Rapid diagnosis of *Chlamydia psittaci* pneumonia with pleural effusion using multiplex PCR-based targeted next-generation sequencing: A case report

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Abstract. *Chlamydia psittaci* pneumonia (CPP) is a zoonotic disease with non-specific clinical and radiographic features, complicating timely diagnosis. Although not common, pleural effusion may indicate more severe disease. Conventional diagnostic methods lack the sensitivity and speed. The present report highlights the utility of multiplex PCR-based targeted next-generation sequencing (mp-tNGS) for rapid etiological diagnosis of CPP, particularly in patients with pleural effusion. A 52-year-old woman and a 64-year-old woman both presented with high fever, cough and systemic symptoms. Inflammatory markers (hs-CRP, IL-6 and ESR) were found to be markedly elevated with normal or mildly elevated white blood cell counts. A chest CT exhibited unilateral consolidations with small ipsilateral pleural effusions. Conventional microbiological tests (stains, cultures and serology) were negative. Bronchoalveolar lavage fluid mp-tNGS rapidly identified *Chlamydia psittaci* (142 and 251 reads per 100,000 sequencing reads, respectively), with a mean turnaround time of 11.35 h (from laboratory receipt to result). Epidemiological history revealed exposure to pigeons and a bird market. Doxycycline therapy led to rapid clinical improvement, fever resolution and notable radiographic clearance. Therefore, pleural effusion can be a feature of CPP and may indicate more severe disease. mp-tNGS proved rapid and decisive when conventional methods failed, enabling timely targeted therapy. A high index of suspicion combined with mp-tNGS is thus key in accurately diagnosing and improving prognosis.

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Introduction

Chlamydia psittaci is an obligate intracellular gram-negative bacterium and an important zoonotic pathogen (1) transmitted to humans through inhalation of aerosolized dried excreta or secretions from infected birds (2). Owing to its non-specific clinical presentation, *Chlamydia psittaci* pneumonia (CPP) is often misdiagnosed, leading to inappropriate antibiotic use and potential antimicrobial resistance (3,4). In China, CPP accounts for ~1-2% of annual community-acquired pneumonia (CAP) cases (5,6), yet it is a notable contributor to severe CAP (7). Chest CT scans typically reveal extensive pulmonary lesions with varying degrees of exudation, consolidation and patchy opacities (1). Despite pleural effusion generally being considered uncommon (8), patients with CPP presenting with pleural effusion are prone to misdiagnosis as other conditions such as parapneumonic effusion, tuberculous pleurisy or malignant pleural effusion.

Although pleural effusion is traditionally considered an uncommon finding in CPP, recent studies (9,10) have suggested that it may occur more frequently than previously recognized, particularly in severe cases. In a cohort of 35 patients with CPP, pleural effusion was present in 68.6 and 51.4% met the criteria for severe pneumonia (9). Similarly, a multicentre study reported pleural effusion in 77.0% of 74 patients, with acute respiratory distress syndrome (ARDS) developing in 55.4% and type I respiratory failure in 52.7% (10). These data indicate that pleural effusion in CPP patients should be considered a marker of a potentially worse prognosis.

However, conventional diagnostic methods, including culture (sensitivity <10%), serology (requiring convalescent-phase titers) and metagenomic nextgeneration sequencing (mNGS), exhibit limitations with regard to speed, cost and host DNA interference (11-14). Multiplex PCR-based targeted nextgeneration sequencing (mp-tNGS) is an advanced technique that covers >95% of clinically relevant pathogens and offers a shorter turnaround time (TAT), reduced cost and lower susceptibility to host DNA (15,16). Recent large-scale diagnostic studies (17,18) have demonstrated that tNGS, including the mp-tNGS used in the present centre, exhibits a diagnostic performance comparable to that of mNGS while offering marked advantages in terms of the TAT and cost. In a prospective cohort of 251 patients with suspected lower respiratory tract infections, mp-tNGS achieved a sensitivity of 86.5% and a specificity of 90.0%, with a mean TAT of 10.3 h and detection costs reduced to ~25% of those of mNGS (17).

Furthermore, tNGS has been shown to have a significantly higher detection rate compared with conventional culture does (75.2 vs. 19.0%; P<0.01) and to guide therapeutic adjustments in ~50% of tested patients (18). These characteristics make mp-tNGS particularly suitable for the timely diagnosis of CPP, a disease in which rapid pathogen identification can directly impact clinical outcomes. However, reports regarding its specific application for detecting *Chlamydia psittaci* in patients with pleural effusion remain limited. Unlike previous reports (19-21) that have focused primarily on the general diagnostic performance of tNGS or mNGS in CPP, the present study was specifically centered around the 'pleural effusion' subgroup. A total of 16 English-language publications were systematically reviewed and 208 published cases of CPP with pleural effusion were aggregated. Therefore, to the best of our knowledge, the present study provided the first quantitative characterization of this high-risk subgroup. In addition, a positive threshold [reads per 100,000 sequencing reads (RPhK) ≥10] was explicitly defined for mp-tNGS in diagnosing CPP and the complete diagnostic-therapeutic trajectory with treatment failures was documented. These features distinguish the present study from prior reports and offer clinically actionable insights. Overall, the present report presents two cases of CPP radiologically characterized by pleural effusion, summarizes their clinical features, reviews previously reported cases with pleural effusion and evaluates the clinical utility of mp-tNGS to inform timely clinical management.

Case report

Case 1. A 52-year-old woman was admitted to the Department of Pulmonary and Critical Care Medicine at Taihe Hospital (Shiyan, China) in June 2025, presenting with a 1-week history of fever that acutely worsened 1 day prior to admission. The patient reported low-grade fever (<38˚C) occurring predominantly in the afternoons and evenings, associated with cough, sputum production, anorexia, fatigue, generalized myalgia and intermittent headache. The patient reported self-administration of over-the-counter medications, including Jizhi syrup (20-30 ml, 3 times daily), Yinqiao granules (10 g, 3 times daily) and Analgin tablets (0.5-1 g as needed, 3 times daily) for ~7 days prior to admission, and proved ineffective. On the day before admission, her temperature spiked to 40˚C, accompanied by nausea and vomiting. An emergency chest CT scan revealed patchy opacities with blurred margins and partial consolidation in the left upper lobe, along with a small left pleural effusion (Fig. 1A). The patient had no notable past medical history of chronic conditions (such as hypertension, coronary heart disease or diabetes) or infectious diseases (including hepatitis B or tuberculosis) and denied any history of smoking or alcohol use.

