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Zhihao Lie

Guangzhou Medical University

Yinghong Hu

Guangzhou Medical University

Jiaqi Chen

Guangzhou Medical University

Zeqi Lai

Guangzhou Medical University

Zixin Fang

Guangzhou Medical University

Huizhong Liu

Guangzhou Medical University

Jingyi Liang

the First Affiliated Hospital of Guangzhou Medical University

Zhiqi Zeng

Guangzhou Medical University

Zifeng Yang

Jeffyah@163.com

the First Affiliated Hospital of Guangzhou Medical University

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**Targeted Next-Generation Sequencing Reveals Co-infection Patterns and
Risk Factors for Severe Pneumonia in Influenza A(H1N1) pdm09**

Zhihao Lie^{1,*}, Yinghong Hu^{2,*}, Jiaqi Chen^{2,*}, Zeqi Lai^{2,*}, Zixin Fang³, Huizhong Liu³,
Jingyi Liang⁴, Zhiqi Zeng³, Zifeng Yang⁴,

1 School of Public Health , Guangzhou Medical University, Guangzhou, China.

2 Nanshan School, Guangzhou Medical University, Guangzhou, Guangdong 511436,
China.

3 KingMed School of Laboratory Medicine, Guangzhou Medical University,
Guangzhou, China

4 State Key Laboratory of Respiratory Disease, National Clinical Research Center
Respiratory Disease, Guangzhou Institute of Respiratory Health, the First Affiliated
Hospital of Guangzhou Medical University, Guangzhou, China.

*These authors contributed equally to this work.

Correspondence should be addressed to Jingyi Liang Ph.D., The First Affiliated
Hospital of Guangzhou Medical University, No. 28, Qiaozhong Middle Road, Liwan
District, Guangzhou, China, email: Jingyi950429@163.com; Zhiqi Zeng Ph.D.,
KingMed School of Laboratory Medicine, Guangzhou Medical University,
Guangzhou, China, email: zeng_zhiq@126.com; Zifeng Yang Ph.D., The First
Affiliated Hospital of Guangzhou Medical University, No. 28, Qiaozhong Middle
Road, Liwan District, Guangzhou, China, email: Jeffyah@163.com.

Abstract

Background: Influenza A(H1N1)pdm09 remains a major cause of seasonal respiratory morbidity, yet predictors of progression to severe pneumonia and the associated co-infection profile remain incompletely characterized. This study aimed to describe the clinical features, laboratory abnormalities, pathogen spectrum, and risk factors for severe influenza A(H1N1)pdm09 pneumonia using targeted next-generation sequencing (tNGS).

Methods: In this retrospective single-center study, hospitalized patients admitted to Panyu Central Hospital of Guangzhou Medical University between January and December 2024 were analyzed. Based on tNGS and clinical classification, 175 patients infected with influenza A(H1N1)pdm09 were included, comprising 92 mild cases and 83 severe pneumonia cases; 121 non-H1N1 respiratory infection cases served as controls. Demographic data, symptoms, comorbidities, blood routine and biochemical indices, and co-infection profiles were compared. Uni- and multivariate logistic regression were used to identify risk factors for severe disease.

Results: Severe pneumonia was more common in older patients and was associated with higher body mass index (BMI), greater rates of hypertension, diabetes, smoking exposure, and longer hospitalization. Compared with mild cases, severe cases showed more frequent cough, dyspnea, nasal congestion/rhinorrhea, neutrophilia, lymphopenia, thrombocytopenia, reduced eGFR, elevated creatinine, liver enzyme abnormalities, and increased erythrocyte sedimentation rate. tNGS revealed distinct

co-infection patterns: mild cases were predominantly bacterial, with *Haemophilus influenzae* as the leading pathogen, whereas severe cases had significantly higher fungal co-infection rates, particularly *Candida albicans* and *Aspergillus fumigatus*, and more Epstein-Barr virus detection. In multivariate analysis, age, BMI, cough, dyspnea, and nasal congestion/rhinorrhea were independent risk factors for severe pneumonia, while headache was protective.

Conclusion: Severe influenza A(H1N1)pdm09 pneumonia is characterized by advanced age, elevated BMI, respiratory symptoms, systemic inflammation, organ dysfunction, and a shift toward fungal/viral co-infection. Early risk stratification and pathogen-directed management may improve outcomes in high-risk patients.

Keywords: Influenza A; targeted next-generation sequencing; pneumonia.

Introduction

Annual seasonal influenza epidemics, caused by influenza virus infections of varying severity, have imposed a substantial global disease burden ^[1]. The global annual incidence of influenza is estimated to be 5-10% in adults and 20-30% in children; however, the mild nature of most cases leads to widespread underreporting and underdiagnosis ^[2]. Nevertheless, the World Health Organization (WHO) attributes 3-5 million cases of severe illness and 290,000 to 650,000 respiratory deaths annually to influenza ^[3]. The global economic burden of influenza—encompassing direct and indirect financial costs, productivity losses from absenteeism, and years of life lost to

mild or severe respiratory illness—is immense, with low-income countries bearing the
brunt of this impact ^[4]. Influenza viruses are classified into types A, B, and C based
on their core protein composition, with influenza A viruses typically being the culprits
behind pandemics and annual epidemics ^[5]. Specifically, the influenza A
(H1N1)pdm09 virus, originating from swine, was first documented in the United
States and Mexico in 2009, subsequently triggering a widespread pandemic that, by
May 11 of the same year, had spread through human-to-human transmission to 30
countries ^[6,7]. Although most influenza A (H1N1)pdm09 virus infections are mild,
self-limiting, and associated with a favorable prognosis, approximately 2% of infected
individuals progress to severe or critical illness, with the mortality rate for critically ill
patients reaching as high as 48.6% ^[8]. Seasonal influenza viruses readily evade host
immunity due to their antigenic drift, leading to regional epidemics each winter and
spring ^[9]. While most infections are mild, the elderly and individuals with chronic
diseases are prone to developing severe disease, which significantly impairs their
quality of life and imposes a considerable economic and healthcare burden ^[10]. This
underscores the urgency and necessity of conducting in-depth research into the
clinical characteristics and risk factors for progression from influenza A (H1N1) virus
infection to severe pneumonia.

