Supplementary Box 1. Conventional microbiological tests used in this study

Conventional Microbiological Test
⑴ Bacterial culture and smear microscopy
⑵ *Mycobacterium tuberculosis* or NTM: Acid-fast staining and cultures T-SPOT.TB GeneXpert MTB/RIF
⑶ PCR test for *Legionella*, *Mycoplasma pneumoniae, Chlamydia pneumoniae* from BALF/sputum
⑷ Serological tests for *Legionella*, *Mycoplasma pneumoniae, Chlamydia pneumoniae* and *Rickettsia*
⑸ Fungal culture and smear microscopy
⑹ Serum (1,3)-β-D-glucan test (G test)
⑺ Serum galactomannan test (GM test)
⑻ BALF GM test
⑼ Capsular polysaccharide antigen of *Cryptococcus* India ink stain for *Cryptococcus neoformans*
⑽ BALF or sputum Grocott's methenamine silver stain
⑾ Serological tests for CMV, EBV, HSV, adenovirus, respiratory syncytial virus, influenza A/B, Parainfluenza virus
⑿ PCR test for CMV、EBV and HSV from serum
⒀ Nucleic acid detection of 13 pathogens in throat swabs: influenza A virus, influenza A H1N1, influenza A H3N2, parainfluenza virus, metapneumovirus, *Mycoplasma pneumoniae*, respiratory syncytial virus, adenovirus, rhinovirus, Boca virus, *Chlamydia pneumoniae*, influenza B virus and coronavirus

Abbreviations: NTM, nontuberculous mycobacteria; MTB, Mycobacterium tuberculosis; RIF, rifampicin; BALF, bronchoalveolar lavage fluid; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HSV, herpes simplex virus.

Supplementary Table 1. Diagnostic performance of mNGS in different types of specimens.

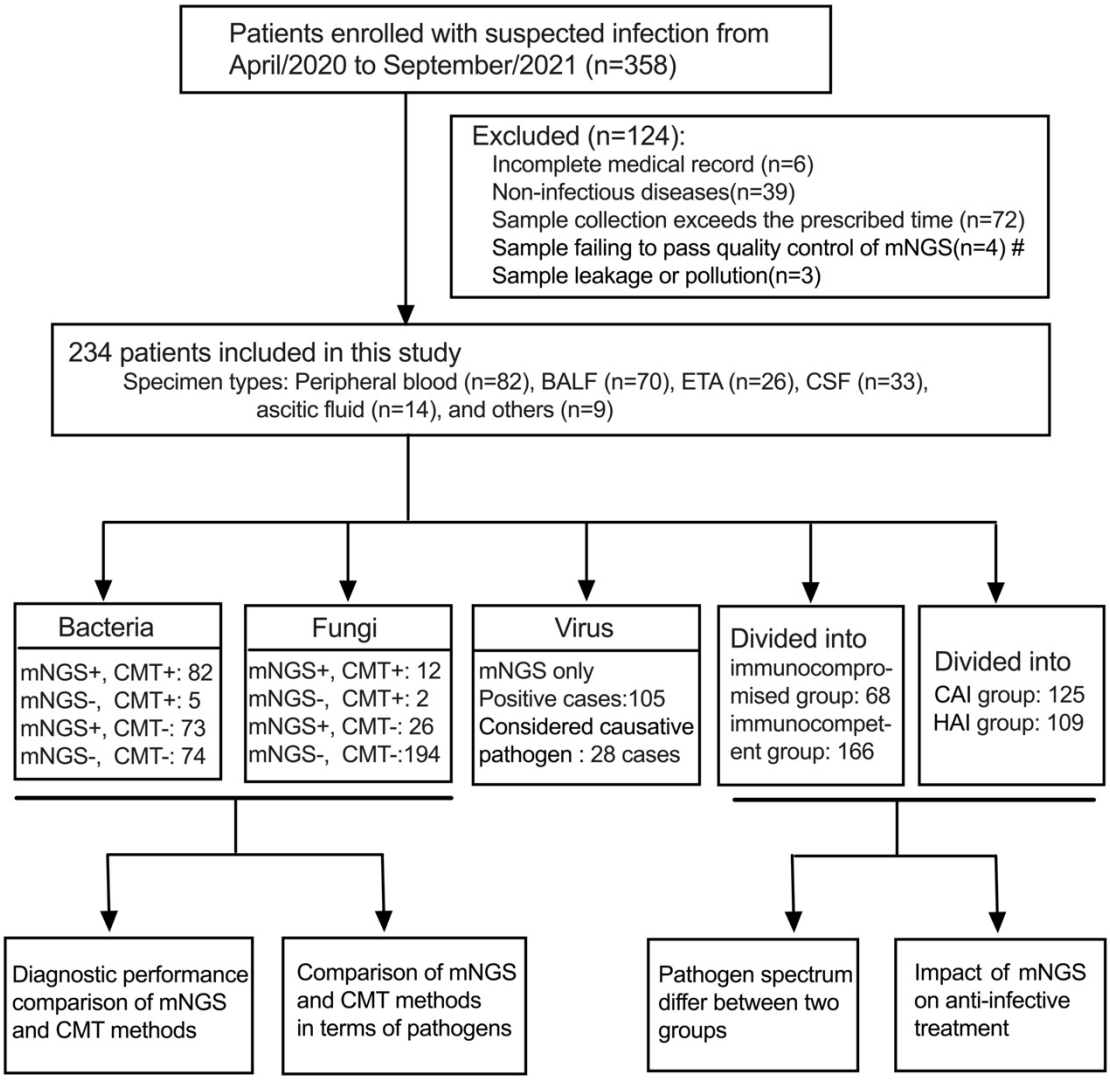
	Bacterial				Fungal			
	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV
Total-mNGS	96.6%	83.1%	90.3%	93.7%	85.7%	93.2%	63.2%	98.0%
Total-CMT	55.2%	92.1%	92.0%	55.8%	42.9%	98.5%	80.0%	92.7%
BALF-mNGS	98.1%	72.2%	91.1%	92.9%	76.9%	59.5%	62.5%	94.4%
BALF-CMT	76.9%	94.4%	97.6%	58.6%	61.5%	94.7%	72.7%	91.5%
ETA-mNGS	100.0%	63.6%	78.9%	100.0%	100.0%	95.8%	66.7%	100.0%
ETA-CMT	80.0%	63.6%	75.0%	70.0%	50.0%	100.0%	100.0%	96.0%
CSF-mNGS	92.3%	100.0%	100.0%	95.2%	100.0%	100.0%	100.0%	100.0%
CSF-CMT	38.5%	100.0%	100.0%	71.4%	NA	93.9%	NA	100.0%
Blood-mNGS	95.2%	85.0%	87.0%	94.4%	100.0%	90.8%	46.2%	100.0%
Blood-CMT	31.0%	95.0%	86.7%	56.7%	16.7%	100.0%	100.0%	93.8%
Ascites-mNGS	92.9%	NA	100.0%	NA	80.0%	100.0%	100.0%	90.0%
Ascites-CMT	28.0%	NA	100.0%	NA	40.0%	100.0%	100.0%	75.0%

