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RESEARCH ARTICLE

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Distinction of clinical features and microbiological methods between *Chlamydia psittaci* and *Legionella pneumophila* pneumonia confirmed by metagenomic next-generation sequencing

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ABSTRACT

Objectives: Detection and diagnosis of *Chlamydia psittaci* (*C. psittaci*) pneumonia is often overlooked due to conventional methods limitations and similarity to other atypical community acquired pneumonia (CAP). Using mNGS, we aimed to distinguish psittacosis from legionellosis for early *C. psittaci* pneumonia diagnosis and better prognosis.
Methods: Thirty-seven patients diagnosed with atypical CAP were enrolled in this retrospective study, including 14 *C. psittaci* pneumonia and 23 *Legionella pneumophila* (*L. pneumophila*) pneumonia. We collected and compared baseline, lab results, radiology imaging, conventional microbiological methods and more importantly, mNGS results of clinical samples, as well as the treatments and prognosis between psittacosis and legionellosis.
Results: Patients with *C. psittaci* and *L. pneumophila* had similar symptoms and were presented with high levels of inflammatory markers. However, patients with *C. psittaci* pneumonia were more likely to have exposure to birds or parrots [11 (78.6%) vs. 2 (8.7%), $p<0.001$], had higher proportions of fever and chill ($p=0.015$ and 0.035), higher levels of hemoglobin and albumin ($p=0.002$ and 0.018) compared with those with *L. pneumophila*. Of 14 *C. psittaci* patients, only one had positive IgM antibody, with no positive cultures. Early identification of pathogens by mNGS method contributed to timely antibiotics' adjustment and better outcomes then, yet with similar hospital mortality between two groups [7.1% (1/14) vs. 34.8% (8/23), $p=0.112$].
Conclusion: Early mNGS detection of atypical pathogens in multiple samples improves on traditional methods, promptly adjust empirical antimicrobial treatment to pathogen-targeted antibiotics, further improve prognosis.

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KEYWORDS
Chlamydia psittaci; Legionella; community-acquired pneumonia (CAP); metagenomic next-generation sequencing (mNGS)

1. Introduction

Community-acquired pneumonia (CAP) remains a common and worldwide infectious cause for admission to the intensive care unit (ICU), and a common cause of morbidity and mortality [1,2]. In many countries, atypical pathogens, like *Legionella pneumophila* (*L. pneumophila*), Mycoplasma pneumoniae, Chlamydia pneumoniae, or Chlamydophila psittaci (*C. psittaci*), are main pathogens of CAP [3], and even could be the most prevalent etiology in China [1]. Of these, C.psittaci is only responsible for less than 1% of the pathogens among CAP patients [4]. Due to the reason that psittacosis-related tests are not typically included in routine microbial diagnostic examinations, and the traditional serological tests and pathogenic cultures often don't provide a definitive result, the detection and diagnosis of *C. psittaci* pneumonia is often underestimated and overlooked. In some patients, the disease may deteriorate rapidly to severe pneumonia, acute respiratory distress syndrome (ARDS), multiple organ failure, and even death [4] due to untimely diagnosis and improper treatments. *L. pneumophila* is another

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atypical pathogen that cause CAP and account for nearly 5~15% of all [2]. The manifestations of *L. pneumophila* pneumonia is often mild or moderate, but when it turns into severe pneumonia, the outcome is usually fatal, with the mortality rates up to nearly 30% [2]. However, the clinical presentations and examination findings of patients with *C. psittaci* and *L. pneumophila* are diverse and similar. The common symptoms are nonspecific from the mild to the severe, including fever, chills, cough, phlegm, dyspnea and fatigue, with some extrapulmonary manifestations [5]. Radiological imaging of patients with *C. psittaci* and *L. pneumophila* is variable and atypical, including ground-glass opacities, consolidation, or pleural effusion [6,7]. All of the above often makes *L. pneumophila* pneumonia confused with those of *C. psittaci*. Consequently, rapid deterioration may occur due to delayed diagnosis and inappropriate antipathogen therapy. Therefore, early and accurate etiological diagnosis is vital in CAP patients.

Recently, the novel method of metagenomic next-generation sequencing (mNGS) has been applied in all potentially infectious diseases [8,9], regardless of microorganism species [10]. It allows thousands to billions of DNA fragments to be simultaneously sequenced [8]. Compared with conventional tests, unbiased sampling is the biggest advantage of mNGS, enabling broad identification of known or unexpected pathogens, or even new organisms [11]. Thanks to the technology of mNGS, the reported number of *C. psittaci* pneumonia cases has been progressively increasing in China [12–18]. However, detailed comparisons of early clinical features between pneumonia infected by *C. psittaci* and *L. pneumophila* based on mNGS results have not been reported yet.

In this study, a retrospective analysis was performed to primarily explore the differences in clinical characteristics, lab results, radiology imaging, serological pathogen results, and more importantly, mNGS results of clinical samples as well as the treatments and prognosis between CAP infected by *C. psittaci* and *L. pneumophila*. Secondly, we also aimed to develop a simple multi-model to guide in early differentiation of *C. psittaci* and *L. pneumophila* pneumonia.

2. Materials and methods

2.1. Study design and participants

A retrospective, monocenter, observational study was conducted at Peking Union Medical College Hospital (PUMCH)from January 2021, to May 2023. A total of 14 patients of CAP due to *C. psittaci* and 23 patients of CAP due to *L. pneumophila* were finally enrolled if they met the following criteria: 1) age ≥ 18 years; 2) diagnosed with atypical CAP according to the current guideline [19,20] and 3) positive results of metagenomic next-generation sequencing (mNGS) examinations from samples of bronchoalveolar lavage fluid (BALF) or serum or sputum. Exclusion criteria were as follows: 1) mNGS examinations were performed more than 72h from admission to the hospital; 2) pregnant; 3) missing medical records. Figure 1 shows the study flowchart. This study was approved by the Ethics Committee of the PUMCH (reference number: K23C3432), and written informed consent was waived due to the anonymized

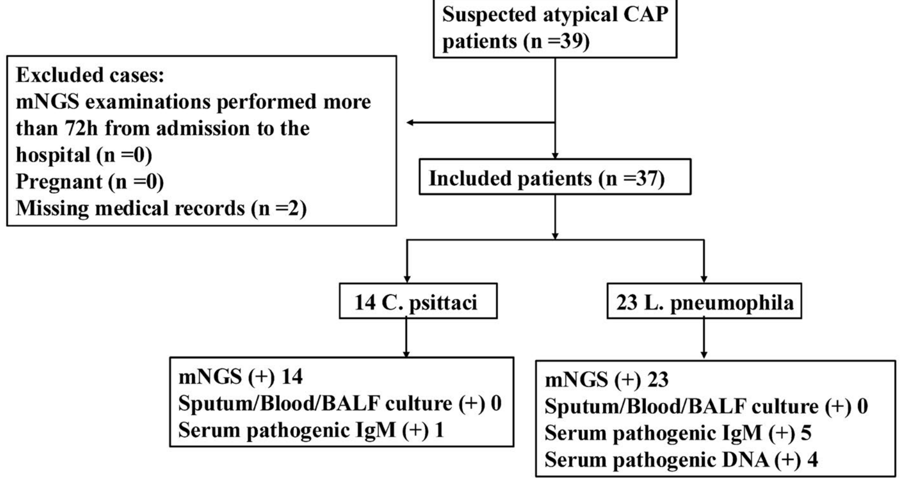

Figure 1. Flowchart of the study.
Note: CAP, community-acquired pneumonia; BALF, bronchoalveolar lavage fluid; *C. psittaci*, *Chlamydia psittaci*; *L. pneumophila*, *Legionella pneumophila*; mNGS, metagenomic next generation sequencing.

retrospective nature of the analysis. The study strictly complied with the Declaration of Helsinki.