Upon admission, physical examination revealed a temperature of 38˚C, heart rate of 107 beats/min, respiratory rate of 20 breaths/min, blood pressure of 107/69 mmHg and a peripheral capillary oxygen saturation of 96% on room air. No jaundice, petechiae or palpable superficial lymphadenopathy was noted. The chest was symmetrical with clear percussion notes and breath sounds; no dry or wet rales were audible.

Laboratory tests upon admission (hospital day 1) revealed a white blood cell count of 6.53×10^9/l with neutrophilia [neutrophils: 85.60% (normal range: 50.00-70.00%); lymphocytes: 8.70% (normal range: 20.00-50.00%); monocytes: 5.50% (normal range: 3.00-10.00%); eosinophils: 0.00% (normal range: 0.44-8.00%) and; basophils: 0.20% (normal range: 0.00-1.00%)]. Inflammatory markers were markedly elevated: High-sensitivity CRP (hs-CRP) concentration: 164.64 mg/l (normal range: 0.00-10.00 mg/l); IL-6 concentration: 212.60 pg/ml (normal range: 0.00-6.60 pg/ml) and; ESR: 83.00 mm/h (normal range: 0.00-20.00 mm/h). The lactate dehydrogenase (LDH) and α-hydroxybutyrate dehydrogenase (HBDH) levels were 309.70 U/l (normal range: 120.00-240.00 U/l) and 209.40 U/l (normal range: 72.00-182.00 U/l), respectively.

Tests for *Mycoplasma pneumoniae* DNA [using reverse transcription (RT)-PCR], influenza A/B virus RNA (RT-PCR), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA (RT-PCR) and tuberculosis IgG antibody (ELISA) were all negative. The RT-PCR protocol for respiratory pathogen detection are as follows: Respiratory specimens (nasopharyngeal swabs, sputum or BALF) collected using sterile synthetic-fiber swabs were transported to the laboratory in viral transport medium at 2-8˚C within 24 h. Total nucleic acid (DNA and RNA) was extracted from 200 μl of specimen using a magnetic-bead-based extraction kit (MagPure Pathogen DNA/RNA Extraction Kit; Magen Biotechnology) on an automated platform, with MS2 phage spiked as an exogenous internal control. One-step; RT-PCR was performed in a 25-μl reaction containing 12.5 μl of 2X master mix (reverse transcriptase, hot-start DNA polymerase, buffer, dNTPs, and Mg^{2+}), 1.0 μl of primer-probe mix targeting specific genes (influenza A/B: M or HA genes; SARS-CoV-2: RdRP or N genes; Mycoplasma pneumoniae: P1 or 16S rRNA gene; and human RNase P gene as an endogenous internal control), and 5 μl of extracted nucleic acid. Thermal cycling was conducted on a real-time PCR instrument as follows: Reverse transcription at 50˚C for 15-30 min, initial denaturation at 95˚C for 10-15 min, followed by 40-45 cycles of 95˚C for 15 sec and 60˚C for 45-60 sec, with fluorescence signal acquisition at the extension step. Each run included positive and no-template controls. Samples were considered positive when target genes exhibited a typical sigmoidal amplification curve with Cq values below the predefined cutoff (Cq ≤38), while the internal control amplified normally (Cq <35).