Abbreviations: BALF, bronchoalveolar lavage fluid; ETA, endotracheal aspirate; CSF, cerebrospinal fluid; mNGS, metagenomic next-generation sequencing; CMT, conventional microbiological testing; PPV, positive predictive value; NPV, negative predictive value; NA, not available.

Supplementary Table 2. Clinical characteristics between immunocompetent and immunocompromised patients

	immunocompetent (n=166)	immunocompromised. (n=68)	P value
Age (years), median (IQR)	58.0 (46.8, 71.0)	54.0 (39, 65.75)	0.032
Gender (female), n (%)	52 (31.3)	25 (36.8)	0.421
Type of immunocompromised status, n (%)			
Chemotherapy or neutropenia	NA	7 (10.3)	NA
Prolonged corticosteroid therapy	NA	20 (29.4)	NA
Hematologic malignancy	NA	13 (19.1)	NA
Immunosuppressive therapy after solid organ transplantation	NA	28 (41.2)	NA
The severity of disease			
SOFA score, median (IQR)	10.0 (8.0, 13.0)	11.0 (8.3, 13.0)	0.284
APACHE II score, median (IQR)	23.0 (20.0, 26.0)	23.0 (20.0, 26.0)	0.592
Invasive mechanical ventilation, n (%)	138 (83.1)	55 (80.9)	0.618
Use of vasopressors, n (%)	104 (62.7)	38 (55.9)	0.336
CRRT or ECMO, n (%)	32 (19.3)	29 (42.6)	<0.001
Laboratory findings			
White blood cell count (10^9/L), median (IQR)	11.21 (7.81, 15.32)	9.4 (5.68, 17.33)	0.215
Lymphocyte count (10^6/L), median (IQR)	800 (500, 1200)	435 (228, 863)	<0.001
C-reactive protein (mg/L), median (IQR)	100.07 (49.0, 200.87)	96.0 (43.05, 146.83)	0.437
Procalcitonin (ng/mL), median (IQR)	1.63 (0.30, 7.42)	0.99 (0.30, 5.02)	0.476
Mixed infection, n (%)	62 (37.3)	31 (45.6)	0.242
Outcomes			
ICU LOS (days), median (IQR)	13 (6.8, 21.3)	13.5 (7.0, 24.8)	0.515
ICU death, n (%)	71 (42.8)	39 (57.4)	0.042
Hospital LOS (days), median (IQR)	21.0 (11.0, 34.25)	28.0 (14.0, 37.8)	0.068
Hospital death, n (%)	74 (44.6）	41 (60.3)	0.029