2.2. Data collection

Baseline clinical data, laboratory tests and imaging findings of each patient upon admission were retrieved from the electronic medical system. 1) Baseline characteristics included data on demographic characteristics (e.g. age and gender), comorbidities, history of bird contact, clinical symptoms and vital signs, and disease severity using Sequential Organ Failure Assessment (SOFA) scores and Acute Physiology and Chronic Health Evaluation (APACHE II). 2) Laboratory tests results included routine blood tests and tests for blood chemistry, liver and kidney function, myocardial injury markers, inflammatory mediators, procalcitonin (PCT) levels, and blood coagulation (e.g. D-Dimer). 3) Imaging findings included the locations and characteristics of lung lesions, e.g. extension of infiltrates, pleural effusion.

Pathogenic examinations included mNGS results, routine pathogenic examinations and serology of respiratory. 1) For mNGS detections, clinical sample of BALF, blood, or sputum were collected by aseptic processing procedures and sent immediately under cryogenic refrigeration to laboratory department of PUMCH. BALF at the lung lesions was obtained through tracheoscopy by an experienced respiratory therapist in accordance with previous practice guideline for qualified BALF specimens [21]. 2) Routine pathogenic examinations included results of microbiological cultures from samples of sputum, blood and/or BALF. 3) Serology of respiratory consisted of the results of serum pathogenic IgM/IgG antibody of *C. psittaci* or *L. pneumophila*.

Treatment methods including respiratory support, empirical antibiotic treatment according to the guidelines [19,20], which were adjusted based on the conventional microbiological tests or mNGS results combined with the inflammatory biomarkers and radiology. Other treatments data including extracorporeal membrane oxygenation (ECMO), continuous renal replacement therapy (CRRT), and the administrations of glucocorticoid, immunoglobulin, or vasopressor administrations.

The following clinical outcomes were assessed: intensive care unit (ICU) admission, mechanical ventilation, the length of hospital stay of survivors, the length of ICU stay, the length of mechanical ventilation, and hospital death.

2.3. Metagenomic next-generation sequencing analysis

mNGS was performed by an experienced technician. The procedure of mNGS included nucleic acid extraction, library construction, sequencing, and bioinformatic analysis [22]. DNA was extracted from specimens of BALF, sputum, or blood which were promptly stored in sterile pipes using a QIAamp® UCP Pathogen DNA Kit (Qiagen) following the manufacturer's instructions. Human DNA was removed using Benzonase (Qiagen) and Tween20 (Sigma) [23]. cDNA was generated using reverse transcriptase and dNTPs (Thermo Fisher).

Libraries were constructed for the DNA and cDNA samples using a Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA) [24]. A Qubit dsDNA HS Assay kit followed by a High Sensitivity DNA kit (Agilent) on an Agilent 2100 Bioanalyzer was utilized to evaluate the library quality. Then, library pools were loaded onto an Illumina Nextseq CN500 sequencer for 75 cycles of single-end sequencing to generate approximately 20 million reads for each library. For negative controls, we prepared peripheral blood mononuclear cell (PBMC) samples with 10^5 cells/mL from healthy donors in parallel with each batch, using the same protocol, and sterile deionized water was extracted alongside the specimens to serve as non-template controls [22,25,26].

Using trimmomatic, low-quality reads, adapter contamination, duplicate reads, and those shorter than 50 bp were removed [26]. Low complexity reads were removed by Kcomplexity with default parameters. Human sequence data were identified and excluded by mapping to a human reference genome (hg38) using Burrows-Wheeler Aligner software [27]. Pathogen lists was selected according to three references: 1) Johns Hopkins ABX Guide; 2) Manual of Clinical Microbiology (12th Edition); and 3) clinical case reports or research articles published in current peer-reviewed journals. The final database consisted of about 13,000 genomes. Microbial reads were aligned to the database with SNAP v1.0beta.18. Virus-positive detection results (DNA or RNA viruses) were defined as the coverage of three or more non-overlapping regions on the genome. For the identification of bacteria, fungi and parasites, a positive detection was reported for a given species or genus if the reads per million (RPM) ratio (RPM-r) was ≥ 5, where the RPM-r was defined as the RPM corresponding to a given species or genus in the clinical sample divided by the RPM in the negative control [24]. In addition, if the species or genus appeared in non-template controls, the RPM of microorganisms sharing a genus or family designation would be reduced to minimize cross-species misalignments among closely related microorganisms. A penalty of 5% was used for species [28]. Hence, non-specific reads would be filtered and only the reads mapped to unique species could be classified to the species level.

For bacteria (except mycobacteria), fungi (except molds) and viruses, a certain microbe was considered as clinically significant microbes (CSMs) when its relative abundance was >30% at the species level with literature-based evidence of pulmonary pathogenicity. Oral commensals were not considered as CSM regardless of the relative abundance unless proven otherwise or strongly suggested by clinical manifestation. Molds were considered to be CSMs when the stringently mapped read number (SMRN) at the species level was >10 and literature supported their possible pathogenicity in the lungs. Mycobacteria were defined as positive in mNGS as long as the SMRN at the species level was >3, given the low possibility for contamination and the difficulty of DNA extraction [29].

The mNGS results included the list of suspected pathogenic pathogens and list of suspected microecology, which both consisted of number of sequences reads, RPM, relative abundance (%), coverage (%), dispersion and sequencing depth. A detailed description of the definition of each mNGS parameter can be found in the Supplementary material.

2.4. Statistical analysis

Continuous variables were expressed as mean ± standard deviation and compared using Student's t-tests in normally distributed data, while non-normally distributed data are expressed as median (interquartile range) and tested using the Mann-Whitney U tests. For categorical variables, data were presented as frequencies and percentages, and compared using Fisher's exact tests due to the small number less than 40 of our study. All the statistical analyses were conducted using SPSS version 27.0 (IBM Corporation, Armonk, New York, USA). A P – value < 0.05 was considered statistically significant.