The global spread of influenza pandemics has highlighted the importance of
establishing efficient pathogen surveillance systems and has spurred an urgent
demand for innovations in pathogen detection technology. Culture methods are widely
used for diagnosing pneumonia due to their cost-effectiveness, ability to quantify

pathogen load, and utility in facilitating antibiotic susceptibility testing. However, cultures are time-consuming and inefficient for detecting atypical pathogens ^[11]. Next-generation sequencing (NGS) offers a rapid and comprehensive approach for detecting respiratory infections, demonstrating superior performance over traditional methods in identifying such pathogens ^[12]. Metagenomic next-generation sequencing (mNGS) can rapidly and unbiasedly identify pathogenic viruses, bacteria, fungi, and parasites, showing promise as a first-line diagnostic tool for severe pneumonia. Its application, however, is constrained by high costs and interference from host genetic material ^[13]. Targeted next-generation sequencing (tNGS), which is based on probe hybridization capture or multiplex PCR amplification, enriches the nucleic acid sequences of multiple pathogenic microorganisms, demonstrating unique application value. By establishing a systematic pathogen screening panel, tNGS achieves comprehensive coverage of pathogens commonly associated with influenza virus co-infections, with its significant technical advantages reflected in its high sensitivity and specificity ^[14]. This technology has shown potential as an ideal surveillance tool in the fields of influenza virus monitoring and mixed-infection diagnosis, providing robust technical support for establishing multi-pathogen joint surveillance networks.

A review of previous research reveals that significant progress has been made both domestically and internationally in studying the clinical features and risk factors of severe pneumonia following influenza A (H1N1)pdm09 virus infection. For instance, research on the manifestations and treatment of influenza A virus with pulmonary co-infections has found that viral pulmonary infections often present with

normal complete blood counts but are frequently accompanied by varying degrees of inflammatory response^[15]. International medical research institutions and universities have a deep foundation in this field. Elsevier, for example, has published extensive discussions on the latest advancements in key clinical influenza issues, pointing out that a logistical challenge for nearly all countries is the immediate availability of influenza diagnostic testing and timely antiviral treatment initiation, especially for high-risk populations^[16]. A landmark study on community-acquired pneumonia (CAP) diagnosed 14 respiratory pathogens and their patterns of infection or co-infection, providing unparalleled insights into the age-specific etiology of CAP^[17]. However, previous studies have often been limited by small sample sizes and the restricted multiplexing capabilities of their pathogen detection methods, which has hindered a more comprehensive analysis of both the clinical characteristics and the risk factors for severe disease caused by the influenza A (H1N1)pdm09 virus.

Therefore, this study employs tNGS technology to perform ultra-multiplex pathogen detection (covering 78 viruses, 80 bacteria, 32 fungi, and 7 atypical pathogens) in patients infected with influenza A (H1N1)pdm09 and a control group. This approach allows for a more representative analysis of the co-infection profiles in patients with influenza A (H1N1)pdm09. Concurrently, by analyzing the distribution and differences in demographic information, complete blood counts, and various biochemical indicators among different populations, this study aims to provide an in-depth investigation into the clinical characteristics and risk factors for the progression of influenza A (H1N1) virus infection to severe pneumonia. The findings are intended

to offer a scientific basis for optimizing influenza prevention and control strategies, as well as the clinical management of severely ill patients.

Materials and Methods

1. General Information

In this retrospective study, hospitalized patients were brought to Panyu Central Hospital of Guangzhou Medical University from January 1, 2024 to December 31, 2024, respectively. 175 patients of the Influenza A (H1N1) pdm09 group were considered, including 83 cases of severe pneumonia (H1N1 pdm09 Severe Pneumonia Group) and 92 cases of mild illness (H1N1 pdm09 Mild Group). 121 patients of non-infected influenza A were randomly drawn from both controls.

Severe Criteria: (1) Meeting any of the following: ①Patients with respiration distress, respiratory rate (RR) ≥ 30 times/min; ②Pulse oxygen saturation $< 93\%$ resting state, no oxygen supply; ③Arterial partial pressure of oxygen (PaO₂) / fraction of inspired oxygen (FiO₂): ≤ 300 mm Hg; (2) Cases that, although not yet meeting the above severe diagnostic criteria, are managed as severe cases: ① Non-infecting patients with lung imaging showing obvious lesions $> 50\%$ within 24~48 hours; ② Age > 60 years of acute chronic disease such as hypertension, diabetes, coronary heart disease, malignant tumors, structural lung disease, cor pulmonale and immunosuppressed populations.

Mild Criteria: Patients with severe criteria that appear to be infected by respiratory infection (shaker throat, low-grade fever, rhinorrhea). Exclusion criteria: (1) Patients

with non-respiratory diseases in the influenza A (H1N1) pdm09 mild group, (2)

Missing key clinical information.

Data was collected primarily on the medical record system, such as population information, laboratory test results, symptoms, physical examination results, underlying disease, time from onset to hospitalization, time duration of hospitalization; Body Mass Index (BMI) was calculated to better understand the relation between each of the populations and physical status.