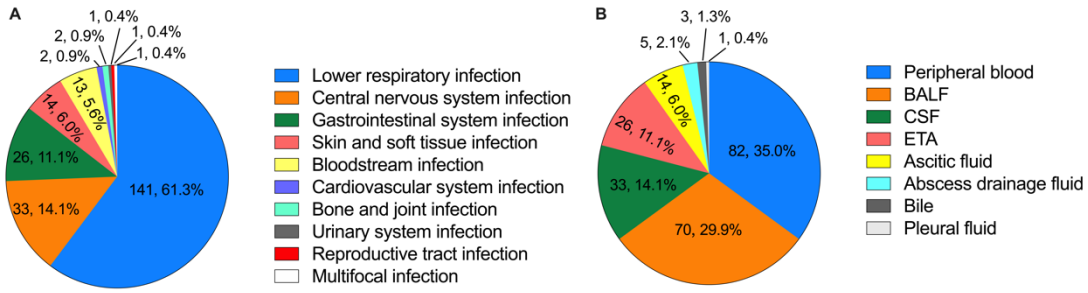
Abbreviations: IQR, interquartile range; SOFA score, Sequential Organ Failure Assessment score; APACHE II score, Acute Physiology and Chronic Health Evaluation II score; CRRT, continuous renal replacement therapy; ECMO, extracorporeal membrane oxygenation; NA, not available; LOS, length of stay.



Supplementary Figure 1. Flow chart of patient selection, classification and comparison.

From 358 patients, a total of 234 were selected for further analysis. Diagnostic performance of bacterial and fungal pathogens using mNGS and CMT methods were compared in a pairwise manner. Differences in the pathogen spectrum between groups were compared, and the impact of mNGS results on antibiotic treatment was discussed in groups. # One sample was excluded because the GC content > 45%, and the other 3 samples were excluded because the low library concentration.

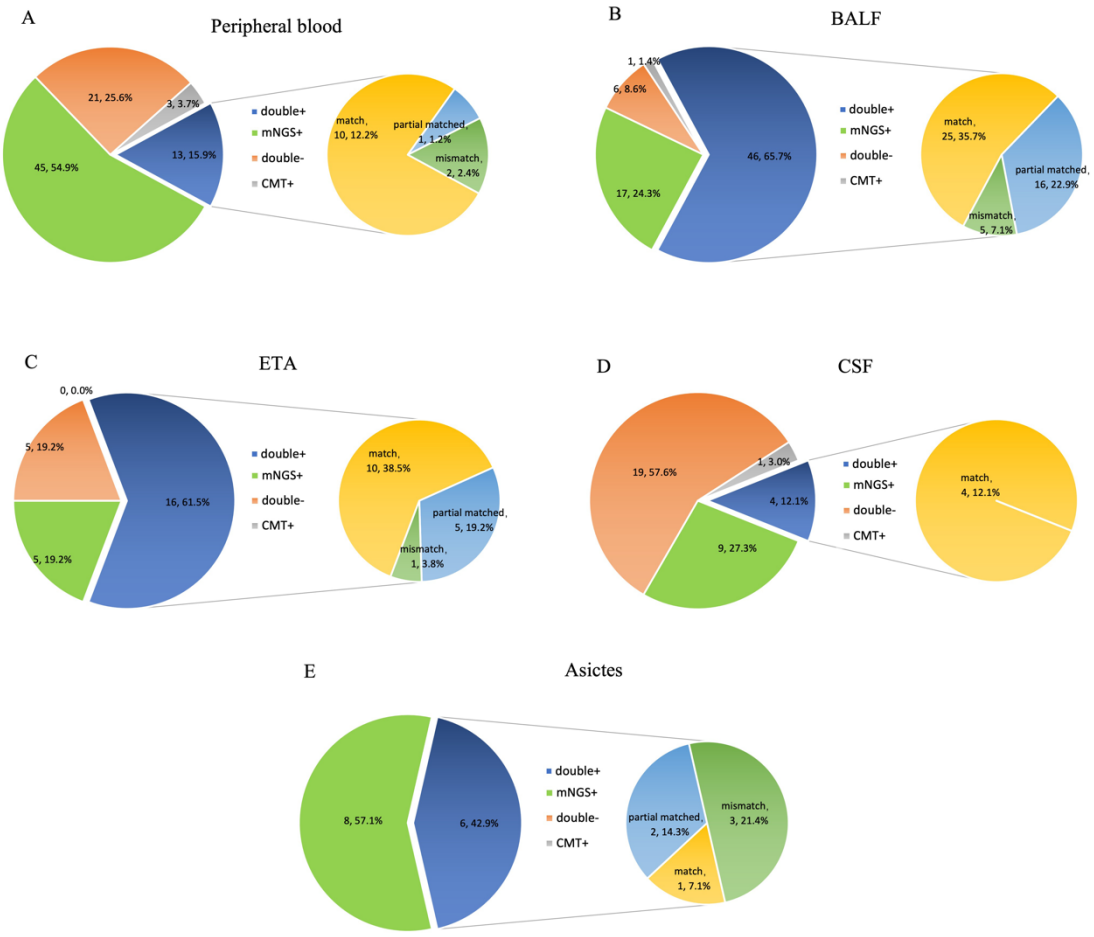
Abbreviations: mNGS, metagenomic next-generation sequencing; CMT, conventional microbiological testing.