3. Results

3.1. Comparison of demographic and clinical characteristics

During the study period, 37 patients with 14 *C. psittaci* and 23 *L. pneumophila* pneumonia were included in the final analysis (Figure 1). Demographic characteristics was provided in Table 1. Of them, patients with

Table 1. Comparison of demographic and clinical characteristics between patients with *C. psittaci* and *L. pneumophila*.

Variable	Total (n = 37)	*C. psittaci* (n = 14)	*L. pneumophila* (n = 23)	P-value
Age, mean ± SD, years	56 ± 17	58 ± 16	56 ± 18	0.967
Age ≥ 65 years, n (%)	12 (32.4)	4 (28.6)	8 (34.8)	1.000
Male, n (%)	25 (67.6)	11 (78.6)	14 (60.9)	0.306
History of contact, n (%)	13 (35.1)	11 (78.6)	2 (8.7)	<0.001*
Comorbidities, n (%)				
Hypertension	14 (38.9)	6 (42.9)	8 (36.4)	0.738
Diabetes	10 (27.8)	4 (28.6)	6 (27.3)	1.000
Coronary heart disease	5 (13.5)	1 (7.1)	4 (17.4)	0.630
Chronic liver disease	2 (5.6)	0 (0.0)	2 (9.1)	0.511
Chronic kidney disease	4 (11.1)	0 (0.0)	4 (18.2)	0.141
Cerebrovascular disease	2 (5.6)	0 (0.0)	2 (9.1)	0.511
Maligancy	6 (16.7)	1 (7.1)	5 (22.7)	0.370
Vital signs, mean ± SD				
Respiration rate, median, breaths per minute	20.3 ± 3.6	21.5 ± 4.3	19.6 ± 2.9	0.155
PaO_2/FiO_2, mm	190.6 ± 126.6	137.1 ± 102.5	216.0 ± 131.4	0.126
Pulse, beats per minute	105.8 ± 20.8	106.8 ± 20.6	105.1 ± 21.5	0.823
Mean arterial pressure, mmHg	93.3 ± 17.6	99.4 ± 20.1	89.2 ± 14.8	0.095
Temperature, ◦C	37.2 ± 1.2	37.8 ± 1.3	36.7 ± 0.9	0.005*
Symptom, n (%)				
Fever	29 (78.4)	14 (100)	15 (65.2)	0.015*
Chills	8 (21.6)	6 (42.9)	2 (8.7)	0.035*
Cough	22 (59.5)	10 (71.4)	12 (52.2)	0.314
Expectoration	20 (54.1)	8 (57.1)	12 (52.2)	1.000
Dyspnea	19 (51.4)	6 (42.9)	13 (56.5)	0.508
Sore throat	7 (18.9)	3 (21.4)	4 (17.4)	1.000
Myalgia	7 (18.9)	2 (14.3)	5 (21.7)	0.687
Fatigue	14 (37.8)	3 (21.4)	11 (47.8)	0.166
Vomit / Nausea	7 (18.9)	3 (21.4)	4 (17.4)	1.000
Diarrhea	3 (8.1)	1 (7.1)	2 (8.7)	1.000
Nervous system symptoms	4 (10.8)	0 (0.0)	4 (17.4)	0.276
Dry rales	3 (8.1)	2 (14.3)	1 (4.3)	0.544
Moist rales	10 (27.0)	4 (28.6)	6 (26.1)	1.000
Disease severity scores, mean ± SD				
SOFA score	6.1 ± 2.1	5.6 ± 2.2	6.5 ± 2.0	0.216
APACHE II score	11.1 ± 6.5	9.7 ± 5.5	12.0 ± 7.0	0.308

Note: APACHE II, Acute physiology and chronic health evaluation; *C. psittaci*, *Chlamydia psittaci*; *L. pneumophila*, *Legionella pneumophila*; PaO_2/FiO_2, partial pressure of oxygen in the arterial blood/fraction of inspired oxygen; SOFA, Sequential Organ Failure Assessment.

C. psittaci pneumonia were more likely to have exposure to birds or parrots [11 (78.6%)] than patients with *L. pneumophila* pneumonia [2 (8.7%)]. In detail, 7/14 patients with *C. psittaci* kept pigeons or parrots at home, 4/14 had neighbors keeping parrots. No significant differences between the two groups were found regarding age, gender, and comorbidities.

As to symptoms and vital signs, fever (78.4%) was the most common symptom among all patients, followed by cough (59.5%), expectoration (54.1%) and dyspnea (51.4%). Compared with patients with *L. pneumophila*, those with *C. psittaci* had higher proportions of fever (100.0% vs. 65.2%, $p=0.015$) and chill (42.9% vs. 8.7%, $p=0.035$), as well as higher levels of temperature upon admission (37.8 ± 1.3 °C vs. 36.7 ± 0.9 °C, $p=0.005$). However, no significant differences were found in PaO_2/FiO_2 ($p=0.126$), SOFA ($p=0.216$) and APACHE II scores ($p=0.308$).

3.2. Laboratory tests and imaging findings upon admission

On admission, both patients with *C. psittaci* and *L. pneumophila* pneumonia had elevated levels of white blood count (WBC), neutrophil percentage, lactate dehydrogenase, NT-proBNP, inflammatory markers (C-reactive protein and erythrocyte sedimentation rate), bacterial infection mediators (procalcitonin), and D-dimer with no significant differences found between groups (Table 2). However, patients with *C. psittaci* had significantly higher levels of hemoglobin (128.2 ± 16.9 vs. 103.0 ± 26.6, $p=0.002$), and albumin (38.5 ± 6.1 vs. 32.7 ± 6.9, $p=0.018$), compared with patients with *L. pneumophila*.

With regard to the imaging changes in patients with *C. psittaci* (Table 2, Figure 2), The most common imaging characteristics on chest CT scans on admission included ground-glass opacity (19/22, 86.4%) and consolidations (13/22, 59.1%). However, left lung

Table 2. Laboratory and radiographic characteristics at admission in patients with *C. psittaci* and *L. pneumophila*.

