Severe community-acquired pneumonia caused by *Legionella gormanii* in combination with influenza A subtype (H1N1) virus in an immunocompetent patient detected by metagenomic next-generation sequencing: A case report

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Abstract. Legionella pneumonia is an atypical form of pneumonia caused by *Legionella gormanii* that can also lead to multiple organ diseases, including acute respiratory distress syndrome and multiple organ dysfunction syndrome. *Legionella gormanii* requires a long incubation period for culture in clinical practice using BCYE medium. The specificity of serum for serological detection is low, resulting in a relatively high rate of missed *Legionella* diagnoses. Contracting the H1N1 virus can lead to the misdiagnosis of *Legionella gormanii*. Metagenomic next-generation sequencing (mNGS) is a novel tool that can rapidly and accurately identify potential *Legionella gormanii* strains. A severe case of community-acquired pneumonia in a 79-year-old patient was reported. The patient was diagnosed with *Legionella gormanii* and influenza A subtype (H1N1) virus using mNGS at The First Affiliated Hospital, Zhejiang University School of Medicine. After anti-Legionella and antiviral therapy, the number of reads identifying *Legionella gormanii* in bronchoalveolar lavage fluid using mNGS decreased from 665 to 112 as the patient's condition gradually improved. A search of PubMed revealed few reports of *Legionella gormanii* in association with the influenza A subtype (H1N1) virus. Patients with severe pneumonia caused by *Legionella* and influenza A subtype H1N1 virus infections should be screened early for infections using methods such as mNGS. This approach enables early and precise treatment, simplifying the administration of antibiotics and enhancing patient outcomes.

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Key words: metagenomic next-generation sequencing, *Legionella gormanii*, community-acquired pneumonia, H1N1, bronchoalveolar lavage fluid

Introduction

The 'Guidelines for Severe Community-Acquired Pneumonia (CAP)' released in the United States in 2019 indicate that the prevalence of atypical pathogens, especially *Legionella pneumophila*, has significantly increased in previous years (1,2). *Legionella pneumophila* is a common atypical pathogen that causes pneumonia and is the leading cause of hospital admission in ~2-15% of patients with CAP (3,4). *Legionella gormanii* is a gram-negative bacterium belonging to the genus *Legionella* that is closely related to Legionnaires' disease in humans. The main clinical symptoms include fever, cough, sore throat, runny nose, limb or joint pain, headache, vomiting and diarrhoea. The main clinical symptoms are not specific, and the prevalence of influenza A subtype (H1N1) is similar to that of *Legionella pneumophila* (5). Older individuals with *Legionella* and H1N1 influenza virus coinfection are prone to misdiagnosing the influenza A subtype (H1N1) virus, which can result in a delay in administering appropriate antibiotic treatment. Importantly, this delay may be associated with worsening morbidity and mortality (6).

Traditional *Legionella* culture methods are time-consuming, and the cultures are susceptible to contamination (7). Clinically, the diagnosis of *Legionella* infection often relies on the detection of urine antigens and anti-*Legionella* antibodies (8). However, the appearance of anti-*Legionella* antibodies is relatively delayed, typically occurring 3 weeks after onset. The clinical specificity of their detection is very poor, and the currently widely used urine antigen test is available only for detecting the *Legionella pneumophila* serogroup (9). To meet the clinical requirements for the rapid diagnosis and timely treatment of *Legionella* infection, metagenomic next-generation sequencing