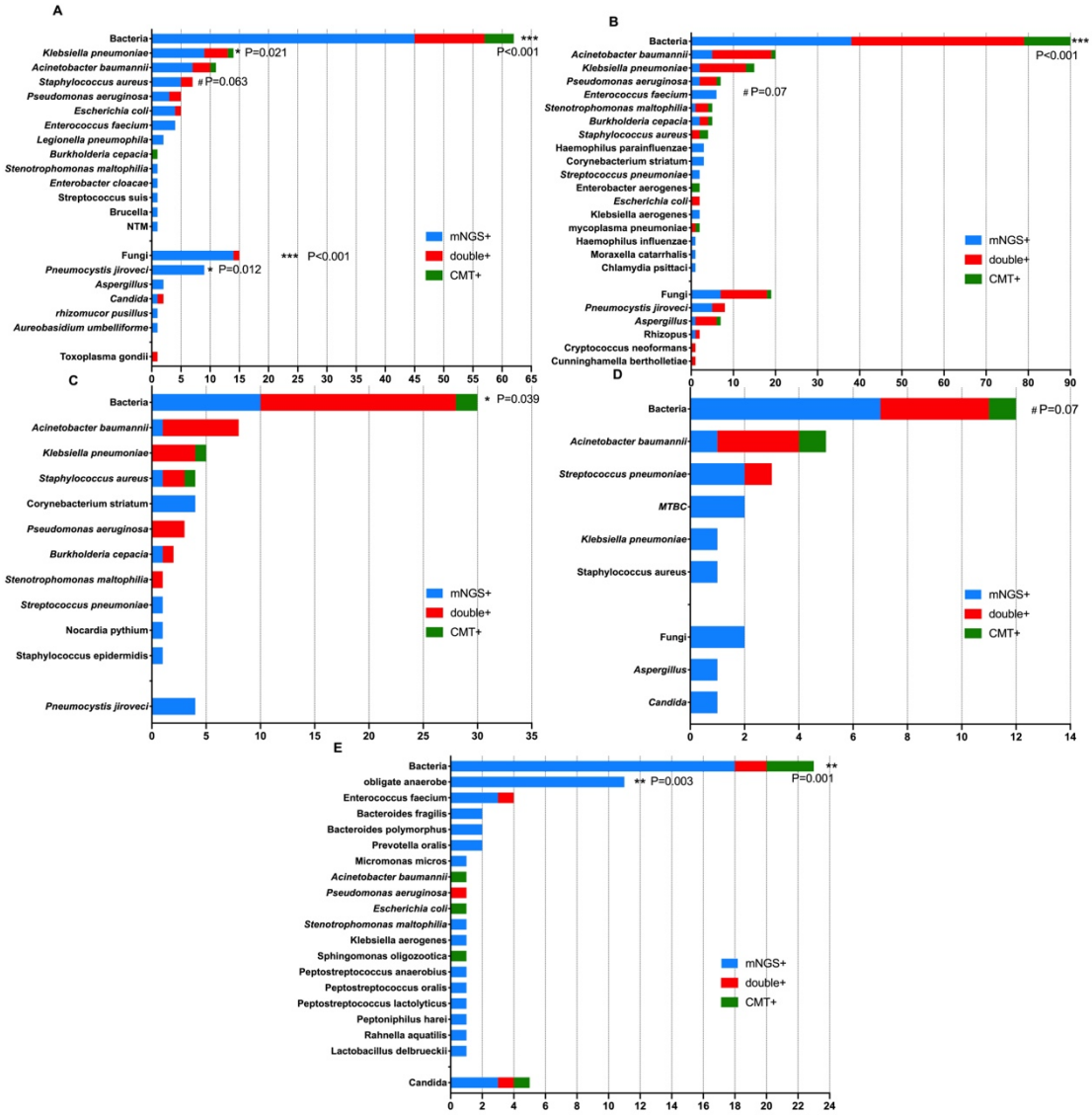
Supplementary Figure 2. The distribution of the infection sites and clinical specimens in the study.

