALF in immunocompromised patients to the CMTs for all types of pathogens(6).

In this study, we attempted to evaluate the diagnostic performance of mNGS of BALF for pneumonia in immunocompromised patients compared with that of CMTs. Moreover, we intended to show diagnostic accuracy rates for different types

of pathogens.

This study received approval from the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (No. 2020-111) and was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent to publish the information were obtained from the personal patients.

Patients And Methods

Patients

From September 2018 to December 2020, all immunocompromised patients with suspected pneumonia admitted to the First Affiliated Hospital of Wenzhou Medical University were retrospectively investigated.

The inclusion criteria of patients were as follows: (1) at least eighteen years old; (2) had an immunocompromised condition;(3) admitted to the Department of Pulmonary and Critical Care Medicine due to suspected pneumonia;(4) had mNGS of BALF within 48 h after admission, and other relevant samples available for the conventional microbiological tests in standard procedures;

Immunocompromised status(7) (≥ 1 of the following risk factors were found): (1) hematologic cancer;(2) chemotherapy during the last three month;(3) chronic steroid (> 0.3 mg/kg/d of prednisone-equivalent for ≥ 3 weeks) or biologic drug use for autoimmune diseases or other immunosuppressive therapy;(4) solid-organ transplant receipt during the last six months;(5) neutropenia;(6) acquired or inherited severe immunodeficiency;

Patients with suspected pneumonia meet the both criteria below: (1) new-onset fever, cough, expectoration or dyspnea; (2) new-onset abnormal chest imaging manifestations;

Methods

Data collection

The following data were recorded: age, sex, underlying diseases and clinical manifestations, illness severity (pneumonia severity index (PSI) score), chest radiology, results of laboratory examination (including microbiological testing) and patient outcomes (regular clinic follow-up or telephone conversation).

Microbiological tests

All patients underwent the operation of bronchoalveolar lavage following a standard safety protocol (8). The BALF specimens were divided into aliquots. One aliquot was set for bacterial and fungal smear (including Gomori methenamine silver (GMS) staining and acid-fast stain) and culture. One aliquot was submitted for the detection of galactomannan antigen and cryptococcus capsular polysaccharide antigen. X-pert MTB/RIF detection of DNA sequences specific for *Mycobacterium Tuberculosis* (MTB) and real-time polymerase chain reaction (PCR) for *cytomegalovirus* (CMV) and *influenza A/B virus*. The other aliquots (5 mL, settled in a sterile sputum container, stored at -20°C) were sent to BGI-Huada Genomics Institute (Shenzhen, China) for detection.

For the other samples, urine was submitted for the detection of antigen of *Legionella pneumophila* and *Streptococcus pneumoniae*. Peripheral blood samples were sent for the detection of the galactomannan antigen to *Aspergillus*, *Cryptococcus capsular polysaccharide antigen* to *Cryptococcus* and immunoglobulin G and M antibodies to *parainfluenza*, *adenovirus*, *CMV*, *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* using commercial enzyme-linked immunosorbent assays (Ani Labsystems) according to the manufacturer's instructions.

mNGS of BALF

The procedure of mNGS for BALF samples includes Nucleic acid extraction, Library construction, Sequencing, and Bioinformatic Analyses according to previous studies(9, 10).

Criteria for a Positive mNGS Result

1. Bacteria (mycobacteria excluded), virus and parasites:

A microbe was considered clinically significant microbes (CSMs) when its coverage rate scored 10-fold greater than that of any other microbes according to Miao's study(11).

2. Fungi:

A microbe was considered CSMs when its coverage rate scored 5-fold greater than that of any other fungus due to its low biomass in DNA extraction (12, 13).

3. Mycobacteria:

MTB was defined as positive when at least 1 read was mapped to either the species or genus level because of the low possibility for contamination and difficulty of DNA extraction(14). Nontuberculous mycobacteria (NTM) were considered when the mapping read number was in the top 10 in the bacteria list because of the low possibility for contamination (15) and low yield rate (16).

Regardless of coverage rate, oral commensals were not defined as CSMs unless they were deemed to be significant by the physicians or proven otherwise(6).