2. Methods

We followed a respiratory pathogen detection workflow: Upper and lower respiratory tract samples (including throat swabs, bronchoalveolar lavage fluid, and sputum) were collected from all subjects within 24 hours of admission and brought cold chain to the clinical microbiology testing center. Sample preparation was performed by manual extraction (sample pretreatment [different ways for different types of samples], enrichment, preparation of negative and positive controls, cell wall disruption, digestion, binding, purification, and final nucleic acid concentration measurement and recording). Sequencing (Respiratory 100) of pathogen microbe was performed with sequencing kits (MH300, MM300, MR100, MH75), and a genetic analyzer (KM MiniSeqDx-CN, NMPA No. 20203220340) manufactured by Guangzhou KingCreate Biotechnology Co., Ltd. The sequence aligning module (bWA v0.7.17, GATK v3.5-0, Picard-tools v1.95) mapped the preprocessed sequence to the human reference

genome (hg19 [GRCh37]) sorting, removing PCR duplicates, and realizing;
bioinformatics analysis was completed after data quality control, variant detection,
and variant annotation. Detectable pathogens included 79 viruses (e.g., Human
Adenovirus, Human Adenovirus Group B), 80 bacteria (e.g, Mycobacterium
tuberculosis complex, Acinetobacter baumannii), 32 fungi (e.g., Candida glabrata,
Aspergillus flavus complex), and 7 abnormal pathogens (e.g. Chlamydia,
Mycoplasma) totaling 198 pathogens. Blood tests conducted within 24 hours after
admission (24 blood routine items, 4 liver function items, 3 kidney function items and
procalcitonin, erythrocyte sedimentation rate, albumin etc.), records of progress, and
summaries of discharge during improvement/death.

3. Observation Indicators

We employed a multidimensional analytical framework to examine differences across
key comparisons: 1) Baseline characteristic differences between the influenza A
(H1N1) pdm09 patient group and the control group; 2) Clinical heterogeneity between
the influenza A (H1N1) pdm09 severe pneumonia group and the mild group.

Study variables included: (1) Demographics: age, sex, and anthropometric measures
(Body Mass Index, BMI);

(2) Laboratory indicators: a) Complete blood count analysis (covering 24
parameters such as white blood cell count, neutrophil percentage); b) Liver function
tests (ALT, AST, AST/ALT ratio, and GGT); c) Renal function profile (blood urea

nitrogen, serum creatinine, estimated glomerular filtration rate [eGFR], uric acid); d)

Other biochemical indicators (serum albumin, erythrocyte sedimentation rate, procalcitonin, etc.);

(3) Clinical characteristics: a) Symptoms: respiratory symptoms (cough, shortness of breath, dyspnea) and systemic symptoms (fever, chills, fatigue, myalgia, headache, etc.); b) Comorbidity analysis (hypertension, diabetes, chronic kidney disease, etc.);

(4) Vital sign parameters: body temperature, respiratory rate, pulse rate, blood pressure;

(5) Disease course-related variables: time interval from symptom onset to admission, length of hospital stay.

4. Statistical Analysis

We used professional statistical analysis software for data processing and inference.

We preprocessed and visualize data using R (v4.3.2; R Foundation for Statistical Computing) and GraphPad Prism 10, and statistical inference with SPSS Statistics 26 (IBM Corp.).

Categorical variables were presented by frequency distribution (n) and proportion (%); inter-group comparisons were performed using Pearson's χ^2 test or Fisher's exact test (applicable when >20% of cells have an expected frequency <5).

Since most continuous variables have non-normal distribution (confirmed by Shapiro-Wilk test), we represented data by median (M) and interquartile range (IQR). We used Mann-Whitney U non-parametric test to compare the comparison between different

groups, and Bonferroni correction for several comparisons. All hypothesis tests are two-sided, with the statistical significance threshold set at $\alpha=0.05$, and the significance level for multiple comparisons adjusted to α/k .

We performed risk factor analysis using univariate and multivariate Logistic regression. We used stepwise forwards approach to select variables entering the model.

Results

1. Grouping and Case Collection Results

From January 1, 2024, to December 31, 2024, a total of 263 patients infected with influenza A (H1N1) pdm09 virus detected at Panyu Central Hospital of Guangzhou Medical University were included in the influenza A (H1N1) pdm09 patient group. Additionally, 150 cases of respiratory infection not infected with influenza A (H1N1) pdm09 virus were included as the control group. After excluding 74 patients with non-respiratory system diseases, a data collection patient list was formed. Following data collection and exclusion of 43 patients with missing key clinical information, 296 cases were ultimately included for subsequent study.

Based on tNGS pathogen detection results, patients were divided into a control group of 121 cases not infected with influenza A (H1N1) pdm09 virus and an influenza A (H1N1) pdm09 patient group of 175 cases infected with the virus. Based on the admitting department (intensive care unit vs. non-intensive care unit) and

clinical diagnosis results, the influenza A (H1N1) pdm09 patient group was further divided into a severe pneumonia group of 83 patients and a mild group of 92 patients, as shown in Figure 1.