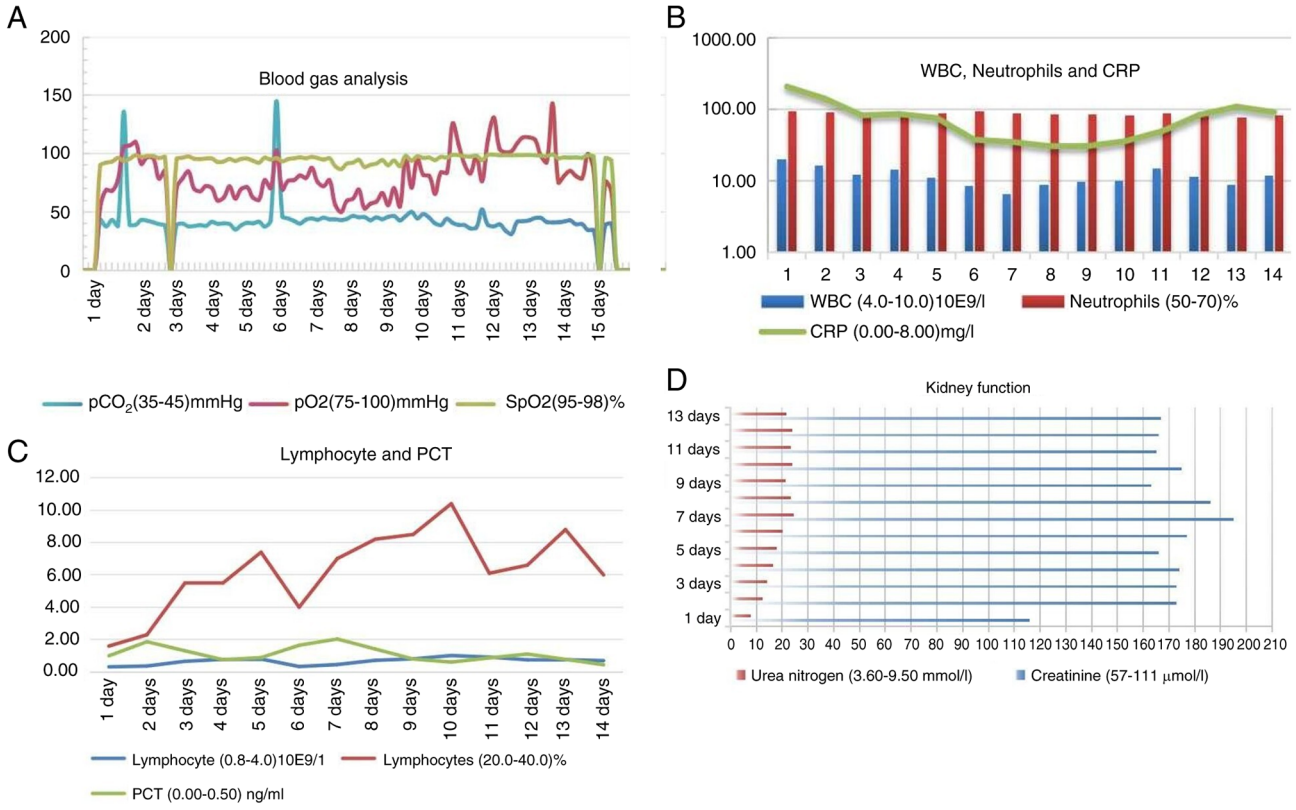

Figure 1. Regularity of inflammatory indicators in patients. (A) Changes in the pCO_2, pO_2 and SpO_2 in the patient's arterial blood before and after receiving ventilator therapy. (B) After treatment, the CRP levels decreased significantly, from 209.34 to a minimum of 30.52 mg/l. (C) Changes in peripheral blood lymphocytes and PCT levels after treatment: PCT decreased significantly, while the lymphocyte count remained low. (D) Urea nitrogen levels consistently exceeded the upper limit of detection, ranging from 7.93 to 24.5 mmol/l, while creatinine levels fluctuated between 116 and 195 μmol/l. WBC, white blood cell; CRP, C-reactive protein; PCT, procalcitonin; pCO_2, arterial partial pressure of carbon dioxide; pO_2, arterial partial pressure of oxygen; SpO_2, arterial oxygen saturation.

(mNGS) is a new tool that can quickly and accurately identify potential pathogens. mNGS was previously highlighted as the most promising method for comprehensively diagnosing infections, particularly severe pneumonia, in the intensive care unit (ICU) (10). In our clinical laboratory, a mNGS platform was build for the diagnosis of infectious disease. The process included nucleic acid extraction, library construction, sequencing, bioinformatics analysis and result interpretation. QIAamp® kits were used nucleic acid extraction in clinical samples. Library construction was performed with the Nextera XT DNA Library Prep Kit (Illumina, Inc.). Sequencing on the Illumina Nextseq CN500 used SE-75 or SE-50 protocols, generating ~20 million reads/sample. Low-quality reads and human sequences were filtered out, followed by microbial database alignment for species identification. Result interpretation guidelines are detailed in the authors' previous studies (11,12). The aim of the present study was to explore the clinical features, diagnosis and treatment of a severe pneumonia patient with co-infection of *Legionella gormanii* and influenza A subtype (H1N1) virus. Our mNGS technology was utilized to promptly and accurately diagnose *Legionella gormanii*, providing patients with the opportunity for early treatment.

Case report

A 79-year-old man with a history of high blood pressure, diabetes, cerebral infarction and tuberculosis was admitted to the ICU on February 28, 2023, at the First Affiliated Hospital, Zhejiang University School of Medicine (day 1), due to repeated fever, cough and sputum over the previous 10 days. At that time, he was diagnosed with CAP and was administered piperacillin-tazobactam (4.5 g q8h) as empirical therapy. The patient's body temperature improved (specific details are unknown), but there was no significant improvement in the symptoms of cough or sputum production. The patient denied travel history, exposure to any cooling systems or other special man-made water system exposure. In addition, blood gas analysis and whole blood lactate measurements revealed the following results: The partial pressure of carbon dioxide was 41.3 mmHg, and the partial pressure of oxygen was 53.8 mmHg. Maintaining adequate oxygenation with nasal high-flow oxygen (maximum oxygen concentration of 85%) remains challenging. The patient exhibited respiratory failure and severe pneumonia and required endotracheal intubation and ventilator-assisted ventilation (PCV-A/C, FiO_2: 35%, SpO_2 90-95%) (Fig. 1A). The pneumonia severity index was >130 points.

Physical examination revealed a temperature of 36.1˚C, a pulse of 108 beats/min, 25 breaths/min, and a blood pressure of 149/110 mmHg. Laboratory investigations revealed a white blood cell count of $19.82x10^9$/l (4.0-10.0), with a neutrophil percentage of 92.8% (50.0-70.0%), a lymphocyte count of $0.32x10^9$/l (0.8-4.0/l), a monocyte count of $1.07x10^9$/l (0.12-1.00/l), a procalcitonin (PCT) concentration