Coverage rate was regarded as the measurement parameter in our study because of the consideration of confounding factors such as pathogen genome size, nucleic acid contamination and total number of sequencing reads (12).

Clinical composite diagnosis as the reference standard

The medical records such as clinical features, laboratory examination, microbiological tests (including mNGS and CMTs), chest imaging and therapeutic response, were reviewed independently by the two physicians who specialize in the management of infection in immunocompromised hosts to determine whether the patients had infectious aetiology or not and identify the pathogens (definite or probable). An in-depth discussion was performed when there was any disagreement between the two physicians and another senior physician was consulted if consensus could not be reached.

Statistical analysis

SPSS 22.0 (IBM Corporation) was used for analysis. Continuous variables were reported as the mean \pm standard deviation (SD) or the median (25th, 75th percentiles) depends on they were normally distributed or non-normal distribution. Categorical variables were presented as numbers (percentages). Determination of microbiological aetiology and clinical composite diagnosis were used as the reference standard. The chi-square test,

McNemar's test or Fisher's exact test was used to compare the diagnostic performance of mNGS and CMTs. All tests were two-tailed and $P < 0.05$ was considered significant.

Results

Patient characteristics

A total of 69 patients including 44 male (64%) were enrolled in this study. The mean age was (57 ± 15) years. Thirty-three patients (48%) received a long-term corticosteroid therapy for solid-organ transplantation or autoimmune diseases and twenty-eight patients (41%) treated with chemotherapy for solid tumors or hematologic malignancy. The median PSI score was (127 ± 51) . Twenty-two patients (32%) received invasive mechanical ventilation and 25 of 69 patients developed septic shock. The clinical features of all patients including demographic characteristic, laboratory findings and prognosis were shown in Table 1.

Table 1
Characteristic of 69 immunocompromised patients

Characteristic	69 patients
Age (year), mean \pm SD	57 \pm 15
Gender, male, n (%)	44(64)
Immunocompromised status, n (%)	
Hematologic malignancy	23(33)
Solid-organ transplantation	14(20)
Solid tumour receiving chemotherapy	5(7)
Immunosuppressive therapy ^a	21(30)
Prolonged corticosteroid therapy ^b	33(48)
Disease severity	
PSI score, mean \pm SD	127 \pm 51
Invasive Mechanical ventilation, n (%)	22(32)
Septic shock, n (%)	25(36)
Laboratory findings	
WBC ($10^9/L$), median (Q1, Q3)	6.58(4.93,11.05)
Neutrophils ($10^9/L$), median (Q1, Q3)	5.66(3.23,9.39)
Lymphocyte count ($10^6/L$), median (Q1, Q3)	0.73(0.35,1.17)
PaO ₂ /FiO ₂ , mean \pm SD	196 \pm 98
Albumin (g/L), mean \pm SD	30 \pm 7
Serum creatinine(mmol/L), median (Q1, Q3)	80(57, 127)
LDH (U/L), median (Q1, Q3)	432(304, 726)
CRP (mg/l), median (Q1, Q3)	88(42,149)
PCT (ng/ml), median (Q1, Q3)	0.27(0.11,1.20)
outcome	
Total 30-day mortality, n (%)	14(20)
Notes: ^a : more than 2 weeks. Underlying diseases include Dermatomyositis (2), Leukemia (1), connective tissue diseases(6), Solid-organ transplantation (9), Hemophilia (1), Chronic nephritic syndrome (1), Thrombocytopenia (1). ^b : Defined as > 0.3mg/kg/d of prednisone-equivalent for ≥ 3 weeks. Underlying diseases include Dermatomyositis (1), Connective tissue diseases(9), Solid-organ transplantation (10), Hemophilia (1), Nephrotic syndrome (6), Thrombocytopenia (1), interstitial lung disease(1), Hematologic malignancy (2), Intracranial tumour(1), Dermatology(1).	
Abbreviations: PSI: pneumonia severity index; WBC: White blood cell; LDH: lactate dehydrogenase; CRP: C-reactive protein; PCT: procalcitonin;	

According to the retrospective review of medical records including mNGS and CMTs, 60 patients were diagnosed with pneumonia including 52 patients with definite pathogens and 8 patients with probable pathogens. Another 9 patients were considered non-infection disease including rejection

of bone marrow with lung involvement (n=2), connective tissue diseases (n=3) or drug (n=2) related interstitial lung disease, lymphoma with lung involvement (n=1) and lung cancer (n=1).