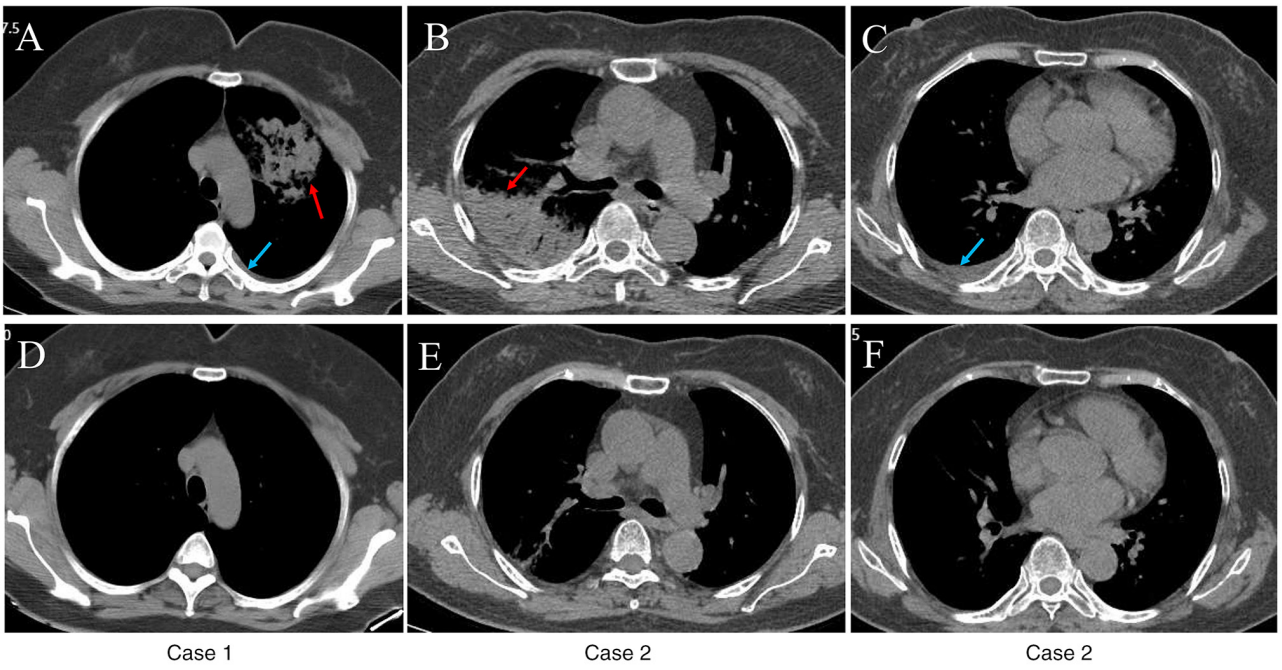

Figure 1. Chest CT radiological features and follow-up changes in the 2 patients with *Chlamydia psittaci* pneumonia. (A) Initial CT (June 2025) revealed patchy opacities with blurred margins and partial consolidation (red arrow) in the left upper lobe, along with a small left pleural effusion (blue arrow) in Case 1. (D) Follow-up CT (July 2025) showing near-complete resolution of the left upper lobe opacities and disappearance of the left pleural effusion in Case 1. (B) Initial CT (October 2025) showing a masslike highdensity opacity (red arrow) with blurred margins in the right upper lobe, an arcshaped area of increased density in the posterior right lower lobe and a (C) small right pleural effusion (blue arrow) in Case 2. (E) Follow-up CT (October 2025) revealing a marked reduction in the size of the masslike opacity in the right upper lobe and (F) complete resolution of the right pleural effusion in Case 2.

Strict contamination prevention measures, including physical separation of workflow areas and use of filter-tipped pipette tips and uracil-DNA glycosylase (UNG), were implemented throughout. A comprehensive respiratory pathogen IgM panel (using indirect immunofluorescence) for respiratory syncytial virus, adenovirus, influenza A/B, parainfluenza, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, Coxsackie B virus and Echovirus was also negative. Urinalysis (through automated dipstick and sediment microscopy) and routine stool testing (through macroscopic examination, microscopic examination and fecal occult blood tests) results were also normal.

Given the persistent high fever (up to 39.2˚C), notably elevated inflammatory marker levels and radiographic evidence of pneumonia (specifically, patchy opacities with blurred margins and partial consolidation in the left upper lobe accompanied by a small ipsilateral pleural effusion on chest CT), empiric therapy with intravenous piperacillin-tazobactam (4.5 g every 8 h) and intravenous methylprednisolone (40 mg once daily) was initiated on hospital day 1.

On hospital day 2, the patient remained febrile (with a peak temperature of 39.5˚C). Arterial blood gas analysis on room air (fraction of inspired oxygen: 21%) revealed a pH of 7.433 (normal range, 7.357.45), a partial pressure of carbon dioxide ($PaCO_2$) of 31.4 mmHg (normal range, 3545 mmHg), a partial pressure of oxygen (PaO_2) of 76.4 mmHg (normal range, 80100 mmHg), a bicarbonate value of 20.5 mmol/l (normal range, 2226 mmol/l), a base excess of -2.6 mmol/l (normal range, -3 to +3 mmol/l) and a lactate concentration of 1.1 mmol/l (normal range, 0.52.2 mmol/l). Bronchoscopy results were normal. Thoracentesis was attempted under ultrasound guidance for diagnostic evaluation of the left pleural effusion; however, no fluid could be aspirated because of the small volume of the effusion. Bronchoalveolar lavage fluid (BALF) was sent for Gram staining (microscopy), acid-fast staining (ZiehlNeelsen stain), microbial culture (semi-automated microbial culture) and mp-tNGS.

The mp-tNGS protocol was as follows: i) mp-tNGS workflow construction: The mp-tNGS workflow, including database integration and primer design, was established based on a previous publication (22) and a study by Yin *et al* (17). The panel covers 198 pathogen targets frequently encountered in clinical practice (a complete list is provided in Table SI). Library construction utilized the tNGS library premix (cat. no. KS5646tNGSJT96; lot no. 2025.07.01; Guangzhou KingCreate Biotechnology Co., Ltd.). Over 300 multiplex amplification primer sets were designed, with additional primers (≥5) included for key pathogens requiring identification or typing, such as the *Mycobacterium tuberculosis* complex and severe acute respiratory syndrome coronavirus 2. A multiplex PCR (The multiplex PCR preamplification of target loci was performed using the RP100™ Respiratory Pathogen Multiplex Testing Kit (Guangzhou KingCreate Biotechnology Co., Ltd.), which contains a high-fidelity hot-start DNA polymerase. The thermal cycling conditions were as follows: Initial denaturation at 95˚C for 3-5 min; 25-35 cycles of denaturation at 95˚C for 15-30 sec, annealing at 60˚C for 30-60 sec and extension at 72˚C for 30-60 sec; and a final extension at 72˚C for 5 min. The amplified products were purified using magnetic beads and quantified using the Equalbit DNA HS Assay Kit (Vazyme Biotech Co., Ltd.) on a Qubit™ 4.0 Fluorometer (Thermo Fisher Scientific, Inc.), with a quality threshold of ≥0.5 ng/μl. Qualified libraries were pooled in equimolar ratios

and subjected to fragment size verification (250-350 bp) using a Qsep100 automated nucleic acid analyzer (BiOptic Inc.). The process was developed and optimized to ensure highsensitivity amplification of target sequences. All of the aforementioned primers targeting pathogens are covered by commercial confidentiality of Guangzhou KingCreate Biotechnology Co., Ltd., therefore the kit instructions did not provide the specific primer sequences; ii) mp-tNGS nucleic acid extraction: BALF samples were mixed with an equal volume of 0.1 M DTT liquefaction reagent, followed by vortexing, shaking and incubation for 3-5 min until complete liquefaction. A total of 1.3 ml liquefied sample was aliquoted, spiked with 13 μl exogenous endogenous reagent and centrifuged at 13,400 x g for 5 min at room temperature. The supernatant was discarded and 500 μl sample was transferred to a bead mill tube from the extraction kit. Then, 50 μl SDS was added, and the mixture was subjected to bead-beating using a sonicator (2,055 x g, 45 sec oscillation, 20 sec pause, 2 intervals with a total oscillation time of 135 sec) for cell disruption. After disruption, the sample was centrifuged again at 13,400 x g for 5 min at room temperature. Nucleic acids were extracted from 400 μl (manual) or 250 μl (automated) supernatant using the MetaPure DNA & RNA Extraction Kit (Guangzhou KingCreate Biotechnology Co., Ltd.), following the manufacturer's instructions; iii) mp-tNGS library construction (23): Library preparation was performed using the RP100™ Respiratory Pathogen Microorganisms Multiplex Testing Kit (Guangzhou KingCreate Biotechnology Co., Ltd.). The specific components and their identifiers used in the present study included the Nucleic Acid Extraction or Purification Reagent (cat. no. KS118-BYTQ-48; lot. 20250501) for nucleic acid extraction, the tNGS Library Premix (cat. no. KS5646-tNGSJT-96; lot. 2025.07.01) and the Respiratory 100 Premix (cat. no. KS608-100HXD96-WX; lot. 202501101) for library construction and the Sequencing Reaction Universal Kit (MR100; cat. no. KS107-CXR; lot. 20250101) for sequencing. All kits were from Gungzhou KingCreate Biotechnology Co., Ltd. and used according to the manufacturer's instructions.