Laboratory findings	Total (n = 37)	*C. psittaci* (n = 14)	*L. pneumophila* (n = 23)	*P*-value
Blood routine, mean ± SD				
White blood cell count, $\times10^9$ /L	10.2 ± 5.1	10.2 ± 4.4	10.2 ± 5.7	0.977
Neutrophil percentage, %	82.3 ± 16.9	81.3 ± 9.3	83.0 ± 20.6	0.775
Lymphocyte percentage, %	10.7 ± 8.6	12.1 ± 6.2	9.7 ± 9.9	0.442
Hemoglobin, g/L	113.1 ± 27.3	128.2 ± 16.9	103.0 ± 26.6	0.002*
Platelet count, $\times10^9$ /L	174.5 ± 90.7	199.2 ± 61.0	158.1 ± 104.2	0.151
Blood biochemistry, mean ± SD or median (IQR)				
Aspartate aminotransferase, U/L	42.5 (23.3, 77.0)	79.0 (37.5, 277.5)	36.0 (21.0, 50.0)	0.158
Alanine aminotransferase, U/L	37.0 (14.0, 55.3)	41.0 (20.0, 78.0)	31.0 (14.0, 49.5)	0.276
Lactate dehydrogenase, U/L	482.3 ± 306.0	425.3 ± 142.8	515.9 ± 370.8	0.378
Total bilirubin, μmol/L	12.9 (9.4, 19.3)	11.5 (9.3, 21.5)	13.9 (9.4, 18.9)	0.889
Albumin, g/L	34.9 ± 7.1	38.5 ± 6.1	32.7 ± 6.9	0.018*
Serum urea nitrogen, mmol/L	6.0 (4.5, 9.9)	6.0 (4.1, 8.0)	6.3 (4.6, 16.0)	0.193
Creatinine, μmol/L	85.5 (59.5, 112.5)	84.0 (66.5, 112.0)	87 (57.0, 146.5)	0.807
Myocardial injury mediators, median (IQR)				
Creatine kinase, U/L	85.0 (26.5, 147.5)	92.5 (43.0, 672.3)	82.0 (22.0, 123.0)	0.308
Creatine kinase isoenzyme MB, U/L	0.7 (0.2, 3.2)	1.1 (0.2, 4.6)	0.7 (0.2, 2.3)	0.701
cTni	2.0 (0.0, 30.5)	0.4 (0.0, 24.0)	2.0 (0.0, 50.0)	0.735
NT-proBNP	1037.5 (335.3, 2419.5)	947.0 (57.0, 2005.0)	1108.0 (353.0, 2670.0)	0.372
Inflammatory mediators, mean ± SD				
C-reactive protein, mg/L	153.8 ± 100.2	159.8 ± 113.0	149.6 ± 93.0	0.775
Erythrocyte sedimentation rate, mm/h	64.4 ± 45.6	60.6 ± 30.9	66.6 ± 53.8	0.826
Bacterial Infection Mediators, median (IQR)				
Procalcitonin, μg/L	0.5 (0.2, 4.2)	0.2 (0.1, 3.0)	0.8 (0.3, 11.7)	0.169
Blood coagulation, mean ± SD or median (IQR)				
D-dimer, μg/mL	2.4 (1.3, 6.5)	3.5 (1.7, 7.3)	2.3 (1.1, 6.4)	0.611
Prothrombin time, sec	13.3 ± 1.9	13.2 ± 1.1	13.3 ± 2.2	0.913
APTT, sec	29.7 ± 5.5	30.3 ± 5.7	29.4 ± 5.5	0.676
International normalized ratio	1.1 ± 0.2	1.1 ± 0.1	1.1 ± 0.2	0.966
Radiographic characteristics				
Location	30 (83.8)	9 (64.3)	21 (95.5)	0.024*
Lesions in left lung, n (%)				
Lesions in right lung, n (%)	33 (91.7)	14 (100)	19 (86.4)	0.267
Lesions in bilateral lungs, n (%)	27 (75.0)	9 (64.3)	18 (81.8)	0.267
Number of lobes involved, medians (IQR)	3 ± 1	3 ± 1	3 ± 1	0.357
Image changes computed tomography scans				
Ground-glass opacity, n (%)	29 (80.6)	10 (71.4)	19 (86.4)	0.394
Consolidation, n (%)	24 (66.7)	11 (78.6)	13 (59.1)	0.292
Pleural effusion, n (%)	20 (55.6)	8 (57.1)	12 (54.5)	1.000
Interstitial changes, n (%)	4 (12.5)	0 (0.0)	4 (18.2)	0.283

Note: APTT, activated partial thromboplastin time; *C. psittaci*, *Chlamydia psittaci*; IQR, interquartile range; *L. pneumophila*, *Legionella pneumophila*; NT-proBNP, N-terminal pro-brain natriuretic peptide.