Comparison of mNGS and CMTs Pathogen Detection Methods

Among 60 patients of confirmed pneumonia, the total detection rate of pathogens by CMTs and mNGS was 26/60 (43%) and 49/60 (82%), respectively. The detection rate reached 54/60 (90%) with a combination of mNGS and CMTs. With regard to bacteria and fungi infection, the detection rate of mNGS was 20/25 (80%) and 31/34 (91%) respectively, which were significantly higher than that of CMTs (9/25 (36%) and 15/34 (44%)) ($P < 0.05$). No significant difference was found in the detection rate of this two methods for virus infection ($P=0.05$). The comparison of different classes of pathogens detection by the CMTs and mNGS was shown in Figure 1.

Comparison of Diagnostic Performance of mNGS and CMTs

With regard to bacterial detection, the diagnostic sensitivity and specificity of CMTs were 36% and 82% respectively. While, mNGS exhibited a diagnostic sensitivity of 80% and a specificity of 73%. mNGS had a higher diagnostic accuracy than CMTs but not statistically significantly different (75% vs. 65%, $P=0.23$). A comparable diagnostic accuracy of mNGS and CMTs was also found for viral infections ($P=0.63$). However, mNGS exhibited a higher diagnostic accuracy for fungal detection than CMTs (78% vs. 54%, $P=0.05$), which mainly because of the high sensitivity of mNGS in patients with *Pneumocystis jirovecii* (100% vs. 28%, $P=0.05$). GMS staining was positive for *Pneumocystis jirovecii* in 7 out of 25 patients with *Pneumocystis jirovecii* pneumonia (PJP), corresponding to a sensitivity of 28% (95% CI 16%-28%). The poor sensitivity for the diagnosis of PJP could be improved to 88% with a combination of GMS staining and Serum (1, 3)-b-D-glucan (BDG), however, the sensitivity and specificity were still lower than that of mNGS. Comparison of diagnostic performance of mNGS and CMTs for the different types of pathogens were showed in Table 2.

Table 2
Comparison of diagnostic performance of mNGS and CMTs in immunocompromised patients

	CMTs					mNGS				
	Sensitivity% (95% CI)	Specificity% (95% CI)	PPV% (95% CI)	NPV% (95% CI)	Accuracy% (95% CI)	Sensitivity% (95% CI)	Specificity% (95% CI)	PPV% (95% CI)	NPV% (95% CI)	Accuracy% (95% CI)
Bacterial pneumonia	36(21-50)	82(73-90)	53(31-74)	69(62-76)	65(54-76)	80(63-92)	73(63-79)	63(49-72)	87(75-94)	75(63-84)
Fungal pneumonia	44(31-57)	63(50-75)	54(38-69)	54(43-64)	54(41-66)	91(79-98)	66(54-72)	72(63-77)	89(73-97)	78(66-85)
<i>Pneumocystis jirovecii</i> ^a	28(16-28)	100(91-100)	100(58-100)	71(66-71)	74(66-74)	100(86-100)	86(79-86)	81(70-81)	100(90-100)	91(82-91)
<i>Pneumocystis jirovecii</i> ^b	88(72-97)	84(75-89)	76(62-83)	93(83-98)	86(74-92)	100(86-100)	86(79-86)	81(70-81)	100(90-100)	91(82-91)
<i>Aspergillus</i> spp.	86(63-99)	97(92-98)	75(41-87)	98(94-100)	96(88-98)	57(23-71)	98(95-100)	80(33-99)	95(92-97)	94(87-97)
Viral pneumonia	53(34-53)	100(95-100)	100(64-100)	89(84-89)	90(81-90)	93(70-100)	83(77-85)	61(46-65)	98(90-100)	86(75-88)
Notes: ^a : detect by Gomori methenamine silver staining only; ^b : detect by a combination of Gomori methenamine silver staining and serum (1, 3)-b-D-glucan(≥ 80 ng/l was defined as positive);										
Abbreviations: mNGS: metagenomic next-generation sequencing; CMTs: conventional microbiological tests; PPV: positive predictive value; NPV: negative predictive value										