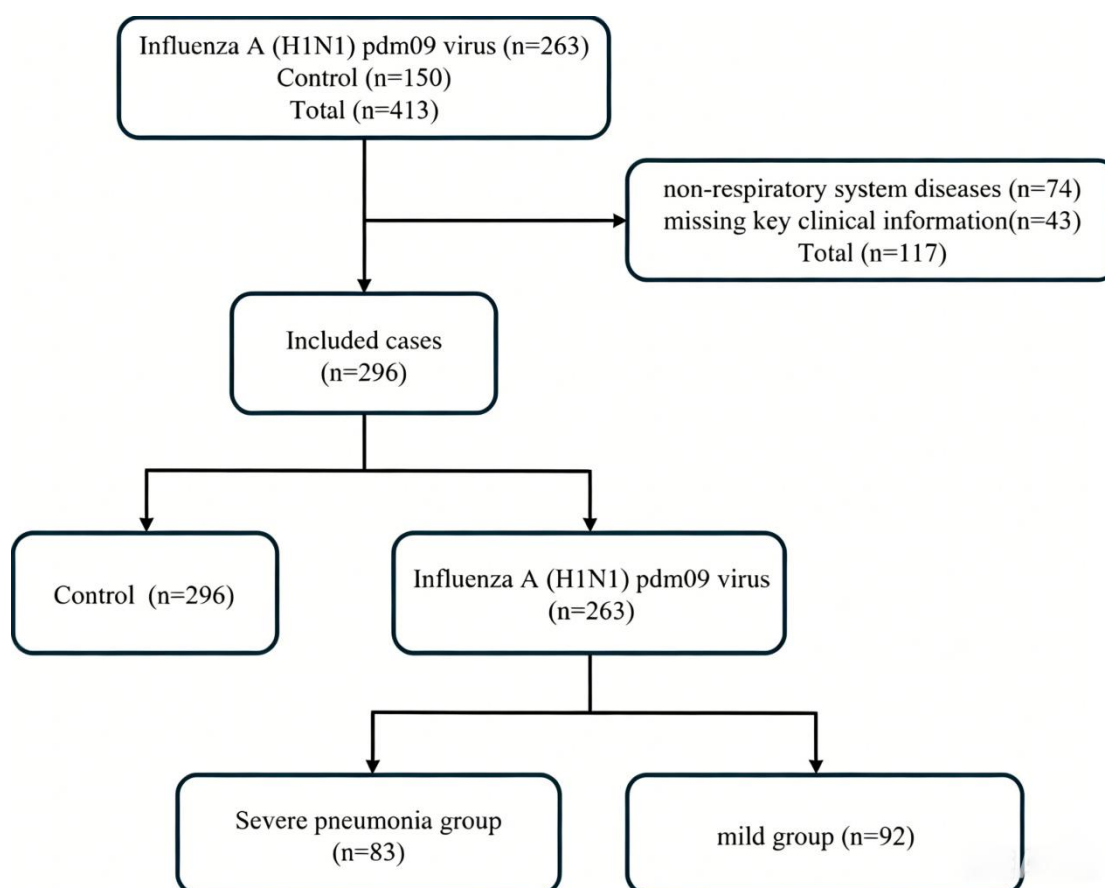


Figure 1. Flowchart of case inclusion

2. Epidemic Trend Analysis of Influenza A (H1N1) pdm09 virus

Temporal epidemiological analysis based on respiratory sample collection times showed significant temporal clustering of the influenza A (H1N1) pdm09 virus. As shown in Figure 2, this viral subtype exhibited a multi-peak distribution characteristic

from epidemiological weeks 9 to 31 (late winter to early summer), reaching an annual epidemic peak during weeks 18-21 (mid-spring).

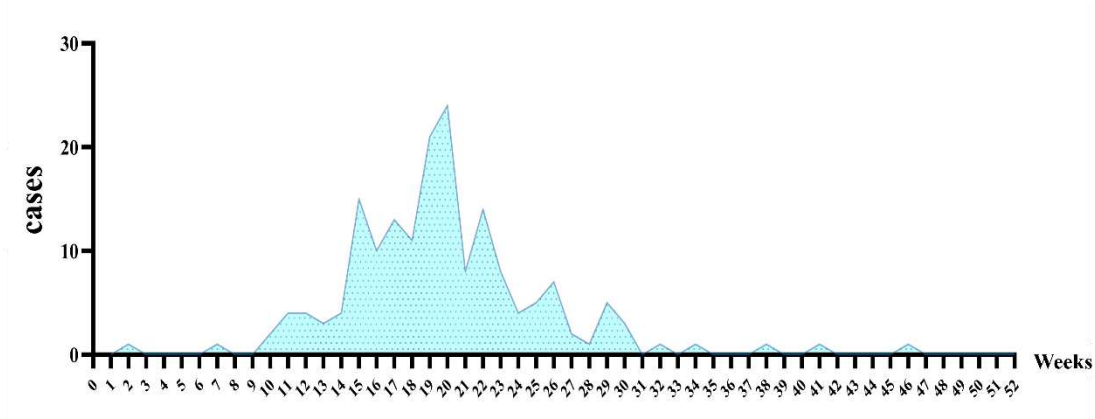


Figure 2. Time distribution of patients infected with Influenza A (H1N1) pdm09 virus

3. Co-infection Characteristics Analysis

Comparative analysis of co-infection characteristics between 92 mild influenza A (H1N1) pdm09 patients and 83 severe pneumonia patients revealed significant differences in infection patterns ($\chi^2=7.89$, $P=0.005$): The rate of single influenza A (H1N1) pdm09 infection in the severe pneumonia group was 16.9% (14/83), significantly lower than the 22.8% (21/92) in the mild group, while the proportion of mixed infections was higher in the severe pneumonia group (77.2% in mild vs. 83.1% in severe).

Pathogen composition analysis showed that bacterial co-infections were predominant in the mild group (57.6%, 53/92), with *Haemophilus influenzae*

accounting for the highest proportion (39.1%, 36/92); the severe pneumonia group showed a surge in fungal co-infection rates (50.6% vs 20.7%, $P<0.001$), with *Candida albicans* becoming the dominant pathogen (22.9%, 19/83), having a detection rate significantly higher than that of the mild group (OR=2.47, 95%CI 1.12-5.44). Notably, the co-infection rates of *Aspergillus fumigatus* (20.5%, 17/83) and Epstein-Barr virus (EBV) (14.5%, 12/83) in the severe pneumonia group were significantly higher than in the mild group ($P<0.05$), suggesting *Aspergillus fumigatus* and EBV are key pathogens of concern (see Figure 3 C, D, E, F).

The control group presented significantly different co-infection characteristics, as detailed in Figure 3 A, B.

The single infection rate reached 68.6% (72/121), with bacterial infections accounting for 54.1% (40/74), characterized by *Haemophilus influenzae* (21.5%, 26/121), *Moraxella catarrhalis* (18.2%, 22/121), and *Pseudomonas aeruginosa* (10.7%, 13/121) as the characteristic pathogens.