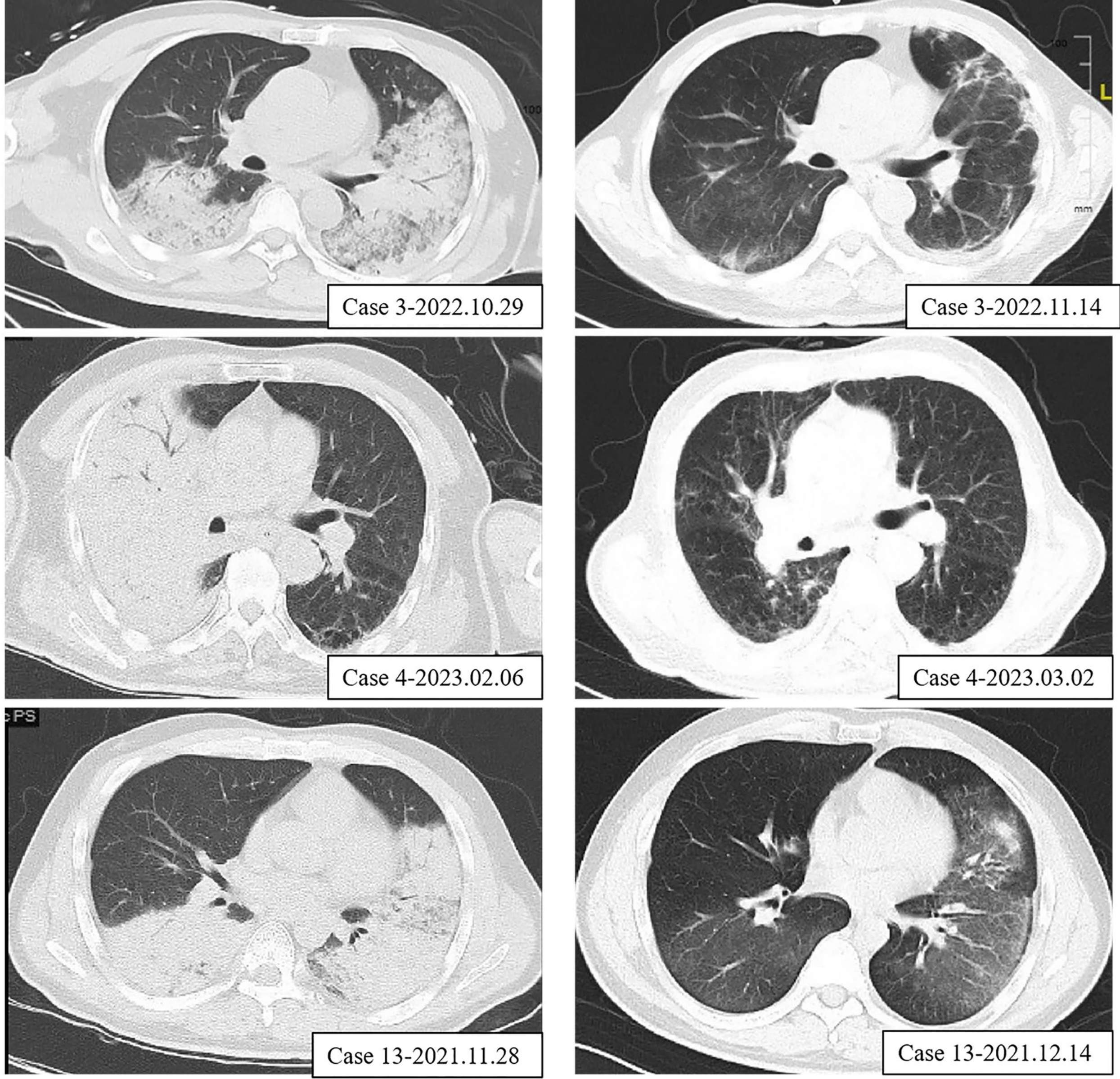

Figure 2. Chest computed tomography images of patients with *Chlamydia psittaci* before and after the treatment of tetracycline.

lesions on chest CT scans were less common in patients with *C. psittaci* than in those with *L. pneumophila* (64.3% vs. 95.5%, $p = 0.024$).

3.3. Pathogenetic findings

Distribution and classification of mNGS DNA results, conventional microbiological culture and IgM antibody for pathogens of patients with *C. psittaci* and *L. pneumophila* were presented in Tables 3 and 4 (other mNGS results for patients with *C. psittaci* in details, see Table S1). For *C. psittaci* detection, compared with conventional methods, of 14 positive mNGS detection for *C. psittaci* sequences, only 1/14 case showed positive staining of IgM antibody for *C. psittaci*, and no positive results was found from routine microbiology culture (Table 3). Besides, the mean time from sample collection to mNGS results was only 38 (24~62) h, which was much shorter than those of microbiology culture [64 (50.5~75) h] and IgM antibody detection [56 (34~111) h], $p = 0.035$, Table 3. Similarly, only 5/23 cases showed positive staining of IgM antibody for *L. pneumophila*, 4/23 showed positive results of *L. pneumophila* DNA of the blood samples, and no positive results were found from routine microbiology culture.

For CAP patients with *C. psittaci* and *L. pneumophila* infection, most of the positive mNGS results were both analyzed from BALF samples (53.3% and 65.4%,

Table 3. Results of mNGS and conventional pathogenic examinations in *C. psittaci* pneumonia patients.

	mNGS results				Microbiological culture				Pathogenic IgM/IgG antibody	
Case number	Type of specimen	Sequence number	Coinfected pathogens	Time to results (h)	Sputum culture	Blood culture	BALF culture	Time to results (h)	Serology of respiratory pathogens	Time to results (h)
Case 1	Sputum	51	/	22	Negative	Negative	Negative	50	CPN-IgG (+)	54
Case 2	BALF + Blood	20 + 54	Corynebacterium striatum, A.baumannii, EBV	50	Negative	Negative	A.baumannii	53	CPN-IgG (+)	33
Case 3	BALF*2	206 + 14	Corynebacterium striatum, A.baumannii	24	A.baumannii	Negative	Negative	78	Negative	34
Case 4	BALF	334	P.aeruginosa	44	Monilia albican	Negative	P.aeruginosa	67	CPN-IgG (+)	50
Case 5	Sputum	2	/	32	Negative	Negative	Negative	64	Negative	30
Case 6	Sputum	15088	/	58	Klebsiella pneumoniae	Negative	Negative	91	CPN-IgG (+)	81
Case 7	Blood	1	Hepatitis GB virus	80	Monilia albican	Negative	Negative	66	Cps-IgG (+)	56
Case 8	BALF	1	P.aeruginosa, K.pneumoniae	100	Negative	Negative	Negative	120	CPN-IgG (+)	126
Case 9	Blood	3	/	62	/	/	/	/	/	/
Case 10	Sputum	3	Human herpesvirus 7 and 6B	62	Negative	Negative	Negative	51	Negative	128
Case 11	BALF	196	/	24	Rapamycin, Near smooth candida	Negative	Rapamycin	72	Negative	126
Case 12	BALF	8	A.baumannii	24	Negative	Negative	Negative	48	CPN-IgG (+), Cps-IgG (+)	81
Case 13	Sputum	15	K.pneumoniae, Staphylococcus aureus, Tropheryma whipplei, Candida albicans, Human herpesvirus 7	24	Negative	Negative	Negative	48	Negative	96
Case 14	BALF	269	/	24	Negative	Negative	Negative	54	CPN-IgG (+), Cps-IgG (+), Cps-IgM(+)	34

Note: *A. baumannii*, *Acinetobacter baumannii*; BALF, bronchoalveolar lavage fluid; CPN, Chlamydia pneumoniae; Cps, *Chlamydia psittaci*; *C. psittaci*, *Chlamydia psittaci*; EBV: epstein-barr virus; IgM, immunoglobulin M; K.pneumoniae, Klebsiella pneumoniae; mNGS, metagenomic next-generation sequencing; *P. aeruginosa*, *Pseudomonas aeruginosa*.

Table 4. mNGS Results in patients with *C. psittaci* and *L. pneumophila*.

Type of specimen	*C. psittaci* ($n = 14$)	Numbers of reads with *C. psittaci* sequences				*L. pneumophila* ($n = 23$)	Numbers of reads with Legionnaires sequences			
		Min	Median	Mean	Max		Min	Median	Mean	Max
BALF	8 (53.3)	1	125	133	334	17 (65.4)	22	619	12248	141040
Blood	2 (13.3)	1	11	11	20	5 (19.2)	6	975	12394	58146
Sputum	5 (33.3)	2	15	3032	15088	4 (15.4)	36	1372	1380	2741
Total	15 (100.0)	1	20	1083	15088	26 (100)	6	693	10604	141040
P-value	0.403	0.476				0.403	0.986			

Note: BALF, bronchoalveolar lavage fluid; *C. psittaci*, *Chlamydia psittaci*; *L. pneumophila*, *Legionella pneumophila*; differences in the number of samples between two groups were calculated by Fisher's exact tests, while differences in the numbers of sequences reads among different types of samples in each group were calculated by Kruskal-*Wallis* test.

respectively), with no differences noted between the two groups ($p = 0.403$, Table 4 and Figure 3A). As to the numbers of *C. psittaci* or *L. pneumophila* sequences reads that detected in different clinical samples, the differences among different clinical samples were non-significant in both groups with the Kruskal-*Wallis* test due to the non-normal distributions ($p = 0.476$ and 0.986, respectively, Table 4 and Figure 3B). Through mNGS testing, we found that some patients were also infected by other pathogens (Figure 4). Compared with patients with *L. pneumophila*, those with *C. psittaci* more likely to be co-infected by Acinetobacter baumannii (3/14 vs. 1/23) and Corynebacterium striatum (2/14 vs. 1/23), Pseudomonas aeruginosa (2/14 vs. 2/23) while less likely to be infected by Cytomegalovirus (CMV) (1/14 vs. 9/23, Figure 4).