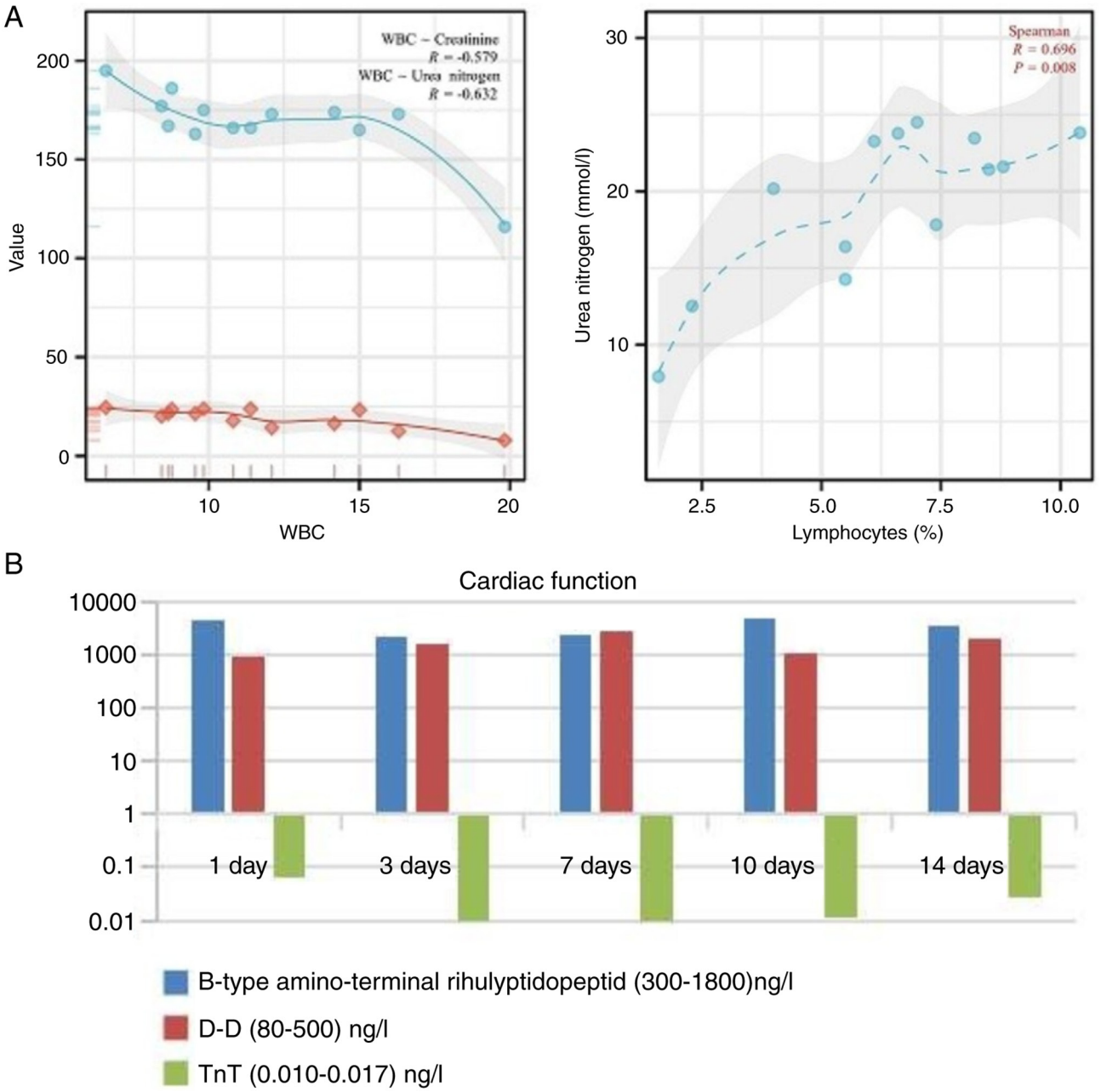

Figure 2. Kidney-related analysis and changes in cardiac markers in patients. (A) Spearman correlation analysis revealed a negative correlation between WBC count and both creatinine and urea nitrogen. Furthermore, there was a positive correlation between the proportion of lymphocytes and urea nitrogen. (B) Heart-related marker testing. WBC, white blood cell.

of 1.00 ng/ml (0.00-0.05 ng/ml) and a C-reactive protein (CRP) level of 209.34 mg/l (0.00-8.00 mg/l) (Fig. 1B and C). The serum creatinine concentration was 116 μmol/l (57-111 μmol/l), and the urea nitrogen concentration was 7.53 mmol/l (3.60-9.50 mmol/l) (Fig. 1D). The indicators of renal function appeared to be within normal limits. The B-type amino-terminal natriuretic peptide level was 4,600 ng/l (300-1,800 ng/l), the D-D level was 917 ng/l (80-500 ng/l) and the TnT level was 0.067 ng/l (0.010-0.017 ng/l) (Fig. 2A and B). Cardiac function indicates significant functional deficiencies. Chest computed tomography (CT) performed on February 28 (day 1) revealed two signs of pneumonia: consolidation in the lower lobe of the right lung and a significant amount of fluid in the chest cavity on both sides (Fig. 3A). Moreover, the bronchoalveolar lavage fluid (BALF) mNGS were conducted to identify the potential pathogen.

On the second day, the BALF mNGS results revealed *Legionella gormanii* and H1N1 influenza infection on March 1 (Fig. 4). DNA mNGS detected 665 sequences that could be mapped to *Legionella gormanii* out of a total of 16,851,224 sequences, with a coverage of 0.892% and 13.856%, respectively (Fig. 4A). RNA mNGS detected 1,458 sequences that could be mapped to H1N1 influenza virus in a total of 6,398,711 sequences, with a coverage of 89.246% (Fig. 4B). The patient was diagnosed with community-acquired pneumonia caused by *Legionella gormanii* and coinfection with the H1N1 influenza virus. The patient received timely