First, cDNA was synthesized from extracted nucleic acids by reverse transcription using the RP100™ Respiratory Pathogen Microorganisms Multiplex Testing Kit (KingCreate Biotechnology Co., Ltd.). The reverse transcription reaction mixture contained the extracted nucleic acid template, a reverse transcription premix [including a modified M-MLV reverse transcriptase with reduced RNase H activity, buffer, dNTPs, and a mixture of random primers and oligo(dT) primers], and nuclease-free water. The thermal cycling program for reverse transcription consisted of primer annealing at 25˚C for 5-10 min, cDNA synthesis at 42-50˚C for 15-45 min and enzyme inactivation at 85-95˚C for 5 min. A no-template control (nuclease-free water) was included in parallel to monitor contamination. The resulting cDNA products were subsequently used for multiplex PCR target amplification. Following library construction, all libraries were quantified using the Equalbit DNA HS Assay Kit (Vazyme Biotech Co., Ltd.) on a Qubit™ 4.0 Fluorometer (Thermo Fisher Scientific, Inc.) to ensure a quality threshold of ≥0.5 ng/μl. Subsequent steps included target region enrichment, two rounds of purification, and adapter ligation to complete library construction. Nuclease-free water (Invitrogen; Thermo Fisher Scientific, Inc.) was used as a non-template control to monitor contamination. Library quantification was performed using the Equalbit DNA HS Assay Kit (Vazyme Biotech Co., Ltd.) on a Qubit™ 3.0/4.0 Fluorometer (Thermo Fisher Scientific, Inc.). All samples met the quality threshold of ≥0.5 ng/μl; otherwise, libraries were re-prepared. Qualified libraries were pooled in equimolar ratios, and fragment size distribution (~250-350 bp) was verified using a Qsep100 fully automated nucleic acid analyzer with a Standard Cartridge Kit (S2). Finally, the pooled library was diluted, denatured and 500 μl was loaded into a KM MiniSeqDx-CN Sequencing Kit for sequencing on the KM MiniSeq Dx-CN Platform (Guangzhou KingCreate Biotechnology Co., Ltd.) and; iv) bioinformatic analysis: Sequencing data were processed using a customized bioinformatics pipeline. Raw reads underwent quality control and adapter trimming using fastp (version 0.20.1) (24) with default parameters. Filtered reads were aligned against a curated mp-tNGS pathogen database using Bowtie2 (version 2.4.1) (25) in very-sensitive mode. The RPhK values were calculated at the species and genus levels. In the present case report, the positive cut-off value for *Chlamydia psittaci* was set at normalized RPhK ≥10. This threshold was established based on the following validation experiments: Serial dilutions of a quantified *Chlamydia psittaci* reference strain (ATCC VR-125) in negative BALF matrix were tested in triplicate. The limit of detection (LoD) was determined as the lowest concentration at which the pathogen was detected in ≥95% of replicates. The LoD for *Chlamydia psittaci* using our mp-tNGS platform was 50-450 CFU/ml, consistently corresponding to RPhK values between 8-15. Based on these data, RPhK ≥10 was conservatively selected as the positive cut-off to ensure analytical sensitivity while minimizing false positives. In addition, negative controls (extraction blanks and no-template controls) processed in parallel in each sequencing run showed no *Chlamydia psittaci* reads, determining the absence of reagent or environmental contamination. Given that *Chlamydia psittaci* is an obligate intracellular bacterium that does not colonize the human respiratory tract (1,2), detection at or above this cut-off was considered clinically significant.

While the conventional microbiological workup results (stains and cultures) were negative, mp-tNGS identified *Chlamydia psittaci* with 142 RPhK and a TAT (defined as the time from laboratory receipt of the BALF sample to the reporting of mptNGS results) of 11.2 h. Subsequent epidemiological inquiry revealed daily exposure to a pigeon flock at the entrance of her residential community.

On the basis of these findings, a diagnosis of CPP was established. Antibiotic therapy was switched to oral doxycycline (100 mg twice daily) for a total of 14 days (7 days during hospitalization and 7 days after discharge). Following this targeted treatment, the patient's fever resolved rapidly and respiratory symptoms markedly improved.

Follow-up laboratory tests demonstrated normalization of the white blood cell differential and a notable decrease in inflammatory markers: A white blood cell count of 4.75×10^9/l (neutrophils: 56.90% and lymphocytes: 31.40%), hs-CRP concentration of 19.51 mg/l, IL-6 concentration of 5.07 pg/ml and ESR of 23.00 mm/h were observed. A later chest CT scan conducted in July 2025 demonstrated marked resolution of the pulmonary infiltrates and complete absorption of the left

pleural effusion (Fig. 1D). Upon admission, the confusion, uremia, respiratory rate, blood pressure and age (CURB)65 score of the patient was 0 and the Pneumonia Severity Index (PSI) was 52 (risk class II), indicating low risk according to conventional scores (26,27). The CURB65 score was calculated according to protocols described by Lim *et al* (26) and the Pneumonia Severity Index (PSI) was calculated according to Fine *et al* (27). The detailed calculation methodology is provided in Table SII. Despite this, pleural effusion, persistent high fever and markedly elevated inflammatory markers despite broadspectrum antibiotics prompted aggressive monitoring during the hospital stay, including daily clinical assessments, repeated laboratory testing, and early bronchoscopy with mptNGS. After discharge, Case 1 was managed according to the standard protocol of Taihe Hospital. The patient attended scheduled outpatient visits at 2 weeks postdischarge, at which the followup chest CT and laboratory tests (as aforementioned) were obtained. The patient did not return to the department for further scheduled visits. Thereafter, the patient was followed by a dedicated telephonebased surveillance system, with regular calls by a designated followup physician to assess symptom status, medication adherence and any signs of recurrence. Case 1 remained completely asymptomatic and stable at the time of the most recent telephone contact in July 2026.