3.4. Treatments and clinical outcome

Respiratory support findings showed similar utilization rates of conventional oxygen therapy, high-flow nasal cannula, noninvasive and invasive ventilation (all

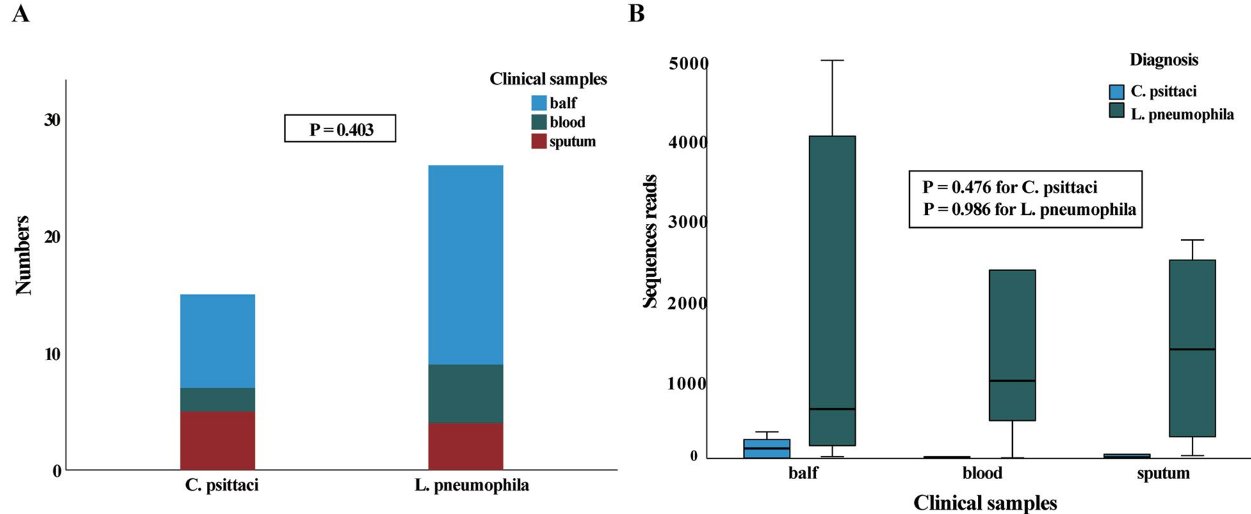

Figure 3. Distribution of clinical samples for mNGS and the numbers of sequences reads in different clinical samples of each group.

Note: A showed the distributions of clinical samples for mNGS testings in patients with *C. psittaci* and *L. pneumophila* infection, with no differences noted between the two groups ($P = 0.403$); while B showed the numbers of sequences reads in different clinical samples of each group, with no significance in both groups ($P = 0.476$ and 0.986, respectively). *C. psittaci*, *Chlamydia psittaci*; *L. pneumophila*, *Legionella pneumophila*.

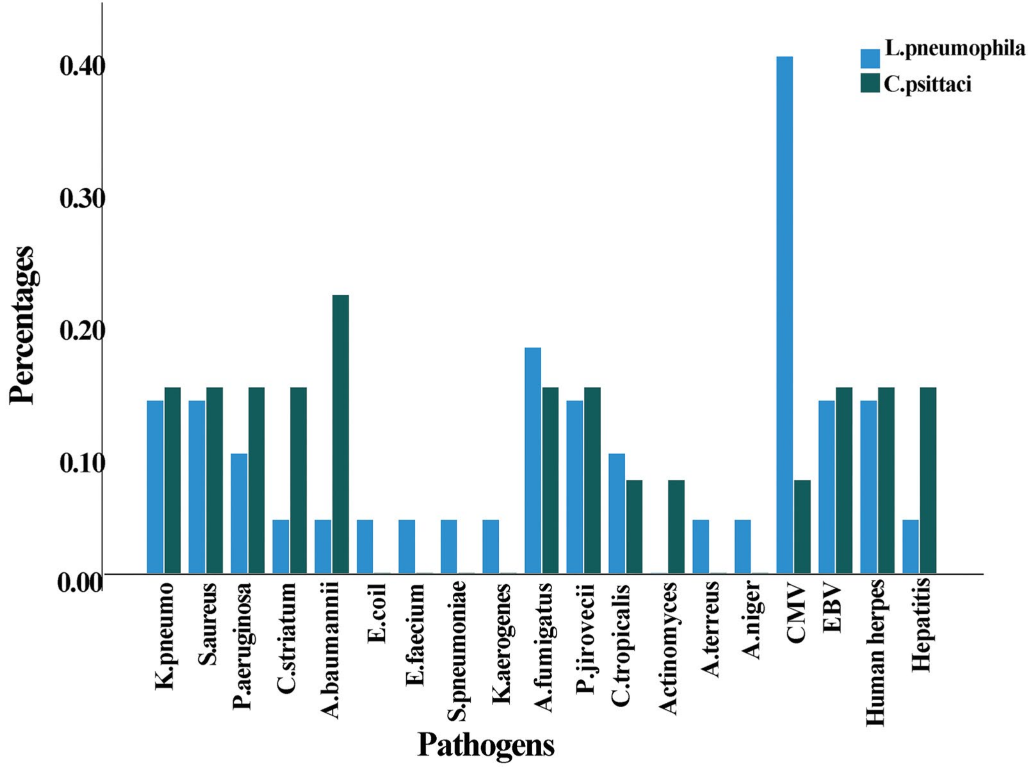

Figure 4. Coinfection with other pathogens based on mNGS method in patients with *C. psittaci* and *L. pneumophila* infection.

Note: *A. baumannii*, *Acinetobacter baumannii*; *A. fumigatus*, *Aspergillus fumigatus*; *A. niger*, *Aspergillus niger*; *A. terreus*, *Aspergillus terreus*; CMV, cytomegalovirus; *C. psittaci*, *Chlamydia psittaci*; *C. striatum*, *Corynebacterium striatum*; *C. tropicalis*, *Candida tropicalis*; EBV, Epstein-barr virus; *E. coli*, *Escherichia coli*; *E. faecium*, *Enterococcus faecium*; *K. aerogenes*, *Klebsiella aerogenes*; *K. pneumoniae*, *Klebsiella pneumoniae*; *L. pneumophila*, *Legionella pneumophila*; *P. aeruginosa*, *Pseudomonas aeruginosa*; *P. jirovecii*, *Pneumocystis jirovecii*; *S. aureus*, *Staphylococcus aureus*; *S. pneumoniae*, *Streptococcus pneumoniae*.