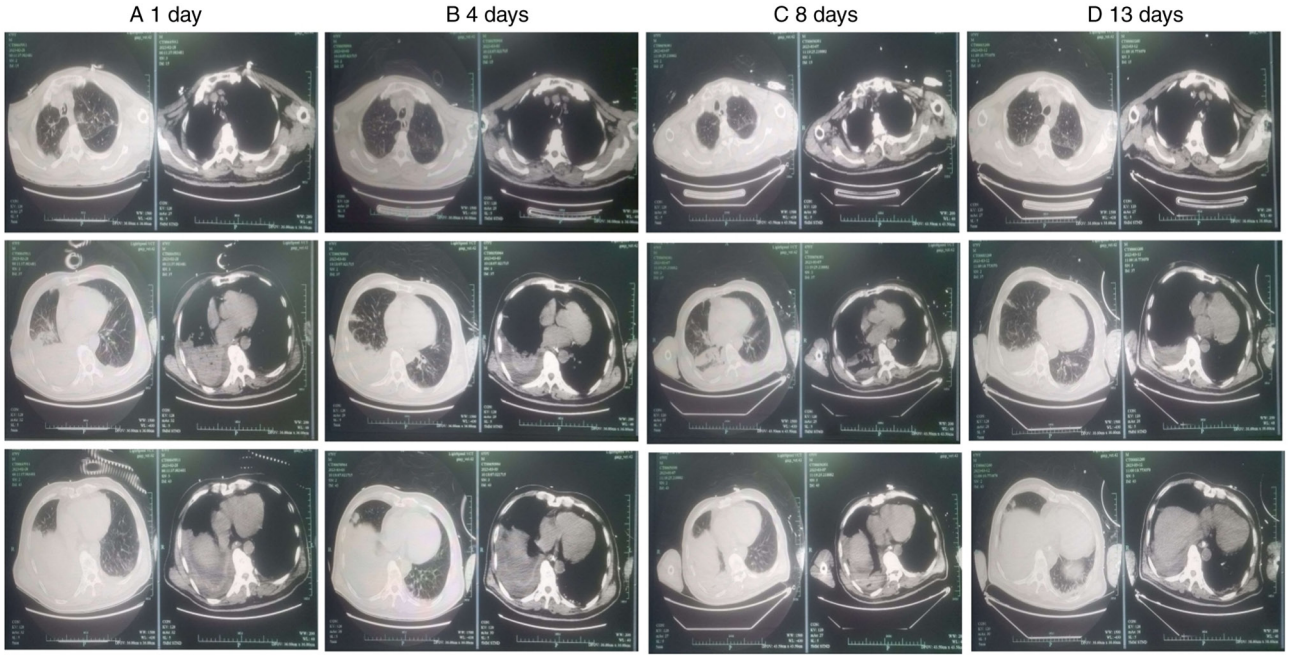

Figure 3. Chest CT results of the patient. (A) CT scan before definitive diagnosis of *Legionella gormanii* combined with influenza A subtype (H1N1): Two pneumonitis changes were observed, including severe consolidation in the lower lobe of the right lung and a small amount of pleural effusion on both sides. (B) First chest CT after treatment (4th day after admission): Two cases of pneumonia were identified, with consolidation in the lower lobe of the right lung revealing slight absorption and a small amount of fluid accumulating in the pleural cavity bilaterally. (C) Second chest CT after treatment (8th day after admission): The inflammation and consolidation in the lower lobe of the right lung were effectively resolved, and a small amount of fluid accumulated in the pleural cavity bilaterally. (D) Third chest CT after treatment (13th day after admission): In the presence of *Klebsiella pneumoniae*, signs of infection were observed in both lungs, with a slight decrease in the function of the left lung and a slight worsening of the severity of the right pleural effusion. CT, computed tomography.

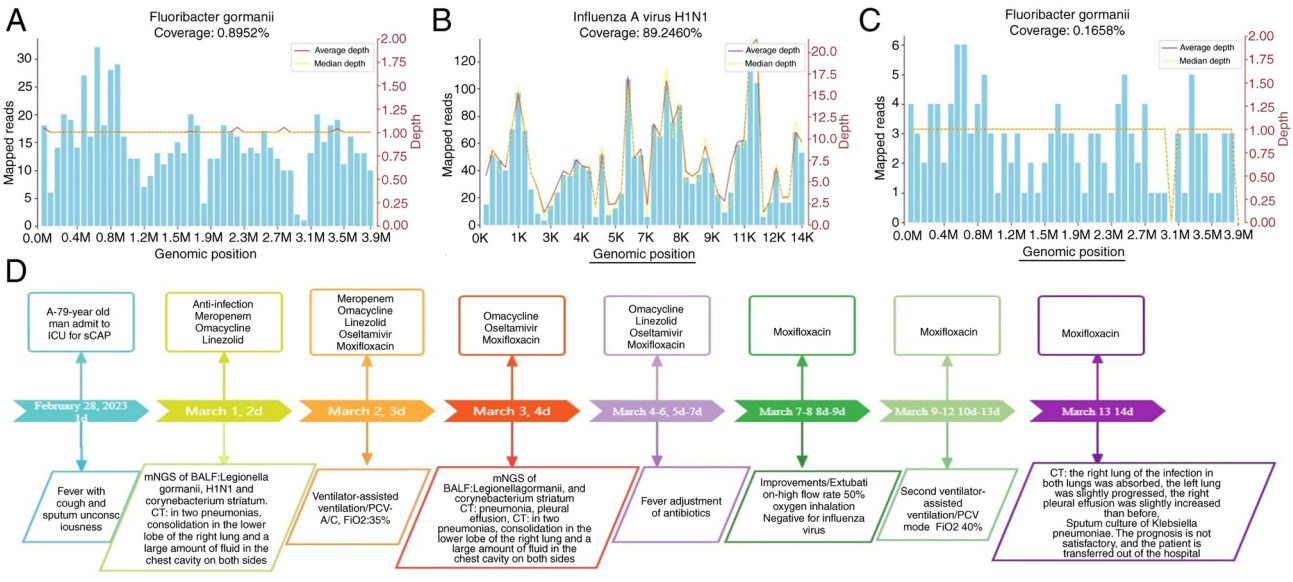

Figure 4. Sequence characteristics of *Legionella gormanii* H1N1 detected by mNGS during treatment. (A) In a total of 16,851,224 sequences, DNA mNGS detected 665 sequences that could be mapped to *Legionella gormanii*; the coverage was 0.892% and 13.856% (green columnar section). (B) RNA mNGS detected 1,458 sequences mapped to H1N1 influenza virus in a total of 6,398,711 sequences, and the coverage was 89.246% (green columnar section). (C) After 5 days of treatment, the number of raw reads of *Legionella gormanii* in BALF was 112, and the coverage was 0.165% (green column). (D) A brief review of the medical history and treatment of this 79-year-old patient. mNGS, next-generation sequencing; BALF, bronchoalveolar lavage fluid; ICU, intensive care unit.

symptomatic treatment, which included an intravenous drip of linezolid (0.6 g Q12H) and oral oseltamivir (75 mg Q12H). When the patient's temperature continued to rise, an intravenous drip of moxifloxacin (400 mg QD) and other treatments were administered (Fig. 5A).

After treatment on the fourth day, the patient's body temperature remained normal (Fig. 5B), and his overall condition improved significantly. The levels of inflammatory indicators, such as PCT, CRP and white blood cells, decreased significantly (Fig. 1B and C). CT imaging revealed two signs of pneumonia:

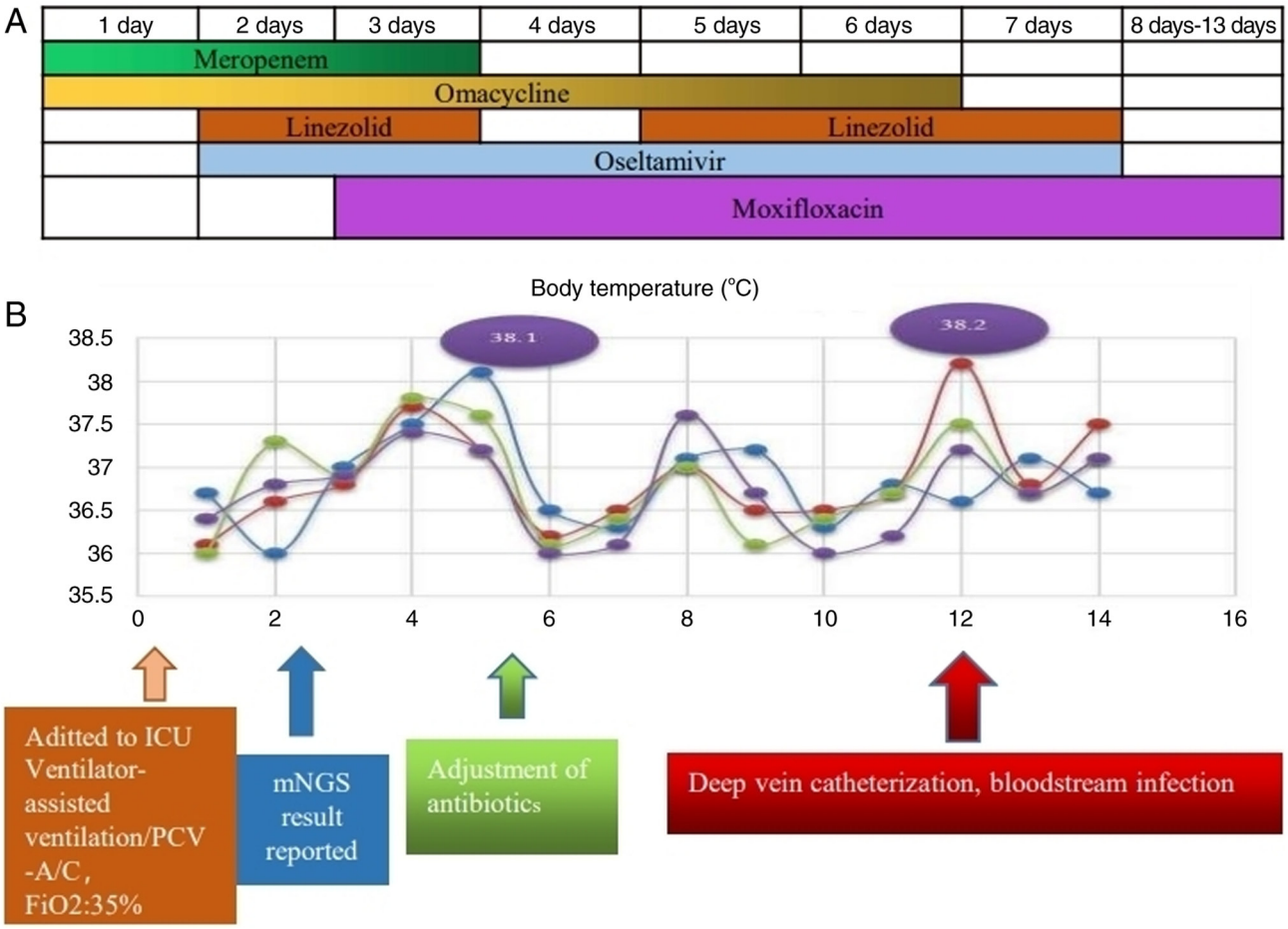

Figure 5. (A) The patient was prescribed antibiotics and antivirals after receiving a confirmed diagnosis. The following intravenous drip was used: Meropenem (0.5 g q6h) for 3 days, omadacycline (0.1 g qd) for 6 days, linezolid (0.6 g Q12h) for 5 days, and moxifloxacin (400 mg qd) for 11 days. Oral: The duration of oseltamivir therapy was 5 days, with a dosage of 75 mg every 12 h. (B) The patient was given a change in body temperature after the corresponding treatment.

Consolidation in the lower lobe of the right lung, which had partially resolved, and a small amount of fluid in the pleural cavity on both sides (Fig. 3B). Tests for β-(1,3)-glucan (BD) and galactomannan were negative. Linezolid treatment was discontinued on the 4th day, and the patient developed a fever on the fifth day, reaching 38.2˚C, but the CRP level did not significantly increase. After 1 week of treatment, the number of raw reads of *Legionella gormanii* in BALF was 112, with a coverage of 0.165%. The virus test result was retested, and the results were negative according to reverse transcription (RT)-PCR. For empirical anti-infective therapy, these results indicated that the treatment was effective, and the chest CT changes were consistent (Figs. 4C and 3C). After antibiotic treatment, the indicators of kidney function damage significantly increased (creatinine 195 μmol/l, urea nitrogen 24.5 mmol/l), and the indicators of heart damage also significantly increased (B-type amino-terminal rihulyptidopeptide 2,450 ng/l, D-D 2,870 ng/l) (Figs. 1D and 2B). The organ function was not promising, and the correlation analysis revealed a significant relationship between white blood cell count, creatinine and urea nitrogen, showing negative correlations (R=-0.596, R=-0.632). Additionally, there was a positive correlation between lymphocyte percentage and urea nitrogen (R=0.696) (Fig. 2A). The impact of drug side effects cannot be disregarded when the impairment of kidney function is associated with the virus, as heart and lung function are closely interconnected. Furthermore, The FACSCanto flow cytometer (Becton Dickinson and Company) and a set of six-colour fluorescently labelled antibodies, including i) ISG1, ii) fluorescein isothiocyanate (FITC)/IgG1, iii) Phycocyanin (PE), iv) CIM FITC/CD8-PE, v) CD3-FITC/CD16^{+}56 PE and CD19-ECD (Becton, Dickinson and Company) were utilized. The Lymphocyte Subsets Test Kit (Becton, Dickinson and Company; cat. no. 662967) was utilized to measure the ratio of T cell subsets (CD3^{+} CD4^{+}, CD3^{+} CD8^{+}), B cells (CD3-CD19^{+}) and the percentage of natural killer (NK) cells (CD3-CD16^{+} CD56^{+}) in peripheral blood, providing relative and absolute values of the detected immune cells. The absolute number of CD45 decreased from 570 to 383, CD3 decreased from 444 to 263, the relative number varied from 51.33 to 61.37%, the absolute value of CD19 decreased from 66 to 29, and the relative value decreased from 44.8 to 2.63%. However, the absolute and relative counts of CD16^{+} and CD56^{+} both increased, from 86 and 2.73 to 33.63% from the original 52. The decrease in T and B cells reflects the poor immune function of the patient, and the increase in NK cells was caused by the elimination effect of *Legionella* and viruses on these foreign pathogens (Figs. 6A and B and 7A-D).