Distribution of Pathogens

The pathogen distribution of 52 patients with identified pneumonia was shown in Figure 2. For the 52 patients with identified pathogens, 33 (63%) were infected by a single pathogen, while 19 (37%) were mixed infection (including 9 cases with 2 pathogens, 9 cases with 3 pathogens and 1 case with more than 3 pathogens). The detection rate of mNGS for mixed infection was significantly higher than that of CMTs (79% vs. 42%, $P < 0.05$) and more co-pathogens could be identified by mNGS. Fungi-virus-bacteria and fungi-virus coinfection were the most common co-pathogens observed in mix-infection patients. The most common pathogen in mixed infections was *Pneumocystis jirovecii* (9/19, 47.4%) and *Cytomegalovirus* (10/19, 52.6%) (Figure 3).

The pathogens detected isolated by mNGS test including 1 case of *Escherichia coli*, 1 case of *Staphylococcus*, 4 cases of *Pseudomonas aeruginosa*, 16 cases of *Pneumocystis jirovecii*, 4 cases of *Cytomegalovirus*, 1 case of *Adenovirus*, 1 case of *Epstein-Barr virus*, 2 cases of *Chlamydia psittaci*, 2 cases of *Legionella* and 8 cases of mixed infection. For CMTs, 2 cases of *Aspergillus*, 3 cases of *Cryptococcus*, 1 case of *Mycobacterium tuberculosis*

complex, 1 case of *Influenza A* and 1 case of mixed infection were detected, which were not identified by mNGS test. Among the 3 cases of cryptococcosis detected by CMTs (PT32, PT40, PT69), the serum and BALF *Cryptococcus capsular antigen* tests were positive and *Cryptococcus neoformans* was identified by BALF culture in 2 cases. *Pneumocystis jirovecii* was identified by mNGS in PT32 and it was considered as respiratory tract colonization at last in combination with relevant clinical data including serum BDG test and imaging. PT69 was diagnosed as mixed infection of *Cryptococcus* and *Aspergillus*, however, none of which was detected by mNGS test (**Additional file 1**).

Discussion

Although many researches have evaluated the performance of mNGS in different patients of different infection type(11, 17), there is still lack of studies on the utility of mNGS of BALF in immunocompromised hosts with pneumonia. In a multicenter prospective study of 329 severe community-acquired pneumonia (SCAP) patients, a higher detection rate (up to 90.3%) and an earlier detection for pathogens by mNGS of BALF than that of CMTs was reported(7). However, immunocompromised cases represented only less than 20% of the patients enrolled. Similarly, pan et al reported an improved detection for opportunistic pathogens by mNGS of BALF in 13 immunocompromised patients(5). On the contrary, peng et al showed a similar diagnostic performance of mNGS of BALF to CMTs for all types of pathogens in 60 immunocompromised patients(6). In this study, a higher microbial detection rate was showed in immunocompromised patients for using mNGS test of BALF than that of CMTs. For the diagnostic accuracy rate, there was no significant difference in bacteria and viruses' infections by using these two methods. However, mNGS had a higher diagnostic accuracy than CMTs for fungal infections, which was not consistent with the study of Peng, mainly because of the low-proportion of aspergillus which was reported to be difficult for detection by mNGS test and high-proportion of pneumocystis in fungal infections in this study.