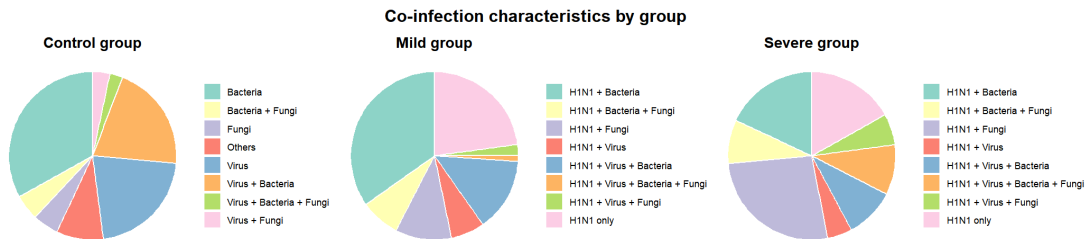


Figure 3. Analysis Results of co-infection characteristics

1. Demographics and Underlying Diseases

1.1. Data Description and Inter-group Difference Analysis

Analysis of baseline characteristics (Table 1) showed no statistically significant differences between the influenza A (H1N1) pdm09 group and the control group in core parameters such as age distribution (median [IQR]: 56 [5-71] vs 11 [5-71] years, $P=0.089$), spectrum of underlying diseases (including hypertension, diabetes, and chronic lung disease, $P>0.05$), smoking exposure rate (16.7% vs 20.7%, $P=0.442$), time from symptom onset to admission (7 [3-10] vs 2 [2-14] days, $P=0.737$), and length of hospital stay (6 [5-9] vs 6 [4-10] days, $P=0.589$).

Notably, the proportion of males in the influenza A (H1N1) pdm09 group was significantly lower than in the control group (55.4% vs 68.6%, $P=0.029$), and both resting pulse rate (95 [82.5-106.5] vs 100 [89-110], $P=0.031$) and BMI (20.96 [16.42-24.22] vs 17.97 [15.15-21.97], $P=0.001$) showed statistically significant inter-group differences.

In contrast, significant statistical differences were observed between the severe pneumonia group and the mild group in age, multiple underlying diseases, body temperature, respiratory rate, pulse, BMI, smoking, and hospital stay ($P<0.05$).

Specifically, compared to the mild group, the severe pneumonia group had more elderly patients (Age >64), generally presented with lower body temperature,

respiratory rate, and pulse, but higher BMI, higher prevalence of hypertension (39.8%) and diabetes (16.9%), higher smoking exposure rate (24.1%), and longer hospital stay.

Table 1. Data description of demographics and underlying diseases and results of inter-group difference analysis

Characteristic s	Patients infected with non- influenza A(H1N1) pdm09 virus (Control group) (N=121)	Patients infected with influenza A(H1N1) pdm09 virus (influenza A (H1N1) pdm09 patient group)			P- valu e	P- valu e’
		Total (N=175)	Mild	Severe		
			symptoms	pneumoni		
			patients	a patients		
			(Mild	(Severe		
Group of	Pneumoni					
Influenza	a Group of					
A (H1N1)	Influenza					
pdm09)	A (H1N1)					
(N=92)	pdm09)					
	(N=83)					
Male,n(%)	83(68.6)	97(55.4)	54(58.7)	43(51.8)	0.029	0.366
Age(y),median (IQR)	11(3-71)	56(5-71)	6(2-57)	67(57-78)	0.089	<0.001
Hypertension,n (%)	24(19.8)	44(25.1)	11(12.0)	33(39.8)	0.326	<0.001
Diabetes,n(%)	14(11.6)	20(11.4)	6(6.5)	14(16.9)	0.999	0.035

Renal insufficiency,n(%)	6(5.0)	10(5.7)	3(3.3)	7(8.4)	0.99 9	0.19 5
T(°C),median(I QR)	36.8(36.6- 37.5)	36.8(36.5- 37.4)	36.9(36.6- 37.7)	36.7(36.5- 37.2)	0.43 5	0.00 7
R(bpm),median (IQR)	22(22-25)	22(20-25)	23(20-28)	22(21-23)	0.20 5	0.04 8
P(bpm),median (IQR)	100(89- 110)	95(82.5- 106.5)	100(86- 110)	91(79-104)	0.03 1	0.00 1
Bp(mmHg),me dian(IQR)	109(96- 138)/ 68(58-78)	120(104.5- 134.5)/ 69(62-80)	109(92- 120)/ 65(58-73)	130(120- 145)/ 74(68-84)	/	/
BMI(Kg/m ²),m edian(IQR)	17.97(15.1 5-21.97)	20.96(16.4 2-24.22)	17.35(14.5 1-21.48)	22.88(20.8 1-25.93)	0.00 1	<0.0 01
Smoking, n (%)	25(20.7)	28(16.7)	8(9.4)	20(24.1)	0.44 2	0.01 3
From onset to admission (d), median (IQR)	7(2-14)	7(3-10)	6(3-10)	7(3-10)	0.73 7	0.89 1
Hospitalization days(d), median (IQR)	6(4-10)	6(5-9)	5(4-8)	7(5-10)	0.58 9	<0.0 01

Note: P-value: Analysis of differences between the influenza A (H1N1) pdm09 patient group and the control group.

P-value': Analysis of differences between the influenza A (H1N1) pdm09 severe pneumonia group and the influenza A (H1N1) pdm09 mild group.

301

302 1.2. Age Stratification Analysis

303 Age stratification analysis was performed, dividing patients into four age groups (0-4
304 years, 5-14 years, 15-59 years, and 60 years and above); results are detailed in Table
305 2.

306 The majority of patients with severe pneumonia infected with influenza A (H1N1)
307 pdm09 were aged 60 years or older (68.7%, 57/83).

308 **Table 2. Age-stratified descriptions and results of intergroup difference analysis**

Age stratification	Patients infected with non-influenza A(H1N1) pdm09 virus (Control group) (N=121)	Patients infected with influenza A(H1N1) pdm09 virus (influenza A (H1N1) pdm09 patient group)		
		Total (N=175)	Mild symptoms patients (Mild Group of Influenza A (H1N1) pdm09) (N=92)	Severe pneumonia patients (Severe Pneumonia Group of Influenza A (H1N1) pdm09) (N=83)
0-4 years old	38	37	37	0
5-14 years old	24	20	20	0
15-59 years old	17	42	16	26
≥60 years old	42	76	19	57

309

310 **2. Clinical Symptoms Difference Analysis**

311 The control group and the influenza A (H1N1) pdm09 patient group did not show
312 many differences in clinical symptom presentation; a statistical difference was only
313 found in fever or chills ($P < 0.05$), with the influenza A (H1N1) pdm09 group showing
314 a higher frequency of fever or chills, reaching 77.1%.