Case 2. A 64-year-old woman was admitted to the Department of Pulmonary and Critical Care Medicine at Taihe Hospital due to a reported 'cough, expectoration and intermittent fever for 5 days'. During a previous 5-day hospitalization at Shiyan People's Hospital (Shiyan, China), the patient developed a cough with white sticky sputum and a high fever, with a maximum temperature of 39.8˚C, accompanied by exertional dyspnea. At this hospital, the patient received empirical antibiotic therapy with intravenous ceftriaxone (2.0 g once daily) and intravenous levofloxacin (500 mg once daily) for 3 days, but the fever and respiratory symptoms did not improve. A chest CT scan conducted in October 2025, revealed a mass-like area of high density with blurred margins in the right upper lung lobe, an arc-shaped area of increased density in the posterior part of the right lower lung lobe and a small amount of right pleural effusion (Fig. 1B and C). The patient was admitted to the Department of Pulmonary and Critical Care Medicine within Taihe Hospital in October 2025. The patient had a history of hypertension and gastric ulcers but denied other medical histories or smoking.

Physical examination revealed no jaundice or petechiae on the skin or mucous membranes and no palpable superficial lymph nodes. The bilateral thoracic cage exhibited no deformity. Lung percussion was clear, breath sounds were clear on auscultation and scattered dry and moist rales were heard in both lungs.

Upon admission, routine peripheral blood tests revealed a white blood cell count of $6.31x10^9$/l [80.80% (normal range: 50.00-70.00%) of neutrophils; 11.60% (normal range; 20.00-50.00%) of lymphocytes; 7.40% (normal range: 3.00-10.00%) of monocytes; 0.00% (normal range: 0.40-8.00%) of eosinophils and; 0.20% (normal range: 0.00-1.00%) of basophils]. The inflammatory marker levels were as follows: An hs-CRP of 152.91 mg/l (normal range: 0.00-10.00 mg/l); an ESR of 46 mm/h (normal range: 0.00-20.00 mm/h) and; an IL-6 concentration of 78.73 pg/ml (normal range: 0.00-6.60 pg/ml). The LDH concentration was 238.70 U/l (normal range: 120.00-240.00 U/l) and the HBDH concentration was 166.20 U/l (normal range: 72.00-182.00 U/l). Arterial blood gas analysis revealed a pH of 7.461, a PaO_2 of 67.6 mmHg and a $PaCO_2$ of 25.8 mmHg (room air), indicating type I respiratory failure.

Given the clinical symptoms and abnormally elevated inflammatory markers of the patient, anti-infective therapy with intravenous piperacillin-tazobactam (4.5 g every 8 h) and intravenous moxifloxacin (400 mg once daily) was initiated upon admission, and nebulization therapy was initiated, consisting of fluticasone propionate inhalation suspension (1 mg, twice daily), ambroxol hydrochloride inhalation solution (30 mg, twice daily) and levalbuterol tartrate inhalation solution (0.63 mg, three times daily), all administered for 5 consecutive days.

Subsequent laboratory tests revealed that results of the influenza A/B virus RNA (reverse transcription PCR), SARS-CoV-2 RNA (reverse transcription PCR) and tuberculosis IgG antibody tests (ELISA) were all negative. IgM antibody testing for a panel of respiratory pathogens (indirect immunofluorescence) for respiratory syncytial virus, adenovirus, influenza A virus, influenza B virus, parainfluenza virus, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, Coxsackie B virus and echovirus was also negative. Urinalysis (automated dipstick and sediment microscopy) and routine stool test (macroscopic examination, microscopic examination and fecal occult blood test) results were normal.

A total of 2 days after the CT scan was performed, electronic bronchoscopy was performed, revealing no abnormalities. Thoracentesis of the right pleural effusion was attempted under ultrasound guidance but yielded no aspirate due to small effusion volume. BALF was sent for Gram staining (microscopy), acid-fast staining (ZiehlNeelsen stain), microbial culture (semi-automated microbial culture) and mp-tNGS using the identical protocol as described in Case 1 (including nucleic acid extraction, library construction, sequencing on the KM MiniSeq DxCN platform and bioinformatics analysis a consistent positive threshold of RPhK ≥10). The only difference was that some reagent kit cat. nos. differed between the two tests, which is a routine laboratory variation and did not affect the protocol or performance. Subsequent laboratory results indicated negative findings from Gram staining, acid-fast staining and microbial culture; however, mp-tNGS detected *Chlamydia psittaci* with 1,251 RPhK, with a TAT (defined as the time from laboratory receipt of the bronchoalveolar lavage fluid sample to the reporting of mptNGS results) of 11.5 h. Further inquiry into the epidemiological history of the patient revealed that they lived near a flower and bird market.

On the basis of the aforementioned findings and exposure history, a diagnosis of CPP was determined. Following diagnosis, targeted anti-chlamydial therapy with oral doxycycline (100 mg twice daily) was added. The patient received a total of 10 days of doxycycline therapy (5 days during hospitalization and 5 days after discharge), along with intravenous human immunoglobulin (2.5 g once daily) administered for 3 consecutive days as adjunctive immunomodulatory therapy. After the addition of doxycycline, the body temperature of the patient rapidly normalized and symptoms of cough and dyspnoea markedly improved.