Pneumocystis jirovecii was the most common opportunistic pathogen in immunocompromised patients(18). It was reported to be responsible for 61.2% of confirmed pneumonia in immunocompromised patients (6) and 44.6% of SCAP in immunocompromised patients in a prospective study(7). Compare to CMTs, mNGS showed remarked advantages for detecting *Pneumocystis jirovecii*. Wang etc (19) showed significantly higher sensitivity of mNGS test than GMS alone for diagnosis PjP (100% vs. 30.7%). In the present study, the poor sensitivity of GMS for diagnosis PJP have be remarkably improved to 88% with the addition of serum BDG test. However, it was still lower than that of mNGS for diagnosis PJP. These results suggest that mNGS is a useful diagnostic tool with good performance for the diagnosis of PJP.

More polymicrobial infections were found in immunocompromised patients in comparison with immunocompetent patients(20). Peng etc(6) reported that more than half of microbiologically confirmed pneumonia having mixed infections. Pathogen detected by CMTs methods is usually a single one, while mNGS can detect multiple pathogens at the same time because of its unbiased detection technology, which has more advantages in the diagnosis of mixed infections(19, 21). In this study, 19 patients (37%) were identified as pulmonary polymicrobial infections. mNGS showed a higher detection rate and broader spectrum for pathogen detection than that of CMTs in mixed infection. Moreover, *Pneumocystis jirovecii* was found to be the most common pathogen in pulmonary mixed infection, which was similar to the previous study(22). Compare to CMTs, mNGS have identified much more co-pathogens of PJP. These results indicated that a remarkable advantage of mNGS method for identify mix pulmonary infection and detect co-pathogens of PJP than CMTs.

Recently, an amount of cases of *Chlamydia psittaci* infection have been reported(23, 24). In our study, we have diagnosed 2 patients of *Chlamydia psittaci* infection solely by mNGS because of the lack of CMTs for *Chlamydia psittaci* and they were improved with a proper treatment. This result shows mNGS is also useful in the case of limited CMTs.

However, it was still a challenge for mNGS to identify *Aspergillus* or *Cryptococcus* because of the difficulty of DNA extraction from the thick polysaccharide cell wall(13, 25). In our study, the diagnosis sensitivity for IPA by CMTs is a litter higher than that of mNGS. As for cryptococcosis, the sensitivity arrived 100% by using CMTs, while only 1 case was identified by mNGS.

Our retrospective study contains certain limitations. Firstly, as a retrospective study, there were selection bias and recall bias which was inevitable. In addition, the lack of RNA sequencing and partial of PCR methods had failed to evaluate the diagnostic value of CMTs and mNGS better especially for virus infection. The interpretation of virus detected by mNGS was depended on the clinician's subjective judgment more than diagnostically confirmed. Finally, it was difficult to distinguish pathogens from colonization to infection due to the unbiased detection of mNGS without unified standard.

Conclusion

mNGS using BALF improved the microbial detection rate of pathogens espically in the case of limited CMTs and exhibited remarkable advantages in detecting PJP and identifying mixed infections in immunocompromised patients. However, it is still a challenge for mNGS using BALF to detect *Aspergillus* or *cryptococcus* infection.

Declarations

Ethics approval and Consent to participate

This study received approval from the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (No. 2020-111) and was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent to publish the information were obtained from the

personal patients.

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Competing interests

The authors report no conflicts of interest in this work.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author on reasonable request.

Authors' contributions

All authors contributed to data analysis, drafting and revising the article, approved the final manuscript, and agreed to be accountable for all aspects of the work.

Consent for publication

Not Applicable.

Acknowledgments

Not Applicable.