315 Comparatively, the severe pneumonia group and the mild group showed statistical
316 differences in multiple clinical symptoms ($P < 0.05$), mainly including cough,
317 shortness of breath or dyspnea, headache, nasal congestion or rhinorrhea.

318 Patients with severe pneumonia were more likely to experience symptoms such as
319 cough, shortness of breath, or dyspnea, with frequencies reaching 96.3% and 73.5%,
320 respectively (see Table 3).

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328 **Table 3. Description of Clinical Symptoms and Results of Intergroup Difference**

329 **Analysis**

Characteristics	Patients infected with influenza A(H1N1) pdm09 virus (influenza A (H1N1) pdm09 patient group)					<i>P</i> - valu e	<i>P</i> - value ,
	Patients infected with non- influenza A(H1N1) pdm09 virus (Control group) (N=121)	Total (N=175)	Mild symptoms patients (Mild Group of Influenza A (H1N1) pdm09) (N=92)	Severe pneumon ia patients (Severe Pneumon ia Group of Influenza A (H1N1) pdm09) (N=83)			
Fever or chills,n(%)	66(54.5)	135(77.1)	74(80.4)	61(73.5)	<0.0 01		0.286
Cough,n(%)	101(83.5)	147(84.5)	68(73.9)	79(96.3)	0.87 2		<0.0 01
Dyspnea or Respiratory distress,n(%)	51(42.1)	94(53.7)	33(35.9)	61(73.5)	0.05 9		<0.0 01
Fatigue,n(%)	20(16.5)	34(19.4)	15(16.3)	19(22.9)	0.54		0.339

Characteristics	Patients infected with influenza A(H1N1) pdm09 virus (influenza A (H1N1) pdm09 patient group)					P- valu e	P- value ,
	Patients infected with non- influenza A(H1N1) pdm09 virus (Control group) (N=121)	Total (N=175)	Mild symptoms patients (Mild Group of Influenza A (H1N1) pdm09) (N=92)	Severe pneumon ia patients (Severe Pneumon ia Group of Influenza A (H1N1) pdm09) (N=83)			
Myalgia,n(%)	5(4.1)	10(5.7)	7(7.6)	3(3.6)	0.60 1	5	0.336
Headache,n(%)	7(5.8)	20(11.4)	16(17.4)	4(4.8)	0.10 5		0.009
Pharyngalgia,n(%)	16(13.2)	29(16.6)	16(17.4)	13(15.7)	0.51 1		0.840
Nasal congestion or Rhinorrhea,n(%)	51(42.1)	56(32.0)	39(42.4)	17(20.5)	0.08 5		0.002
Nausea and	9(7.4)	8(4.6)	5(5.4)	3(3.6)	0.31		0.723

Characteristics	Patients infected with influenza A(H1N1) pdm09 virus (influenza A (H1N1) pdm09 patient group)					P- valu e	P- value ,
	Patients infected with non- influenza A(H1N1) pdm09 virus (Control group) (N=121)	Total (N=175)	Mild symptoms patients (Mild Group of Influenza A (H1N1) pdm09) (N=92)	Severe pneumon ia patients (Severe Pneumon ia Group of Influenza A (H1N1) pdm09) (N=83)			
vomiting,n(%)						9	
Diarrhea,n(%)	4(3.3)	5(2.9)	2(2.2)	3(3.6)	0.99 9		0.669

Note: P-value: Analysis of differences between the influenza A (H1N1) pdm09 patient group and the control group.

P-value': Analysis of differences between the influenza A (H1N1) pdm09 severe pneumonia group and the influenza A (H1N1) pdm09 mild group.

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332 3. Laboratory Test Indicators Difference Analysis

333 3.1. Blood Routine Test Results

Infection-related leukocyte parameters mainly include White Blood Cell Count (WBC), Neutrophil Count (NEUT), Lymphocyte Count (LYMPH), Monocyte Count (MONO), Eosinophil Count (EOS), and Basophil Count (BASO); their descriptive analysis results are detailed in Table 4.

Results for indicators such as WBC, NEUT, LYMPH, MONO, and EOS showed statistical differences between the influenza A (H1N1) pdm09 group and the control group ($P < 0.05$); specifically, WBC, LYMPH, and EOS were significantly lower in the influenza A (H1N1) pdm09 group than in the control group, while NEUT and MONO were higher.

Between the severe pneumonia group and the mild group, statistical differences were only observed in NEUT and LYMPH results ($P < 0.05$); specifically, the severe pneumonia group had higher NEUT levels but lower LYMPH levels compared to the mild group.