$p > 0.05$, Table 5) between patients with *C. psittaci* and *L. pneumophila*. *C. psittaci* pneumonia patients required significantly lower amounts of glucocorticoids (14.3% vs. 69.6%, $p = 0.002$) and immunoglobulins (7.1% vs. 47.8%, $p = 0.013$) than *L. pneumophila* pneumonia patients. However, the vasopressor use, continuous renal replacement therapy and extracorporeal membrane oxygenation administration were similar in both groups (all $p > 0.05$).

For antimicrobial treatments, due to unknown etiologies upon admission, empirical antibiotic therapies were administered before mNGS in 14 *C. psittaci* pneumonia patients but showed no improvement even with upgraded antibiotics. Then the antibiotics was timely switched to doxycycline (50.0%), azithromycin (35.7%) or other tetracycline after *C. psittaci* infection was confirmed by mNGS, 13/14 patients with *C. psittaci* pneumonia gradually recovered (Figure 2) and finally discharged. When *L. pneumophila* was established, the antibiotic therapy was adjusted to quinolones including moxifloxacin (45.5%), levofloxacin (18.2%), etc, 15/23 slowly recovered and finally discharged. From what have been mentioned above, it could be concluded that with the early identification of pathogens by mNGS method, timely antibiotics' adjustment could be performed, and the outcomes would also be better then. Compared with patients with *L. pneumophila*, more patients with *C. psittaci* received azithromycin (23.1% vs. 4.5%, $p = 0.134$) and minocycline (23.1% vs. 0.0%, $p = 0.044$) before diagnosis.

Regarding clinical outcomes (Table 5), the duration of IMV was higher for patients with *C. psittaci* compared with those with *L. pneumophila* [7 (6, 9) vs. 1 (1, 5), $p = 0.006$], which suggesting the more severe conditions of respiratory function of patients with *C. psittaci*. In addition, during hospitalization, no differences between these two groups were observed in ICU admission, IMV, duration from onset of illness to

Table 5. Treatments and prognosis of patients with *C. psittaci* and *L. pneumophila*.

Variable	Total ($n = 37$)	*C. psittaci* ($n = 14$)	*L. pneumophila* ($n = 23$)	*p*-value
Respiratory support mode, n (%)				
No respiratory support	7 (18.9)	3 (21.4)	4 (17.4)	1.000
Conventional oxygen therapy	28 (75.7)	10 (71.4)	18 (78.3)	0.705
High-flow nasal cannula	11 (29.7)	5 (35.7)	6 (26.1)	0.713
Noninvasive ventilation	3 (8.3)	1 (7.7)	2 (8.7)	1.000
Invasive ventilation	20 (54.1)	7 (50.0)	13 (56.5)	0.745
Other supportive treatments, n (%)				
Glucocorticoids	18 (48.6)	2 (14.3)	16 (69.6)	0.002*
Immunoglobulin	12 (32.4)	1 (7.1)	11 (47.8)	0.013*
Vasopressor	21 (56.8)	6 (42.9)	15 (65.2)	0.305
Continuous renal replacement therapy	9 (25.0)	3 (21.4)	6 (27.3)	1.000
Extracorporeal membrane oxygenation	2 (5.6)	1 (7.1)	1 (4.5)	1.000
Empirical antibiotic therapies before diagnosis, n (%)				
Moxifloxacin	22 (62.9)	10 (76.9)	12 (54.5)	0.282
Ceftalidime	12 (34.3)	6 (46.2)	6 (27.3)	0.292
Meropenem	14 (40.4)	5 (38.5)	9 (40.9)	1.000
Ertapenem	7 (20.0)	1 (7.7)	6 (27.3)	0.220
Cefoperazone sulbactam sodium	3 (8.6)	2 (15.4)	1 (4.5)	0.541
Piracillin tazobactam	7 (20.0)	2 (15.4)	5 (22.7)	0.689
Imipenem cilastatin	7 (20.0)	1 (7.7)	6 (27.3)	0.220
Azithromycin	4 (11.4)	3 (23.1)	1 (4.5)	0.134
Minocycline	3 (8.6)	3 (23.1)	0 (0.0)	0.044*
Vancomycin	7 (20.0)	1 (7.7)	6 (27.3)	0.220
Pathogen-targeted antibiotic therapies after diagnosis by mNGS, n (%)				
Moxifloxacin	14 (38.9)	4 (28.6)	10 (45.5)	0.485
Levofloxacin	4 (11.1)	0 (0.0)	4 (18.2)	0.141
Doxycycline	7 (19.4)	7 (50.0)	0 (0.0)	< 0.001*
Minocycline	4 (11.1)	4 (28.6)	0 (0.0)	0.017*
Azithromycin	11 (30.6)	5 (35.7)	6 (27.3)	0.716
Ceftalidime	9 (25.0)	2 (14.3)	7 (31.8)	0.432
Meropenem	10 (27.8)	3 (21.4)	7 (31.8)	0.706
Cefoperazone sulbactam sodium	6 (16.7)	3 (21.4)	3 (13.6)	0.658
Piracillin tazobactam	5 (13.9)	2 (14.3)	3 (13.6)	1.000
Imipenem cilastatin	5 (13.9)	2 (14.3)	3 (13.6)	1.000
Voriconazole	9 (25.0)	1 (7.1)	8 (36.4)	0.062
Clinical outcomes, n (%)				
Intensive care unit admission	24 (64.9)	9 (64.3)	15 (65.2)	1.000
Invasive mechanical ventilation	20 (54.1)	7 (50.0)	13 (56.5)	0.745
Duration of invasive mechanical ventilation	4 (1, 7)	7 (6, 9)	1 (1, 5)	0.006*
Time from illness onset to hospital admission	7 (4, 28)	7 (5, 8)	7 (3, 43)	0.360
Hospital stay	19 (10, 31)	15 (5, 23)	24 (12, 52)	0.057
ICU stay	10 (5, 14)	12 (7, 14)	9 (1, 15)	0.519
Hospital death	9 (24.3)	1 (7.1)	8 (34.8)	0.112

Note: *C. psittaci*, *Chlamydia psittaci*; ICU, intensive care unit; *L. pneumophila*, *Legionella pneumophila*.

hospital admission, hospital stay, and ICU stay (all $p > 0.05$). The final outcomes were also similar, with hospital mortality rates of 7.1% (1/14) in patients with *C. psittaci* and 34.8% (8/23) in patients with *L. pneumophila* ($p = 0.112$).