Routine peripheral blood tests revealed normalization of the white blood cell differential percentages: A white blood cell count of $4.41x10^9$/l [neutrophils: 45.8% (normal range: 50.00-70.00%); lymphocytes: 38.30% (normal range: 20.00-50.00%); monocytes: 10.80% (normal range: 3.00-10.00%); eosinophils: 3.60% (normal range: 0.40-8.00%); basophils: 0.50% (normal range: 0.00-1.00%)]. The levels of inflammatory markers, such as hs-CRP (20.84 mg/l, normal range: 0.00-10.00 mg/l), IL-6 (5.00 pg/ml; normal range: 0.00-6.60 pg/ml) and ESR (15.00 mm/h, normal range: 0.00-20.00 mm/h) notably decreased or normalized. A follow-up chest CT scan after 9 days (in October 2025) of treatment revealed that the mass-like high-density shadow with blurred margins in the right upper lobe was significantly smaller in size than before and the right pleural effusion had disappeared (Fig. 1E and F). Upon admission, the CURB65 score of the patient, calculated according to Lim *et al* (26), was 0 and PSI, calculated according to Fine *et al* (27), was 64 (risk class II), indicating low risk according to conventional scores. The detailed calculation methodology is provided in Table SII. Despite this, pleural effusion, persistent high fever and markedly elevated inflammatory markers despite broadspectrum antibiotics prompted aggressive monitoring during the hospital stay, including daily clinical assessments, repeated laboratory testing, and early bronchoscopy with mptNGS. After discharge, Case 2 were managed according to the standard protocol of the Department of Pulmonary and Critical Care Medicine at Taihe Hospital. The patient attended scheduled outpatient visits after 9 days (October 2025) of treatment, at which the follow up chest CT and laboratory tests (as aforementioned) were obtained. The patient did not return to the department for further scheduled visits. Thereafter, the patient was also followed by a dedicated telephone-based surveillance system, with regular calls by a designated follow up physician to assess symptom status, medication adherence and any signs of recurrence. Case 2 remained completely asymptomatic and stable at the time of the most recent telephone contact in July 2026 (Case 2 declined further imaging due to radiation concerns but reported sustained good health without any respiratory symptoms).

Literature review

A comprehensive search of the PubMed (https://pubmed.ncbi.nlm.nih.gov/), Google Scholar (https://scholar.nq69.top/) and Web of Science databases was conducted to identify relevant studies published from January 1, 2000, to May 1, 2026, using the following search terms: (*Chlamydia psittaci* OR psittacosis OR parrot fever) AND (pneumonia OR community-acquired pneumonia) AND (pleural effusion OR parapneumonic effusion). In addition, references from retrieved articles meeting the inclusion criteria were manually searched. The exclusion criteria included studies: i) Not written in English; ii) reviews without original case data; iii) systematic reviews or metaanalyses; iv) reports with insufficient clinical or therapeutic details or; v) cases without a determined microbiological diagnosis of *Chlamydia psittaci*. The extracted data included patient characteristics (geographic region, age, sex, epidemiological history, clinical manifestations, severity indicators, laboratory parameters, radiological findings, diagnostic methods, treatment details and clinical outcomes).

A total of 208 published cases of CPP with pleural effusion were identified across 16 Englishlanguage publications (including case reports, case series and retrospective studies) (9,10,19-21,28-38). The cohort included 113 men (54.3%) and 95 women (45.7%) on the basis of available sex data. The ages ranged from 10-85 years, with a median age of 58 years. A total of 182 patients (87.5%) had a documented history of exposure to poultry or birds.

The most common clinical manifestations found were fever (100%), cough (85.6%), dyspnea (61.5%), fatigue (48.1%), expectoration (44.2%) and headache (28.8%). Radiologically, pleural effusion was present in all included cases (100%, by inclusion criterion), followed by pulmonary consolidation (89.9%), patchy shadows (70.7%), air bronchogram (55.3%) and groundglass opacities (41.3%). Pleural effusion was unilateral in ~60% of the patients and bilateral in 40%, with smallvolume effusions being the most common finding.

Laboratory findings revealed that CRP levels were elevated in all patients (100%), with values ≥70 mg/l in reported 82.4% of the patients. White blood cell counts were normal or mildly elevated in 88.9% of the patients. LDH was elevated in 67.5% of the tested patients and both the IL6 concentration and the ESR were elevated in the majority of patients for whom data were available. Pleural fluid analysis was performed in 19 patients (9.1%) and consistently revealed a lymphocytepredominant exudative effusion with elevated ADA levels.

The most common diagnostic method was mNGS of BALF, which was positive in 96.6% of the patients. tNGS was used in 7.2% of the cases. All patients received tetracyclinebased therapy (doxycycline or minocycline) or alternative targeted antibiotics (quinolones and macrolides), with doxycycline as the most frequently prescribed firstline agent. Empirical fluoroquinolones were used in some patients and 88% improved after targeted therapy adjustment.

Patients with CPP and pleural effusion exhibited more severe disease compared with those without effusion did. Among the collected cases, 51 patients (24.5%) developed type I respiratory failure, 45 patients (21.6%) required intensive care unit admission and 34 patients (16.3%) required invasive mechanical ventilation. In a multicentre study of 74 patients with CPP (10), pleural effusion was present in 77.0%, ARDS in 55.4%, type I respiratory failure in 52.7% and a mortality rate of 8.11%. In a cohort of 35 patients with CPP (9), 68.6% had pleural effusion and 51.4% met the criteria for severe pneumonia. In a series of 14 patients with severe CPP (39), all had respiratory failure and invasive mechanical ventilation, with a 21.4% mortality rate. In another cohort of 57 patients (38), the severe group exhibited significantly higher rates of pleural effusion (87.0 vs. 41.2%; $P<0.001$) and multivariate analysis revealed elevated CRP as an independent risk factor for severe disease.

Among the 208 cases, clinical improvement or cure was achieved in 194 patients (93.3%), with 14 mortalities (6.7%) reported across studies. The mortality rate in a multicentre study by Liu *et al* (10) was 8.11% (6/74) and 21.4% in a severe cohort by Zhang *et al* (39) (3/14). All surviving patients exhibited resolution or marked reduction of pleural effusion on followup imaging. These findings demonstrate that the presence of pleural effusion in CPP is consistently associated with more severe disease, pronounced hypoxia

and an increased risk of invasive ventilation and mortality, underscoring the importance of rapid diagnosis and timely initiation of targeted therapy. Details of the reviewed studies are provided in Table SIII.