Table 4. Description of Blood Routine Test Results and Intergroup Difference Analysis

Examination	Patients infected with influenza A(H1N1) pdm09 virus (influenza A (H1N1) pdm09 patient group)					
	Patients infected with non- influenza A(H1N1) pdm09 virus (Control group) (N=121)	Total (N=175)	Mild symptoms patients (Mild Group of Influenza A (H1N1) pdm09) (N=92)	Severe pneumon ia patients (Severe Pneumon ia Group of Influenza A (H1N1) pdm09) (N=83)	P- val ue	P- valu e'
WBC($10^9/L$),median(IQR)	8.58 (6.12- 11.09)	7.19 (5.33- 10.08)	6.71 (5.19- 9.53)	7.78 (5.50- 10.16)	0.015	0.112
NEUT($10^9/L$),median(IQR)	65.3 (45.2- 75.8)	69.9 (51.0- 81.3)	60.7 (43.5- 75.5)	73.9 (63.8- 86.7)	0.041	<0.001
LYNPH($10^9/L$),median(IQR)	25.8 (15.7- 45.4)	20.0 (10.9- 36.0)	27.1 (15.1- 43.6)	16.1 (7.0- 23.3)	0.009	<0.001

MONO($10^9/L$),median(IQR)	6.5 (5.1-7.9)	7.6 (5.7-10.3)	7.6 (5.9-10.4)	7.7 (5.5-10.3)	<0.001	0.470
EOS($10^9/L$),median(IQR)	1.8 (0.7-3.5)	0.3 (0.1-1.8)	0.5 (0.1-1.8)	0.2 (0.0-1.8)	<0.001	0.354
BASO($10^9/L$),median(IQR)	0.2 (0.1-0.3)	0.1 (0.1-0.3)	0.1 (0.1-0.2)	0.2 (0-0.3)	0.064	0.502
%NEUT($10^9/L$),median(IQR)	4.81 (3.09-7.63)	5.15 (2.86-7.47)	3.99 (2.12-6.54)	5.84 (3.44-8.43)	0.883	0.001
%LYNPH($10^9/L$),median(IQR)	1.98 (1.33-3.34)	1.44 (0.86-2.19)	1.73 (1.15-2.73)	1.04 (0.60-1.80)	<0.001	<0.001
%MONO($10^9/L$),median(IQR)	0.52 (0.41-0.71)	0.55 (0.37-0.78)	0.55 (0.36-0.77)	0.58 (0.38-0.80)	0.620	0.762
%EO($10^9/L$),median(IQR)	0.14 (0.05-0.23)	0.03 (0.01-0.10)	0.04 (0.01-0.10)	0.02 (0.00-0.12)	<0.001	0.346
%BASO($10^9/L$),median(IQR)	0.01 (0.01-0.03)	0.01 (0.00-0.02)	0.01 (0.00-0.02)	0.01 (0.00-0.02)	0.005	0.487

RBC($10^{12}/L$),median(IQR)	4.48 (3.90-4.95)	4.55 (4.10-4.89)	4.66 (4.27-5.02)	4.37 (3.83-4.74)	0.64 2	0.00 1
HGB(g/L),median(IQR)	121 (109-135)	126 (114-134)	125 (114-132)	127 (108-141)	0.13 2	0.25 5
HCT(%),median(IQR)	36.8 (33.7-40.5)	37.9 (34.3-41.1)	37.7 (34.4-39.9)	38.5 (34.1-42.2)	0.18 7	0.21 2
MCV(fL),median(IQR)	84.3 (80.6-89.2)	85.9 (81.1-90.4)	82.3 (78.0-86.9)	89.2 (85.8-92.3)	0.21 0	<0.001
MCH(pg),median(IQR)	27.9 (26.3-29.1)	28.4 (26.7-30.1)	27.0 (25.5-28.6)	29.6 (28.0-30.6)	0.07 3	<0.001
MCHC(g/L),median(IQR)	328 (320-334)	330 (324-337)	330 (323-338)	330 (325-337)	0.038	0.87 8
RDW-SD(fL),median(IQR)	39.4 (37.3-42.3)	40.4 (38.3-43.6)	38.7 (36.9-41.1)	42.5 (40.0-45.0)	0.046	<0.001

RDW-	13.1	13.3		13.3		
CV(%),median(I	(12.6-	(12.6-	13.2 (12.7-	(12.6-	0.15	0.78
QR)	13.7)	14.0)	14.3)	14.0)	7	6
PLT(10 ⁹ /L),medi	290	226 (162-	249 (177-	202		
an(IQR)	(213-	304)	320)	(149-	<0.001	0.005
	384)			273)		
PDW(fL),median	8.5 (8.0-	8.9 (8.4-	8.7 (8.1-	9.4 (8.6-	0.001	<0.001
(IQR)	9.3)	9.7)	9.4)	10.2)		
MPV(fL),median	0.248	0.209	0.219	0.194		
(IQR)	(0.196-	(0.155-	(0.166-	(0.150-	<0.001	0.041
	0.313)	0.261)	0.276)	0.248)		
PCT(%),median(I	15.8	15.9	15.8 (15.5-	16.0		
QR)	(15.5-	(15.6-	16.1)	(15.8-	0.003	<0.001
	16.1)	16.3)		16.3)		
P-	16.5	19.5		21.7		
LCR(%),median(I	(13.2-	(15.7-	17.8 (14.5-	(17.4-	0.001	0.001
IQR)	22.6)	25.3)	23.4)	28.7)		

Note: P-value: Analysis of differences between the influenza A (H1N1) pdm09 patient group and the control group.

P-value': Analysis of differences between the influenza A (H1N1) pdm09 severe pneumonia group and the influenza A (H1N1) pdm09 mild group.

3.2. Biochemical Indicator Test Results

Detailed description and analysis of various biochemical indicator test results are shown in Table 5.

Between the influenza A (H1N1) pdm09 group and the control group, significant statistical differences were only observed in CREN, eGFR, ALT, AST, and GGT ($P < 0.05$).

Specifically, the influenza A (H1N1) pdm09 group had higher levels of CREN, ALT, AST, and GGT than the control group, while only eGFR was lower.

In comparison, more differences in biochemical indicator results were observed between the severe pneumonia group and the mild group.

Specifically, analysis results for UREA, CREN, UA, ALT/AST, GGT, and ESR were all higher in the severe pneumonia group, while eGFR and ALB results were comparatively lower.