After 13 days of treatment, the CRP index increased. CT imaging revealed significant inflammatory infiltrative changes in the lungs. However, the function of the left lung

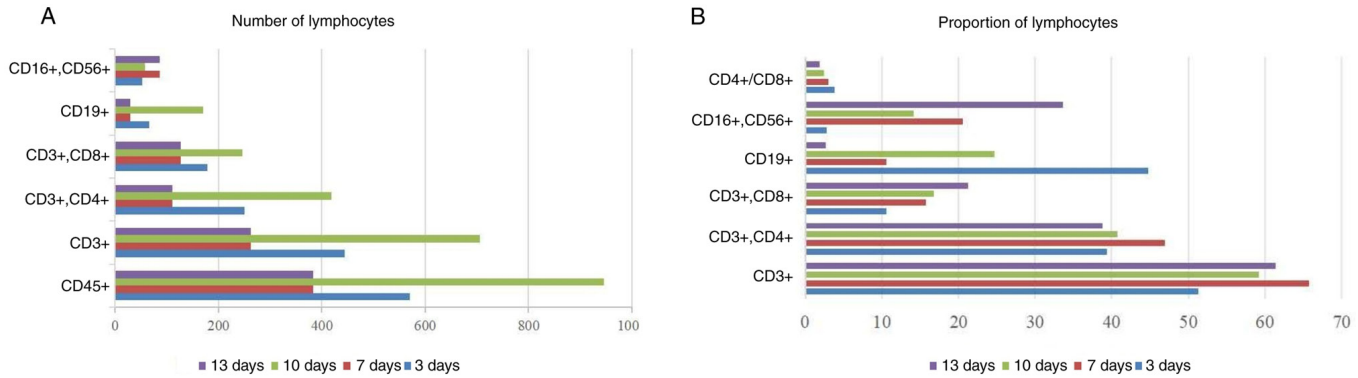

Figure 6. Immune cell changes. (A) and (B): Therapeutic tests related to a patient's immune function involve the use of flow cytometry techniques. The number of lymphocytes significantly increased on the 10th day of treatment. However, when *Klebsiella pneumoniae* is introduced, it disrupts the body's immune function, leading to a decrease in the number of lymphocytes, which remains within the normal range.

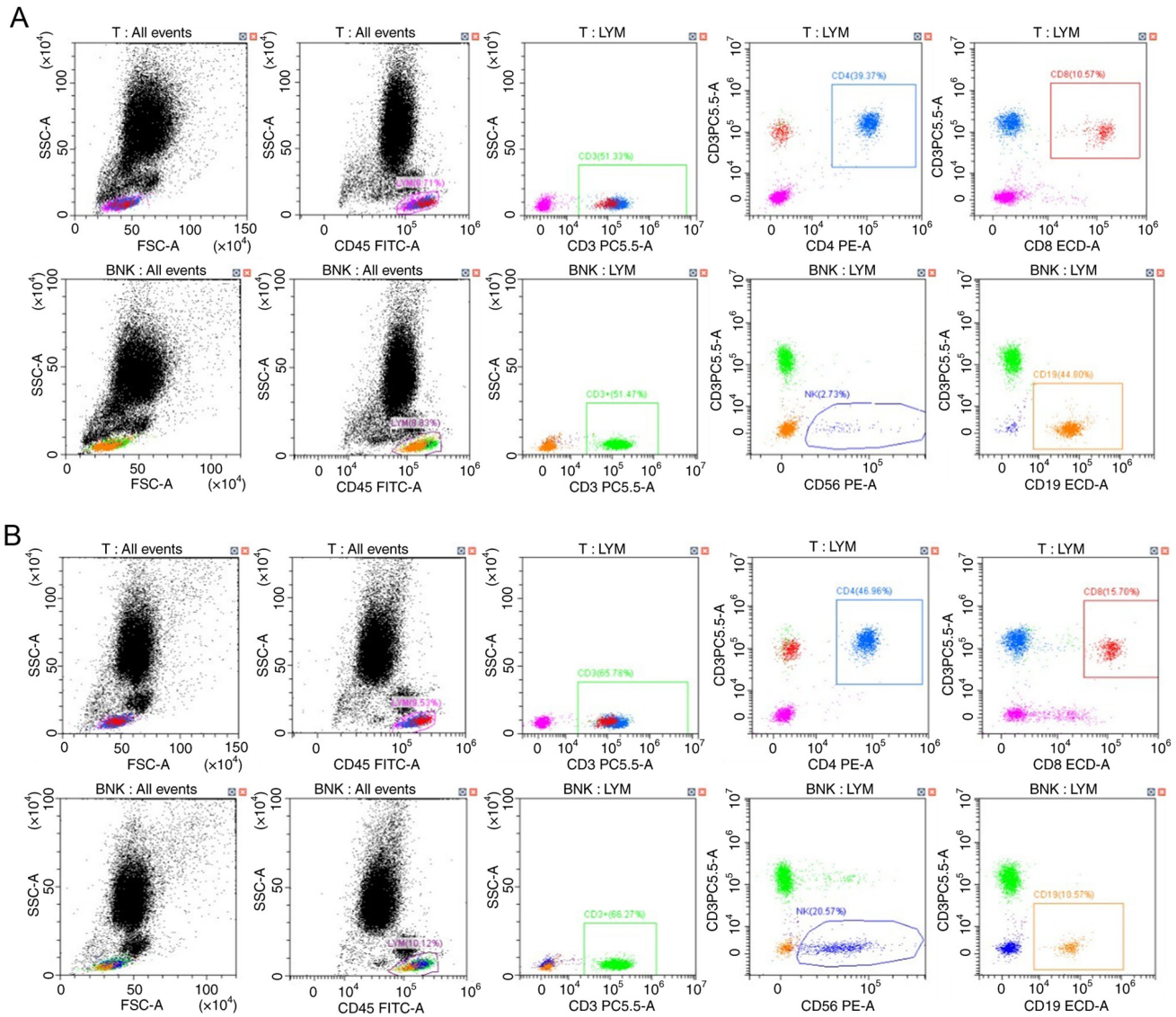

Figure 7. Continued.