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Figures

Comparison of CMTs and mNGS

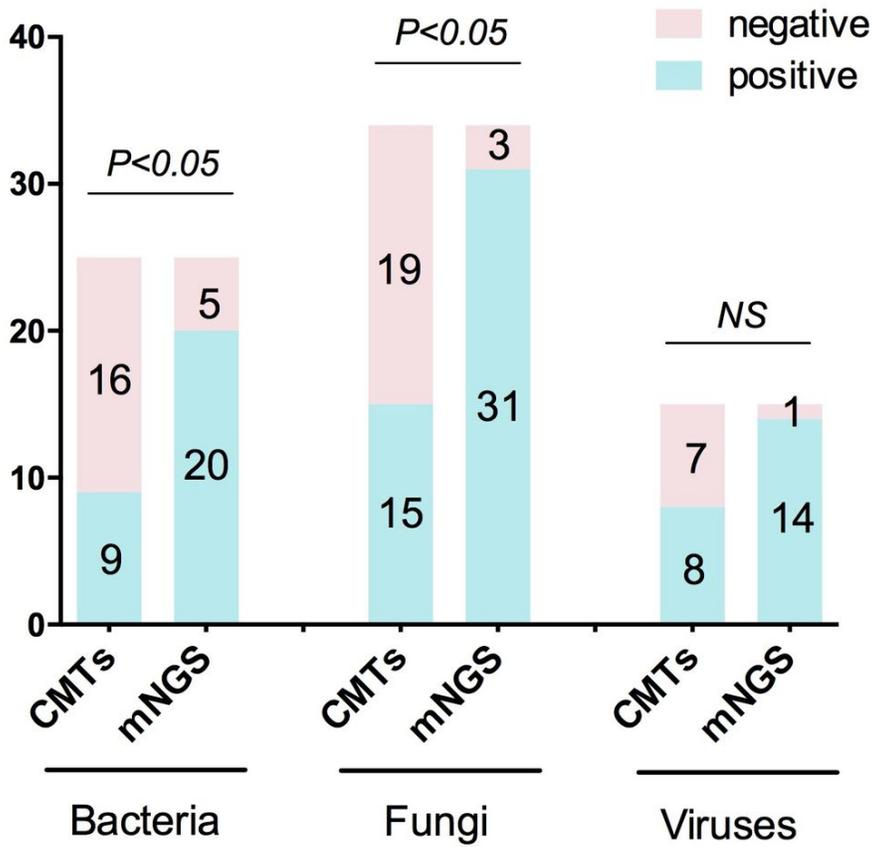


Figure 1
 Comparison of CMTs and mNGS test for the different classes of pathogens.
Notes: The number of positive samples (y-axis) for pairwise mNGS and CMTs is plotted against the bacteria, fungus and virus groups (x-axis).
Abbreviations: mNGS: metagenomic next-generation sequencing;
 CMTs: conventional microbiological tests