4. Discussion

4.1. Distinction of clinical features between psittacosis and legionellosis

The overall demographic characteristics between CAP patients infected by *C. psittaci* and *L. pneumophila* was similar, yet patients with *C. psittaci* exhibited more contact with birds or parrots. As to clinical symptoms and laboratory results, it was observed that both *C. psittaci* and *L. pneumophila* infections could cause a pronounced inflammatory response, including increased WBC, neutrophils, CRP and PCT. Recent small sample clinical studies have also shown the presence of significant increases in CRP, PCT, WBC, and neutrophils with *C. psittaci* [7,17,30,31], which was consistent with our results. However, in the present study, patients with *C. psittaci* infection had higher proportions of fever and chill, as well as higher levels of hemoglobin and albumin than those with *L. pneumophila* infection, which might due to the higher percentage of past history of coronary heart disease and chronic kidney disease in the latter group (see Table 1). Radiologic chest images due to atypical pathogens seem to be a diagnostic challenge. Our results showed that both patients with *C. psittaci* and *L. pneumophila* infections exhibited ground-glass opacities, consolidations, and pleural effusions on imaging with no significant difference noted between the groups. However, patients with *L. pneumophila* infection showed a higher percentage of left lung involvement. And the number of lobes involved between the two groups was nearly the same. But in a previous study, patients with *L. pneumophila* admitted to the ICU presented with diffusions of more lobes than those with *C. psittaci* [32]. The inconsistence of this might due to the lower ICU rate of only 65% in our study.

4.2. Strengths of mNGS method

More importantly, our study shed light on the advantages of mNGS by comparing with conventional methods. 1) Pathogenic culture was more time-consuming and usually could not detect atypical pathogenic microorganisms (Table 4), while serology of respiratory pathogens was more likely to exhibit false negative results [33], compared with mNGS. A study also showed that CAP patients' overall microbial detection rate was 90.3% for mNGS versus 39.5% for conventional tests [34]. In the present study, no positive culture and low rates of positive pathogenic IgM antibody of *C. psittaci* or *L. pneumophila* pneumonia patients were observed, which was consistent with previous findings [6,35] and might due to the antibiotic use before serological testing. 2) The mNGS method can be used to quickly obtain etiological results with high sensitivity. A previous study showed that the sensitivity and specificity of mNGS were 50.7% and 85.7% among infectious diseases [36]. And it can also detect microorganisms from various samples like blood, BALF, or sputum (Table 4), which was also verified in previous studies [24,25,34]. 3) The mNGS method could help promptly adjust tetracycline-based antimicrobial therapy (see Table 5), reduce the time to diagnose, and shorten the course of psittacosis [7,37,38].

4.3. Coinfection with other pathogens based on mNGS method

Through mNGS testing in the present study, besides *C. psittaci* and *L. pneumophila*, some other pathogens were also detected. 3/14 patients with *C. psittaci* infection were co-infected with Acinetobacter baumannii, while 2/14 with Corynebacterium striatum and Pseudomonas aeruginosa. Coinfection with other pathogens in patients with *C. psittaci* pneumonia based on mNGS were also observed in multiple studies [5,16,35,39,40], including various kinds of bacteria, fungi, and virus. In a retrospective analysis enrolling 12 *C. psittaci* pneumonia patients in China [41], one was co-infected with Pseudomonas aeruginosa and Acinetobacter baumannii. Another study using mNGS method showed the presence of Corynebacterium striatum in 3/27 severe *C. psittaci* pneumonia patients [7]. However, patients with *L. pneumophila* infection were more likely to be co-infected with CMV (9/23 vs. 1/14, Figure 4), which occurred frequently in immunosuppressed patients [42]. In our study, 2/9 with coinfection of CMV had malignant tumor, 7/9 had more than one comorbidity of coronary heart disease, chronic kidney disease, diabetes mellitus, or hypertension, which might contribute to the high rate of CMV coinfection.

4.4. Treatments and prognosis of C. psittaci pneumonia

The optimal antibiotic therapy for *C. psittaci* pneumonia was doxycycline or other tetracycline like minocycline and azithromycin. Small sample studies also

showed that conditions of psittacosis gradually improved and recovered after tetracycline were added [17,30,31]. In the present study, the prognosis of patients with *C. psittaci* was similar to that of *L. pneumophila*, with mortality hospital rates of 7.1% (1/14) for psittacosis, which was consistent with previous findings [7,31,32,43]. The only one psittacosis patient who died received the first dose of doxycycline 3 days after ICU admission, and exhibited improved condition then. However, the death occurred 12 days after ICU admission due to the direct cause of severe intra-abdominal hemorrhage and indirect causes of severe pneumonia, multiple organ dysfunction, etc. ICU rate of psittacosis in our study was 65%, with the median hospital stay of 15 (5, 23) days. In a recent retrospective study in China [43], the ICU rate and hospital stay for *C. psittaci* pneumonia patients were 50% and 14 (10, 17) days, respectively, which were consistent with those in the present study.

4.5. Limitations

This study has some limitations due to its monocentric design and retrospective nature. First, the results may not apply to other settings and the sample size is limited, which introduced some biases and more validation of the method is needed. A larger sample size was believed to be better to conduct the multivariable analysis to distinguish patients with *C. psittaci* and *L. pneumophila*. Then, the factor of smoking history should also be considered. Second, as there are no standard molecular and serological clinical diagnostic kits in China, all patients with psittacosis in our study were diagnosed using mNGS, which may underestimate *C. psittaci* pneumonia incidence. Third, the identification of *C. psittaci* and *L. pneumophila* by mNGS testing does not rule out coinfection with other pathogens which might also affect the clinical presentations, making the research results less typical. However, this is, by far, the first and largest study to compare clinical characteristics of psittacosis with legionellosis based on the results of mNGS detection.

5. Conclusion

Though patients with *C. psittaci* and *L. pneumophila* had similar symptoms and imaging findings, detection of atypical pathogens by mNGS in multiple clinical samples can make up for the deficiencies of conventional microbiological methods, promptly adjust empirical antimicrobial treatment to pathogen-targeted antibiotics, might further improve prognosis.

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Authors contributions

Study design: Jing Yang and Huadong Zhu; Acquisition of data: Dong Zhang, Qiwen Yang and Lin Shi; Analysis and interpretation of data: Lin Shi; Drafting the manuscript: Lin Shi; Revision of the manuscript: Jing Yang and Huadong Zhu; Technical support: Dong Zhang, Qiwen Yang; Study supervision: Jing Yang. All authors have read and approved the final version of the manuscript.

Ethical approval

The study was approved by the Medical Ethics Committee of Peking Union Medical College Hospital (reference number: K23C3432). Due to the anonymized retrospective nature of the analysis, written informed consent was waived. All methods were performed in accordance with relevant guidelines and regulations.

Consent to publish

For case 3, 4 and 13, whose radiological results were displayed in Figure 2, images had been obtained from these participants with oral or written informed consent.

Disclosure statement

The authors report no conflict of interest.

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Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request if permitted by Peking Union Medical College Hospital.

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