had slightly deteriorated. The severity of the right pleural effusion also slightly increased, and sputum culture revealed *Klebsiella pneumoniae* (Fig. 3D). Spearman correlation analysis was conducted using SPSS 20 software (IBM Corp.). There was no significant improvement in cardiopulmonary function (Fig. 2B). The patient's condition remained

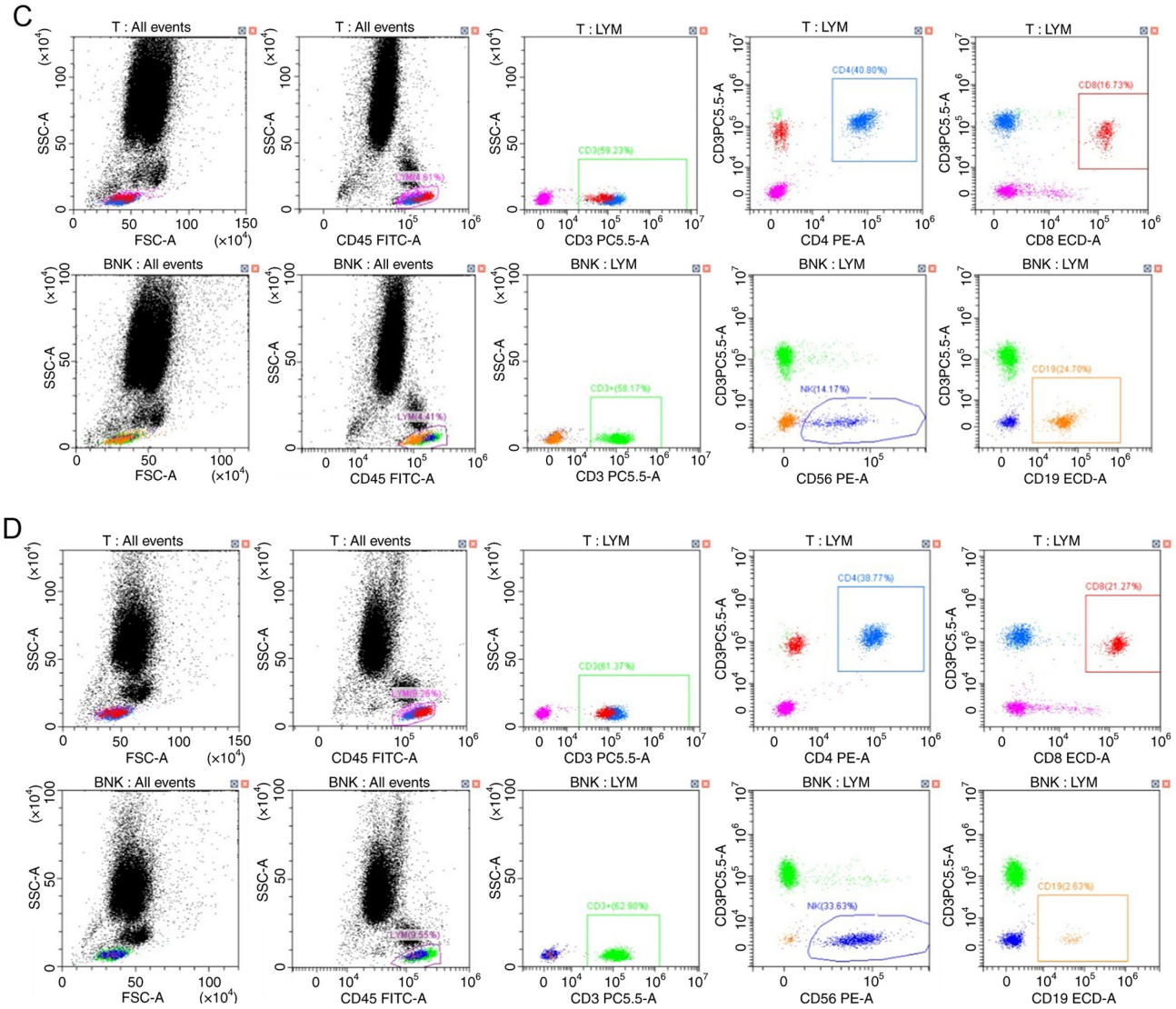

Figure 7. The number of lymphoid subsets was measured by flow cytometry. (A) Number of lymphocytes on the third day of the disease course. (B) Number of lymphocytes on the seventh day of the disease course. (C) Number of lymphocytes on the tenth day of the disease course. (D) Number of lymphocytes representing the thirteenth day of the disease course. FACSCanto flow cytometry was used (T lymphocyte antibody reagents included CD3-FITC antibody, CD8-PE antibody, CD45-perCP antibody, CD4-APC antibody; B lymphocyte antibody reagents including CD3-FITC anti-CD16 + CD56-PE anti-CD45-Percp anti-CD19-APC antibody) were used to automatically set the gate to detect $CD3^+$ T cells $CD4^+$ T in whole blood samples Percentage of cells, ($CD3^+$ $CD4^+$) $CD8^+$ T cells ($CD3^+$ $CD8^+$) B lymphocytes ($CD19^+$) and NK cells (CD3-$CD16^+$ 56^+) in total lymphocytes and their absolute values, the absolute values were measured by body Product method $CD3^+$ $CD4^+$ ratio is calculated value. NK, natural killer.

unstable due to the lack of significant improvement in their immune system, compounded by the underlying primary disease. In this scenario, new pathogens associated with nosocomial infections and multiple antibiotic-resistant strains have emerged, leading to no apparent improvement following treatment. Consequently, the patient's family requested discharge. The treatment process is illustrated in Fig. 4D.

Discussion

To understand the clinical features, diagnosis and treatment of *Legionella gormanii* in conjunction with influenza A subtype (H1N1) virus, which causes a high-risk, low-epidemic infectious disease, the present case report introduced a patient with *Legionella gormanii* and H1N1 influenza virus coinfection, which led to severe pneumonia. mNGS technology was utilized to promptly and accurately diagnose *Legionella gormanii*, providing patients with the opportunity for early treatment.