(A) Pie chart demonstrated infection type of patients based on retrospective diagnosis.

(B) Pie chart demonstrated specimen types submitted for testing.

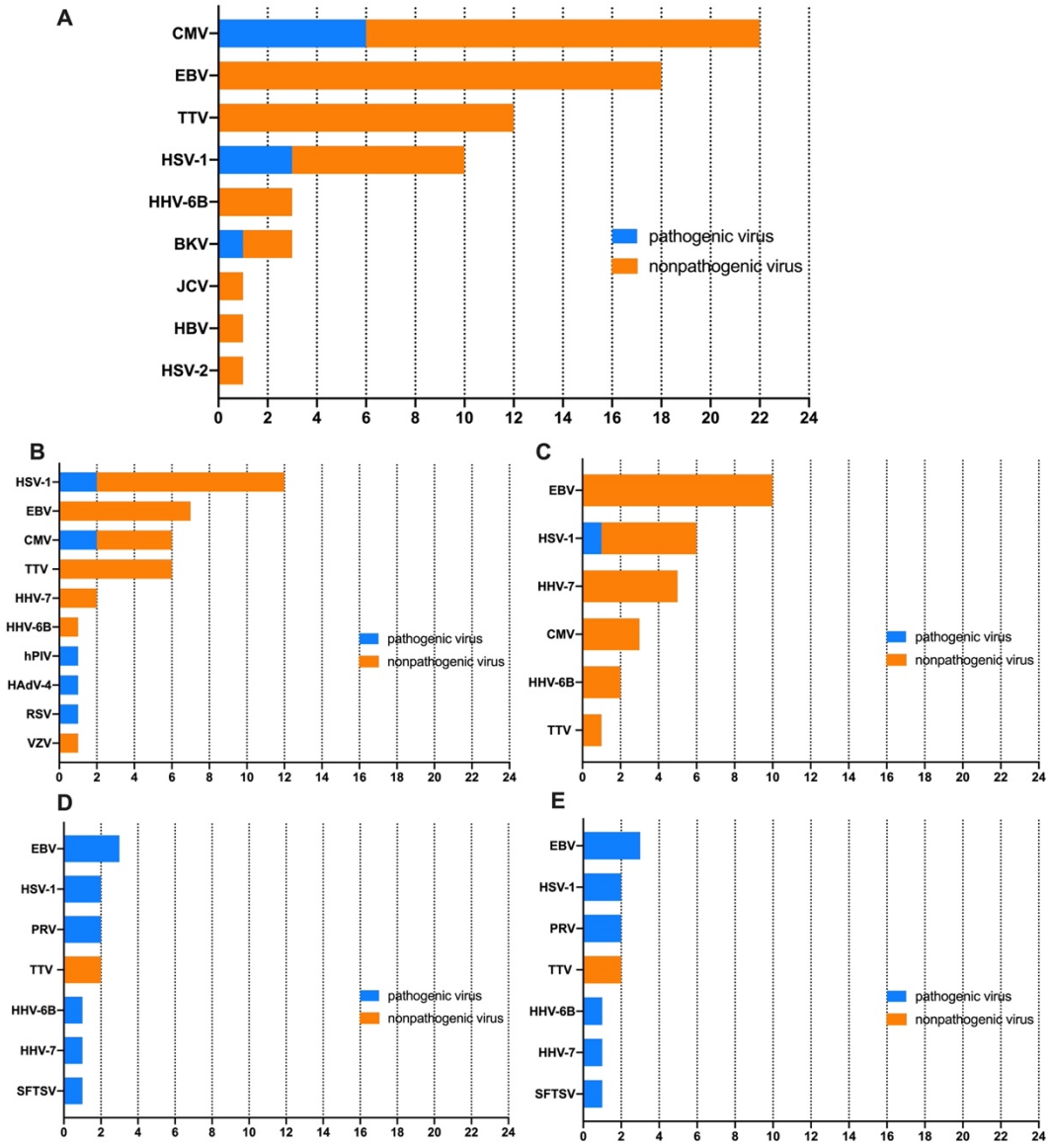


Supplementary Figure 3. Concordance analysis between mNGS and CMT method for bacterial and fungal detection in peripheral blood **(A)**, BALF **(B)**, ETA **(C)**, CSF **(D)**, ascites **(E)**. Abbreviations: mNGS, metagenomic next-generation sequencing; CMT, conventional microbiological testing; BALF, bronchoalveolar lavage fluid; ETA, endotracheal aspirate; CSF, cerebrospinal fluid.