Discussion

CPP accounts for ~1-2% of CAP cases annually in China (5,6); however, among patients with severe pneumonia admitted to the intensive care unit, this percentage can be as high as 8% (13). The incubation period for CPP is generally 5-14 days (2), with clinical manifestations being nonspecific and often presenting as influenza-like symptoms or pneumonia, including fever, chills, fatigue, cough and myalgia (5). In the present case series, the observed clinical symptoms, including high fever, cough, sputum production, anorexia, fatigue, generalized myalgia and headache, may be confused with those of influenza and other forms of CAP. The lack of improvement in the patients following initial antimicrobial therapy at outside hospitals is consistent with the commonly reported clinical presentation of *psittacosis* infection (9,10,40).

Chest CT scan findings in patients with CPP typically include extensive pulmonary lesions accompanied by varying degrees of exudation, consolidation and patchy shadows (41). By contrast, the radiological presentation in the present case series, patchy high-density opacities in unilateral lungs accompanied by ipsilateral pleural effusion, constitutes a less common imaging pattern compared with the aforementioned typical features. Atypical pneumonia caused by pathogens such as *Legionella*, *Mycoplasma*, *Rickettsia* or respiratory viruses may also be accompanied by pleural effusion (42), which typically presents as a small volume of exudative fluid ipsilateral to the solid pulmonary infiltrates and often resolves spontaneously with effective treatment of the pneumonia (43). Therefore, CPP cannot be definitively diagnosed on the basis of radiological features alone. An analysis of clinical features and prognosis in atypical pneumonia by Zhao *et al* (33) indicated that patients with concomitant pleural effusion had more severe disease and higher mortality. Integrating the findings from the present case series with the relevant literature on CPP reveals that patients who present with pleural effusion generally experience more severe illness. Physiologically, arterial blood gas analysis revealed more pronounced hypoxia, leading to a markedly increased risk of developing type I respiratory failure and requiring invasive ventilatory support, as illustrated in the present *Case 2*.

The underlying reasons for this aggravated clinical course are multifactorial. CPP is typically characterized by rapid progression, a high incidence of severe cases and an exceptionally high frequency of hypoxemia, with ~50% of patients progressing to respiratory failure necessitating high-flow oxygen therapy or even mechanical ventilation (44). In this context, the presence of pleural effusion, even if small in volume, can further compromise gas exchange and exacerbate the degree of hypoxemia, thereby precipitating or worsening respiratory failure (45).

Studies have indicated that patients with CPP often do not exhibit markedly elevated white blood cell counts, which tend to be lower compared with those observed in patients with other types of pneumonia, whereas the levels of inflammatory markers such as CRP are notably elevated (46,47). This conclusion is consistent with the present case series, in which patients exhibited normal white blood cell counts but notably elevated hs-CRP levels, IL-6 levels and ESRs. However, the diagnostic value of these inflammatory markers is limited, as their elevation is not specific to *Chlamydia psittaci* infection and lacks pathogen-directed specificity. In agreement with previous reports and studies (40,41,47), *Case 2* demonstrated marked elevations in LDH and HBDH levels. These abnormal laboratory findings likely reflect tissue and cellular damage induced by the inflammatory cascade of CPP (48,49).

Epidemiological data have indicated that ~90% of CPP cases involve a history of poultry contact (50), with exposure to sick poultry leading to diagnostic clue clues. Therefore, systematic elicitation of occupational exposure and bird-keeping history is key in clinical diagnosis. All the patients in the present report had had contact with pigeons or birds, which significantly increased the risk of *Chlamydia psittaci* exposure. The course of their illness was highly consistent with the typical infection route of this pathogen.

Currently, laboratory diagnosis of CPP relies on primarily three categories of methods: Serological testing, pathogen culture and nucleic acid amplification techniques. Among serological assays, commonly used clinical methods include pathogen isolation, ELISA, complement fixation test and microimmunofluorescence assay (51). The complement fixation test requires a convalescent-phase serum antibody titer that is ≥4 times higher than the acute-phase titer or an IgM titer >1:32 for diagnosis, but this method cannot differentiate between *Chlamydia* species (14). Direct immunofluorescence antibody staining of respiratory specimens (such as sputum, throat swabs or BALF), while useful for rapid screening, has marked limitations in both sensitivity and specificity (1). Compared with serological testing, nucleic acid amplification techniques (including PCR) demonstrate superior diagnostic performance but are generally recommended only for determining the pathogen during psittacosis outbreaks (52).

With the rapid development of molecular biology, techniques such as mNGS are now widely used for detecting pathogenic microorganisms in respiratory samples and some reports or studies on the application of mNGS in the diagnosis of CPP have been published (6,40,53,54). mp-tNGS is a high-throughput sequencing technology that uses numerous primer sets to simultaneously amplify specific target gene sequences from the nucleic acids of a sample through multiplex PCR, after which the resulting amplicons are then sequenced, enabling subsequent bioinformatic analysis to identify the targeted pathogens (17,22). While the primary focus of the present case series was to demonstrate the clinical application and utility of mp-tNGS, a comparative perspective underscores its potential advantages. Traditional methods such as culture and staining, as evidenced in the present cases, often yield to deliver timely results for *Chlamydia psittaci* (1,11). mNGS, while beneficial in unbiased pathogen detection, presents challenges including high sequencing costs, significant interference from abundant host nucleic acids which can reduce pathogen detection sensitivity and long turnaround times (typically 16-24 h) (16,55). By contrast, mp-tNGS employs targeted amplification, which enriches pathogen sequences, markedly reduces host background and