Legionella is a significant cause of community-acquired pneumonia, with ~90% of reported cases attributed to *Legionella pneumophila*, 79% of which are caused by the *Legionella pneumophila* serogroup (13). Human infection with *Legionella pneumophila* primarily occurs through the inhalation of aerosols containing pathogens (14). However, the symptoms of H1N1 influenza are constantly evolving, especially in older individuals with underlying medical conditions. Older individuals with *Legionella* and H1N1 influenza virus coinfection are prone to misdiagnosis, which can delay the administration of antibiotic treatment.

Previous cases were reviewed of misdiagnosis and missed diagnoses of Legionella pneumonia (Table I) (12). According to the relevant literature, the patient tested positive for the *Legionella* antigen in his urine. However, there were also

Table I. A review of clinical information on Legionella gormanii combination influenza A subtype (H1N1) virus syndrome cases reported in recent years.

Case/Sex/Age	Symptoms and Inducement	Clinical feature	Pathogen (detection method)	Medication	Length of stay	Assisted ventilation	Outcome	References
The present case/ Male/79	Repeated fever with cough and sputum during the previous 10-day	Respiratory failure. Severe pneumonia. High blood pressure. Diabetes	mNGS of BALF: *Legionella gormanii* H1N1	Meropenem Omacycline Linezolid Oseltamivir Moxifloxacin	14 days	Trachea cannula	Transfer to hospital for rehabilitation	The present study
Case1/Male/59	Hot/cold sweats and high fever shower in the work's changing rooms were not widely used	Pulmonary infiltrates atrial flutter with rapid ventricular response	Urinary legionella antigen: *Legionella gormanii* RT-PCR: NH1N1	Oseltamivir rifampicin clarithromycin ciprofloxacin	14 days		Live	Schofield *et al*, 2010 (16)
Case 2	Sore throat without obvious	Exacerbations of chronic bronchitis, asthma and congestive heart failure	Urinary legionella antigen: *Legionella gormanii* RT-PCR: NH1N1	Oseltamivir other antibiotics			Live	Burk *et al* 2010 (5)
Case 3		Pneumonia after returning from a 1-week travel abroad	Legionella serology (single titer): *Legionella gormanii* RT-PCR: NH1N1	Oseltamivir other antibiotics			Live	Caterina *et al*, 2010 (12)

mNGS, next-generation sequencing; BALF, bronchoalveolar lavage fluid; RT-PCR, reverse transcription polymerase chain reaction.

instances of false positive results in urine tests, and the testing process was relatively time-consuming. There are several crucial factors to consider in the misdiagnosis of *Legionella pneumonia*. First, the clinical manifestations of *Legionella pneumoniae* infection are non-specific, and the diagnosis is based on laboratory testing for pathogens. Second, molecular diagnostic techniques, with a sensitivity of 70-80% and high specificity of 99-100%, have revealed a high prevalence of respiratory viruses in cases of atypical bacterial infections. Compared with 16S rRNA gene sequencing (Cloning library sequencing; Applied Biosystems; Thermo Fisher Scientific, Inc.), mNGS offers greater classification resolution and has been utilized in pneumonia diagnosis, outbreak tracking, infection control monitoring and pathogen detection (15).

Severe community-acquired pneumonia was definitively diagnosed, which was caused by *Legionella gormanii* in combination with influenza A subtype (H1N1) in an immunocompetent patient, as detected by mNGS. Fluoroquinolones or macrolides are considered first-line options for patients with *Legionella pneumonia*, while combination therapy is recommended for critically ill or immunocompromised patients. The initial treatment for the H1N1 virus was 75 mg of oseltamivir twice daily, and the patient was relocated to an isolated room in accordance with the hospital's infection control policy. After 10 days of antibiotic treatment, the patient's overall condition significantly improved, and the levels of inflammatory biomarkers decreased. CT imaging twice demonstrated that the pleural effusion had significantly decreased, indicating absorption, and the RT-PCR test result was negative. On days 11-14, the patient had a fever and elevated levels of the inflammatory marker CRP. Kidney function damage significantly increased (Fig. 1D and F). CT imaging of the chest revealed enlarged pneumonia opacities on both sides and increased pleural effusions on both sides, while sputum culture indicated the presence of drug-resistant *Klebsiella pneumoniae*. The occurrence of *Klebsiella pneumoniae* is mainly due to endogenous infection in the hospital, primarily through self-contact transmission of pulmonary gram pathogens within the patient's body (16,17). These changes predict worsening of the condition and a poor prognosis.

The worsening of the patient's condition is likely due to the presence of multiple underlying diseases, such as hypertension and diabetes. Additionally, the patient was definitively diagnosed with Legionella pneumonia (6). The appropriate treatment requires a significant amount of time, which can delay the recovery process and cause the patient to miss the optimal treatment window. Third, the patient's inflammatory markers did not decrease to normal levels, and her immune function continued to deteriorate. Fourth, there was no significant recovery from severe cardiac function injury, as indicated by high levels of B-type amino-terminal rihulyptidopeptidase, and the elevated D-dimer suggested that the patient's peripheral blood circulation had not improved (18).

In summary, the present study aimed to investigate the clinical characteristics, diagnosis, and management of *Legionella gormanii* in conjunction with the influenza A subtype (H1N1) virus. This combination results in a high-risk, low-epidemic infectious disease. MNGS technology was utilized to promptly and accurately diagnose *Legionella gormanii*. Patients who respond to antibiotic and antiviral treatments. mNGS may be a high-resolution and sensitive assay for the diagnosis and surveillance of *Legionella* infection. Further research and exploration are still needed to understand the pathogenic mechanism of *Legionella* and to evaluate the effectiveness of antibiotics.

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Availability of data and materials

The data generated in the present study may be found in the in the National Center for Biotechnology Information (NCBI) under the ascension number PRJNA1116256 or at the following URL: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1116256/.

Authors' contributions

DH designed the present study. SL performed the sample and data detection. DH, SL and YZ analysed the data. SL wrote the manuscript and participated in the literature collection and evaluation. DH and SL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The studies involving human participants were reviewed and approved (approval no. IIT20220714A) by the Institutional Review Board of the First Affiliated Hospital, Zhejiang University School of Medicine (Hangzhou, China).

Patient consent for publication

Written informed consent was obtained from the patient for the publication of the present case report and any accompanying images. The patient came from the First Affiliated Hospital, Zhejiang University School of Medicine.

Competing interests

The authors declare that they have no competing interests.

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