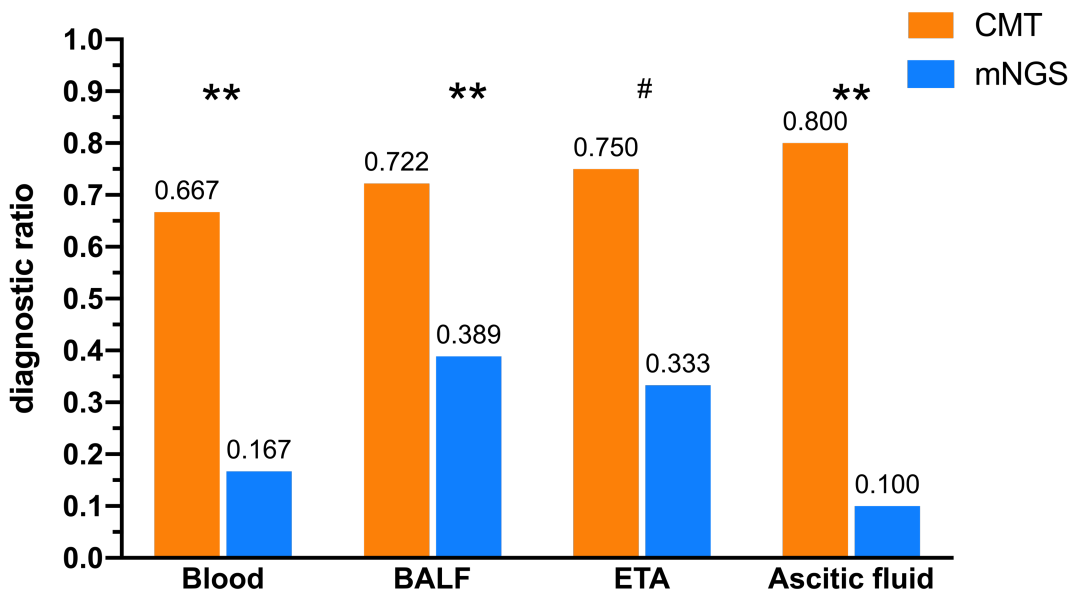


Supplementary Figure 4. The overlap of positivity between mNGS and CMT methods for specific species in peripheral blood **(A)**, BALF **(B)**, ETA **(C)**, CSF **(D)**, ascites **(E)**.