allows for faster, more cost-effective sequencing runs. As demonstrated in prior studies (15,17), mp-tNGS consistently achieves an average TAT of 10.3 h. This rapid TAT is a key differentiator, enabling same-day or next-morning diagnosis, which directly informed timely therapeutic adjustments in all the present patients. In the present two cases, the TATs for mp-tNGS analysis of BALF samples were 11.2 h and 11.5 h, which are comparable to those reported by Yin *et al* (17) and notably shorter compared with the 16-24 h reported for mNGS (17). Furthermore, on the basis of the rapid results of mp-tNGS testing, the time from admission to diagnosis was notably shortened in the two cases, thereby preventing potential progression to severe pneumonia due to delayed diagnosis. Therefore, mp-tNGS offers both timely diagnostic guidance for targeted therapy and a more economical option for patients. Considering the timeliness of diagnosis and the prevention of CPP from developing into severe pneumonia, it is necessary to adopt a more rapid detection method. Huang *et al* (20) reported 12 cases of CPP diagnosed by mNGS, highlighting its clinical and imaging features. While this study underscores the value of mNGS in detecting this elusive pathogen, the present cases specifically demonstrates the advantages of mp-tNGS, a technique that employs targeted multiplex PCR amplification prior to sequencing. Compared with the mNGS approach used by Huang *et al* (20) mp-tNGS in the present cases achieved a mean TAT of 11.35 h, markedly shorter compared with the 16-24 h typically reported for mNGS (16,17), thereby enabling same-day therapeutic adjustments. The rapid diagnostic capability of mp-tNGS, as shown, facilitates the early initiation of precise antimicrobial therapy (such as doxycycline). This can curtail the duration of inappropriate broad-spectrum antibiotic use, potentially reduce the length of hospital stay, prevent progression to severe (and more costly) pneumonia and mitigate the long-term societal costs associated with antimicrobial resistance (3,4). Therefore, while a formal cost-benefit analysis was beyond the scope of this clinical case series, the combined advantages of reduced direct testing cost and profound positive impact on clinical management pathways position mp-tNGS as a potentially cost-effective solution for the diagnosis of challenging respiratory infections such as psittacosis.

Evidence-based medical research has determined that the mortality rate for untreated *Chlamydia psittaci* infection can reach 15-20%, whereas appropriate treatment can notably reduce it to ~1% (56). For the aetiological treatment of CPP, tetracyclines (including doxycycline) and fluoroquinolones (such as moxifloxacin), which exert antibacterial effects by inhibiting pathogen nucleic acid metabolism and protein synthesis, are recommended as first-line therapeutic options (39). In a study by Chen *et al* (40) analysing 50 patients with CPP, doxycycline monotherapy was administered in 66% (33/50) of cases; quinolone monotherapy in 14% (7/50); a combination of doxycycline and azithromycin in 4% (2/50); and a combination regimen of doxycycline and quinolones in 4% (2/50), with all regimens achieving marked radiological improvement and favorable clinical outcomes. In the present case series, the rapid diagnostic results from mp-tNGS enabled timely initiation of targeted antibiotic therapy in all patients. Notably, all patients achieved complete resolution of pulmonary lesions and significant clinical cure within 10 days.

It should be acknowledged that biochemical and microbiological characterization of the pleural effusion (including Light's criteria, pH, glucose, lactate dehydrogenas, adenosine deaminase or mptNGS of pleural fluid) was not available in the present cases. The reason, as clarified was that thoracentesis was attempted in both patients but failed to aspirate any fluid because of the small volume of the effusion, a common clinical scenario for small parapneumonic effusions (estimated volume: <300-500 ml) that often do not provide a safe or accessible fluid pocket for diagnostic sampling, even under ultrasound guidance (43). Notably, even when thoracentesis is successful in patients with psittacosisrelated pleural effusion, the findings are typically nonspecific. Xu *et al* (28) reported three cases of psittacosis pneumonia with pleural effusion in which the pleural fluid was characterized as having lymphocytepredominant exudative effusions with elevated ADA levels, features that overlap notably with tuberculous pleurisy and lack diagnostic specificity for *Chlamydia psittaci*. Sheng *et al* (58) reported a similar a case of CPP with parapneumonic effusion in which pleural fluid analysis yielded only nonspecific lymphocytic exudative findings, while a definitive diagnosis was achieved through mNGS of BALF (58). Consistent with these observations, a review by Sahn (42) on atypical pneumonia revealed that effusions in this context 'rarely provide a definitive diagnosis' and that the atiologic organism 'usually is not necessary to establish the diagnosis' through pleural fluid analysis (43). Furthermore, a recent study comparing blood and BALF samples for psittacosis diagnosis demonstrated that BALF performed improved due to a higher pathogen DNA load in the lower respiratory tract (12,57). Therefore, the absence of pleural fluid analysis did not compromise the diagnostic validity of these cases. A definitive diagnosis was securely established by mptNGS of BALF, which remains the most reliable molecular approach for detecting *Chlamydia psittaci* in lower respiratory tract infections. In addition, the rapid clinical response to doxycycline and complete radiographic resolution of both pulmonary infiltrates and pleural effusion on followup CT support the classification of these effusions as small, uncomplicated parapneumonic effusions secondary to CPP.

In conclusion, CPP exhibits complex, variable and non-specific clinical and radiographic manifestations, including the presence of pleural effusion, which may indicate greater disease severity. High clinical suspicion should be maintained for patients with a history of avian exposure (such as that to parrots or poultry) who present with high fever, cough, pleural effusion on imaging and associated symptoms such as headache, sore throat, myalgia and chills, particularly when laboratory findings reveal markedly elevated hs-CRP, ESR and IL-6 levels and normally increased LDH and HBDH levels; normal white blood cell counts; and negative conventional microbiological tests. In such scenarios, mp-tNGS emerges as an economical, rapid and reliable diagnostic tool for CPP. Its timely application can help prevent diagnostic delays and potential progression to severe disease.

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Availability of data and materials

The data generated in the present study may be found in the NCBI Sequence Read Archive under accession number PRJNA1420488 or at the following URL: https://www.ncbi.nlm.nih.gov/bioproject/1420488.

Authors' contributions

YJ and LH contributed equally to the present study. YJ and YW were involved in the conception and design of the present study. LH and HW drafted the manuscript and performed data acquisition and analysis/interpretation. JW and TR made notable contributions to data interpretation and critically revised the manuscript for important intellectual content. TR and HW acquired clinical data and collected laboratory and radiology information. JW and YW assisted in updating patient follow-up information and conducting the literature search. YJ, LH and HW confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Written informed consent was obtained from both patients for publication of the present case report and all accompanying images.

Competing interests

The authors declare that they have no competing interests.

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