Distribution of Pathogens

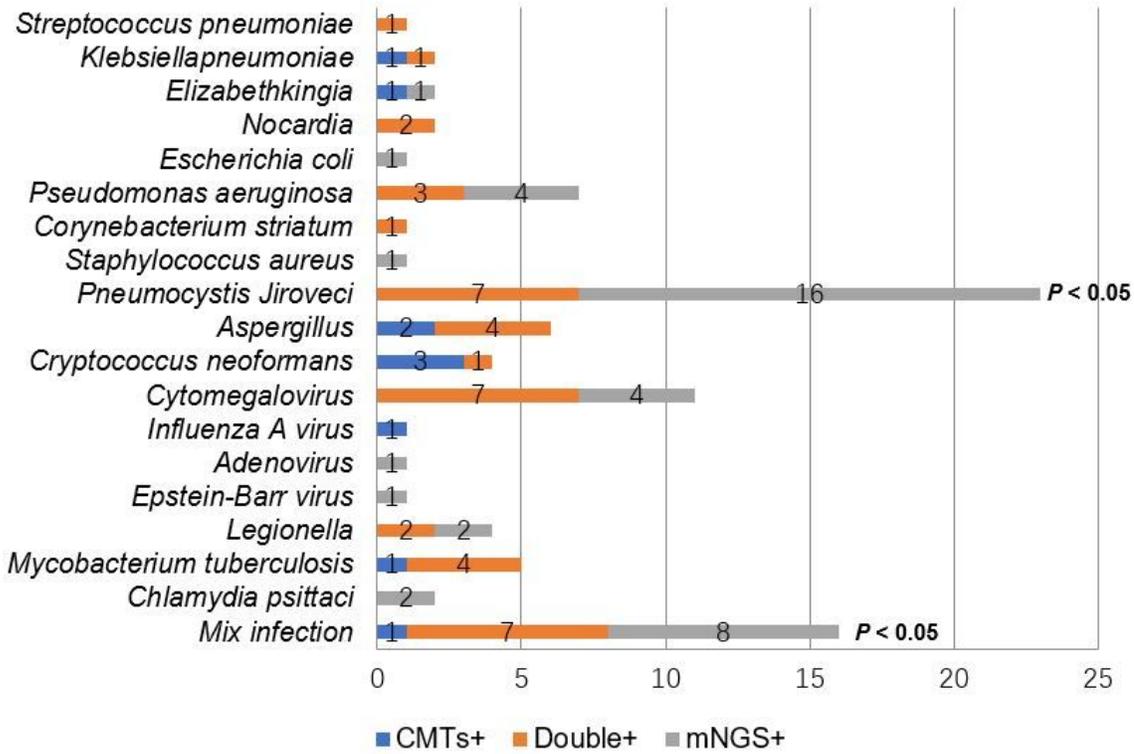


Figure 2

Distribution of pathogens identified in immunocompromised patients using CMTs versus mNGS.

Abbreviations: mNGS: metagenomic next-generation sequencing; CMTs: conventional microbiological tests;

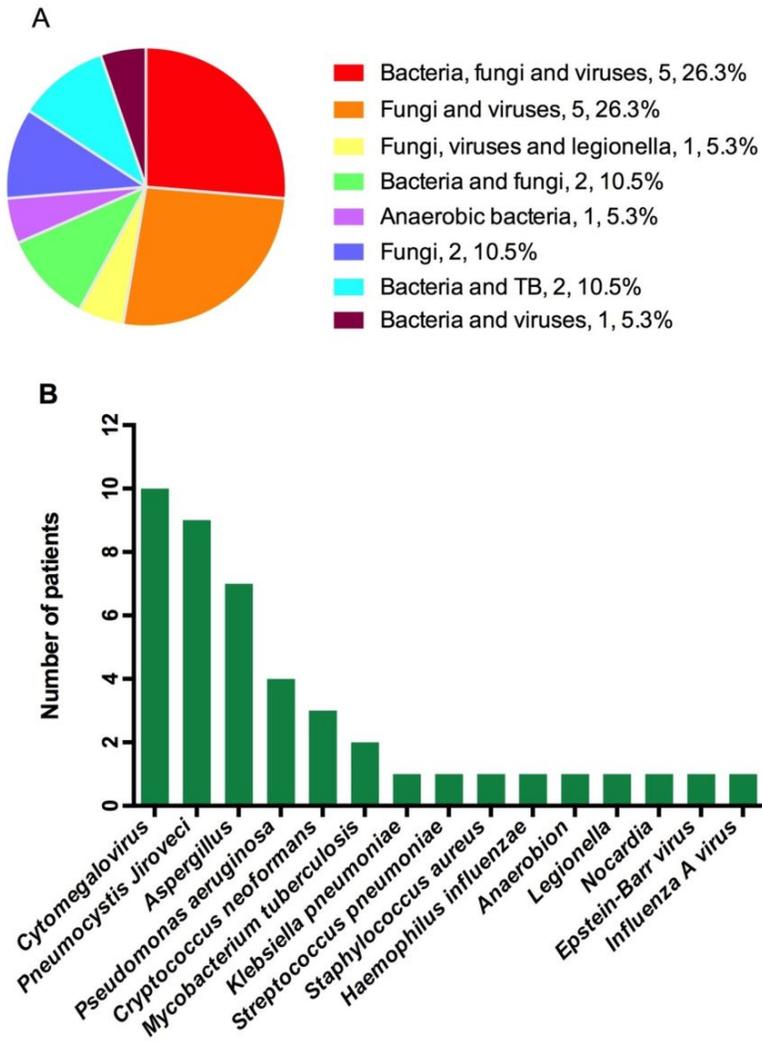


Figure 3

Distribution of pathogens identified in immunocompromised patients with mixed infection.

Notes: (A) Percentage of patients with mixed infection for various pathogens.

(B) Pathogen spectrum of immunocompromised patients with mixed infection.

Abbreviations: TB: Mycobacterium tuberculosis.

Supplementary Files

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