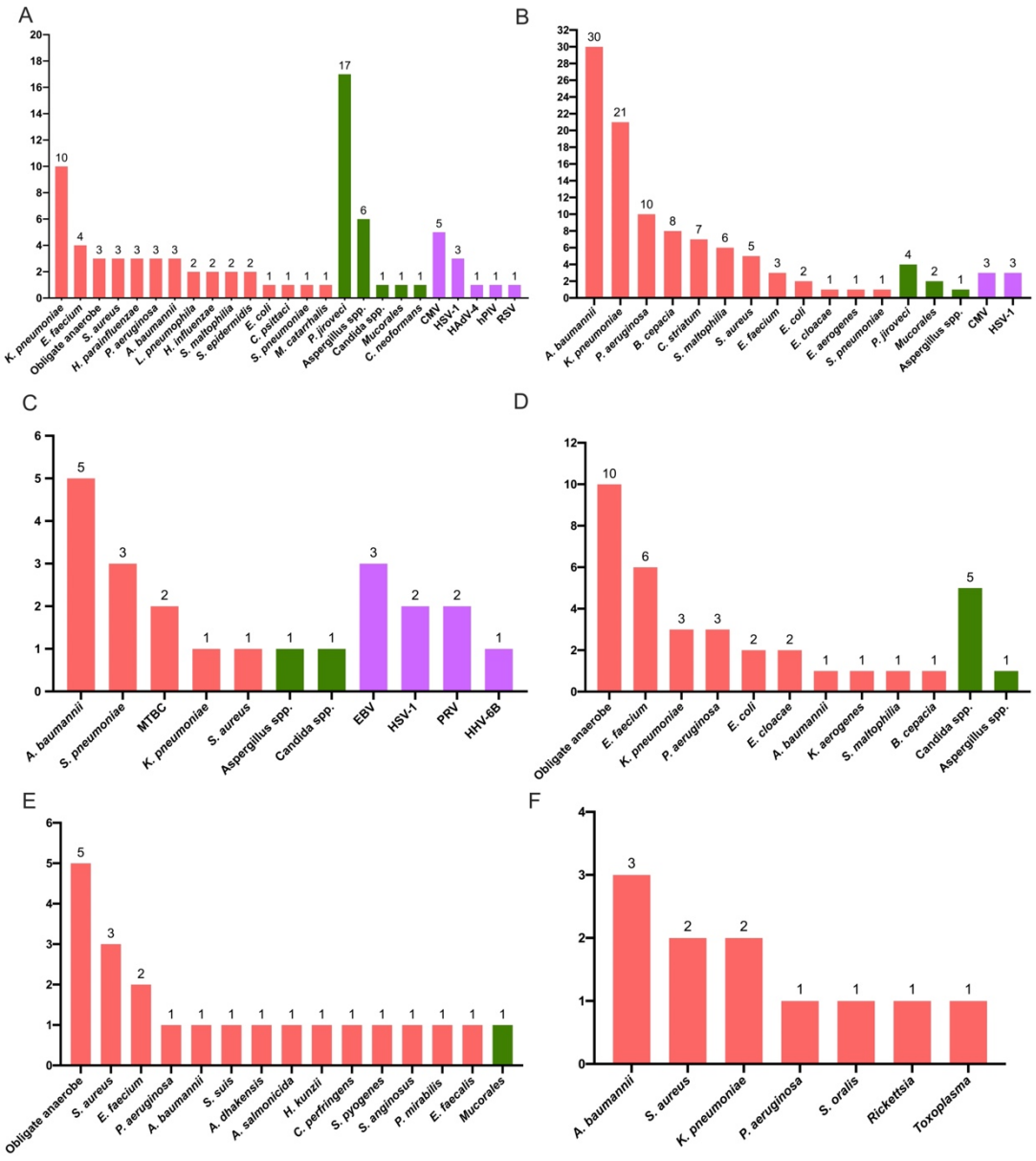
* The pathogens were observed to have a higher positive detection by mNGS than by CMT methods with statistical difference (P<0.05). ***The pathogens were observed to have a higher positive detection by mNGS than by CMT methods with statistical difference (P<0.001). # The pathogens were observed to have a higher positive detection by mNGS than by CMT methods, although the difference was not significant (P>0.05). Abbreviations: mNGS, metagenomic next-generation sequencing; CMT, conventional microbiological testing.



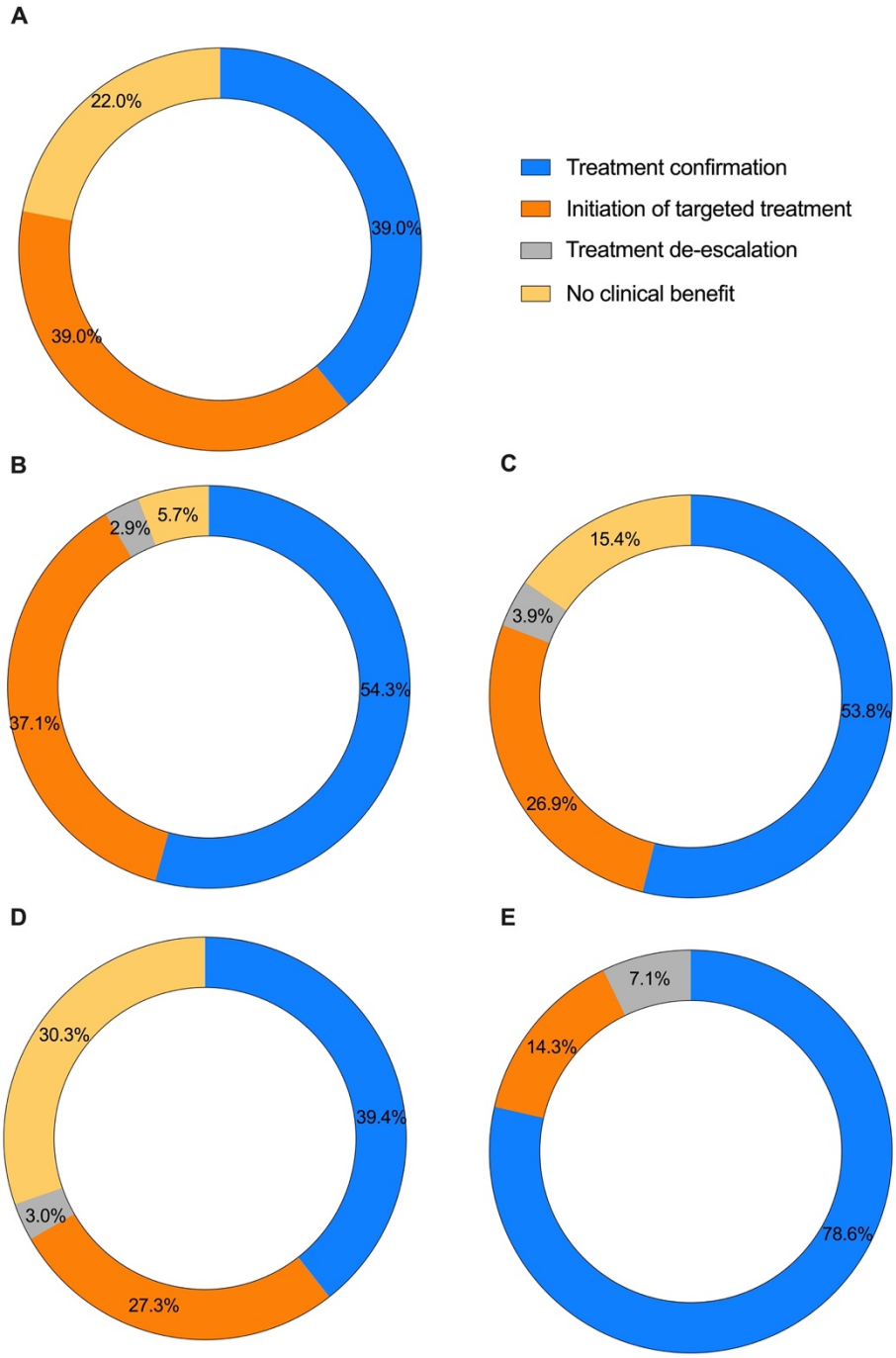
Supplementary Figure 5. Detection of viruses in different specimen types by mNGS method. **(A)** Peripheral blood; **(B)** BALF; **(C)** ETA; **(D)** CSF; **(E)** Ascites. Abbreviations: mNGS, metagenomic next-generation sequencing; BALF, bronchoalveolar lavage fluid; ETA, endotracheal aspirate; CSF, cerebrospinal fluid; HSV-1, Herpes simplex virus type 1; CMV, Cytomegalovirus; EBV, Epstein-Barr virus; TTV, torque teno virus; hPIV, human parainfluenza virus; PRV, Pseudorabies virus (suid herpesvirus 1); SFTSV, Severe fever with thrombocytopenia syndrome virus; HAdV-4, human adenovirus type four; BKV, BK polyomavirus; HHV-6B, human herpesvirus 6B; VZV, varicella-zoster virus; JCV, JC polyomavirus; HBV, hepatitis B.



Supplementary Figure 6. Comparison of the diagnostic ratio of mixed infection between mNGS and CMT in different kinds of specimens. ** P value < 0.01. # The diagnostic ratio of mixed infection by mNGS method was higher than that by CMT method in ETA, although the difference was not significant (P=0.10). Abbreviations: mNGS, metagenomic next-generation sequencing; CMT, conventional microbiological testing.



Supplementary Figure 7. Pathogen Spectrum in the different disease groups. (A) Community-acquired pneumonia. (B) Hospital-acquired pneumonia. (C) Central nervous system infection. (D) Gastrointestinal system infection. (E) Skin and soft tissue infection. (F) Bloodstream infection. Abbreviations: CMV, Cytomegalovirus; HSV-1, Herpes simplex virus type 1; HAdV-4, human adenovirus type four; hPIV, human parainfluenza virus; RSV, respiratory syncytial virus; EBV, Epstein-Barr virus; PRV, Pseudorabies virus (suid herpesvirus 1); HHV-6B, human herpesvirus 6B.



Supplementary Figure 8. Impact on antibiotic treatment of mNGS in various specimens. The clinical impact was divided into four aspects, which were showed by four different colors. **(A)** Peripheral blood; **(B)** BALF; **(C)** ETA; **(D)** CSF; **(E)**Ascities. Abbreviations: mNGS, metagenomic next-generation sequencing; BALF, bronchoalveolar lavage fluid; ETA, endotracheal aspirate; CSF, cerebrospinal fluid.