Table 5. Description of Biochemical Parameter Results and Intergroup

Difference Analysis					
Examination	Patients infected with non-influenza A(H1N1)	Patients infected with influenza A(H1N1) pdm09 virus (influenza A (H1N1) pdm09 patient group)			<i>P</i> -value
		Total	Mild	Severe	
			symptoms	pneumoni	

	pdm09 virus (Control group) (N=121)	(N=175)	patients (Mild Group of Influenza A (H1N1) pdm09) (N=92)	a patients (Severe Pneumon ia Group of Influenza A (H1N1) pdm09) (N=83)		
UREA(mmol/L) ,median(IQR)	4.0(3.7-5.1)	4.3(3.5- 5.7)	3.6(2.9-5.4)	4.8(3.6- 6.1)	0.69 5	<0.0 01
CREN(μmol/L), median(IQR)	58(55-91)	80(66- 108)	74(54-84)	84(72- 124)	0.02 0	<0.0 01
eGFR(ml/min/1. 73m²), median(IQR)	138.18(70.2 1-146.56)	76.86(49. 30-92.84)	87.17(67.22 -119.89)	62.00(46. 76-82.77)	0.00 1	<0.0 01
UA(μmol/L),me dian(IQR)	272(242- 345)	308(239- 412)	248(202- 385)	310(275- 398)	0.94 2	0.00 2
ALT(U/L),medi an(IQR)	14(11-25)	23(14-39)	32(15-35)	20(15-38)	0.00 3	0.09 6
AST(U/L),medi an(IQR)	23(20-29)	36(19-50)	40(22-47)	30(19-59)	0.03 9	0.33 2
ALT/AST,media n(IQR)	0.52(0.45- 1.14)	0.81(0.44- 1.02)	0.80(0.41- 1.25)	0.82(0.52- 0.87)	0.57 3	0.00 1

GGT(U/L),median(IQR)	22(10-40)	24(16-37)	18(14-24)	25(23-37)	0.002	<0.001
ALB(g/L),median(IQR)	40.2(33.1-45.5)	37.3(30.9-38.4)	38.4(38.2-40.7)	34.2(29.7-37.3)	0.059	0.006
ESR(mm/h),median(IQR)	22(18-62)	45(30-79)	31(21-63)	49(39-81)	0.458	0.009
PCT(ng/ml),median(IQR)	0.13(0.04-0.22)	0.13(0.04-0.92)	0.26(0.05-0.54)	0.10(0.04-0.87)	0.236	0.988

Note: P-value: Analysis of differences between the influenza A (H1N1) pdm09 patient group and the control group.

P-value': Analysis of differences between the influenza A (H1N1) pdm09 severe pneumonia group and the influenza A (H1N1) pdm09 mild group.

366

367 **4. Risk Assessment of Influencing Factors**

368 The results of the risk factor analysis are shown in Table 6.

369 Univariate Logistic regression models showed that the risk of progression to
370 severe pneumonia in influenza A (H1N1) pdm09 infected patients was significantly
371 positively associated with age (per 1-year increase: OR=1.066, 95%CI 1.047-1.086,
372 P<0.001), underlying diseases (Hypertension: OR=4.860, 95%CI 2.555-10.476,
373 P<0.001; Diabetes: OR=2.908, 95%CI 1.062-7.965, P=0.038), smoking history
374 (OR=3.056, 95%CI 1.261-7.403, P=0.013), and metabolic indicators (per 1-unit
375 increase in BMI: OR=1.302, 95%CI 1.190-1.425, P<0.001).

After adjustment by multivariate modeling, only age (per 1-year increase: OR=1.107, 95%CI 1.063-1.154, P<0.001), cough (OR=20.567, 95%CI 3.468-121.959, P<0.001), shortness of breath/dyspnea (OR=3.450, 95%CI 1.008-11.802, P=0.048), nasal congestion or rhinorrhea (OR=8.777, 95%CI 1.455-52.934, P=0.018), and BMI (per 1-unit increase: OR=1.195, 95%CI 1.040-1.372, P=0.012) remained independent risk factors, while headache (OR=0.022, 95%CI 0.003-0.157, P<0.001) was a protective factor.

Table 6. Univariate and Multivariate Logistic Regression Analyses of Influencing Factors

Factor	Univariate logistic analysis		Multivariate logistic analysis	
	OR(95%CI)	P-value	OR(95%CI)	P-value
Hypertension	4.860(2.555-10.476)	<0.001	1.121(0.297-4.231)	0.866
Diabetes	2.908(1.062-7.965)	0.038	0.658(0.132-3.277)	0.609
Smoking	3.056(1.261-7.403)	0.013	0.558(0.159-1.960)	0.363
BMI	1.302(1.190-1.425)	<0.001	1.195(1.040-1.372)	0.012
Age	1.066(1.047-1.086)	<0.001	1.107(1.063-1.154)	<0.001

Discussion

This study provides a comprehensive comparison between severe and mild influenza A(H1N1)pdm09 pneumonia, identifying key clinical, laboratory, and microbiological features associated with disease severity. Influenza A(H1N1)pdm09 remains the most prevalent subtype. Although most infections are mild, older adults and individuals with chronic diseases are prone to severe forms of illness^[8], resulting in impaired quality of life and substantial clinical and economic burden. While previous studies have examined the clinical characteristics of influenza A(H1N1)pdm09 in China, systematic comparisons focusing specifically on the differential features and determinants of severe pneumonia versus mild disease remain limited. The present analysis helps address these gaps by providing a detailed characterization of clinical profiles and severity-associated factors.

Marked differences emerged in co-infection patterns. Mild cases were predominantly characterized by single-pathogen infection, whereas increasing disease severity was associated with a marked rise in mixed bacterial, fungal, and viral co-infections. *Haemophilus influenzae* accounted for most bacterial co-infections in mild disease, while severe pneumonia was associated with a notable rise in fungal co-infection, particularly *Candida albicans*. This trend likely reflects the combined influence of immunosuppressive medication, influenza-induced innate immune dysfunction, and heightened vulnerability to secondary bacterial or fungal pathogens^[18]. Severe cases also displayed elevated rates of *Aspergillus fumigatus* and Epstein–Barr virus (EBV) co-infection, accompanied by greater bacterial diversity. Such heterogeneity in pathogen profiles may aggravate lung injury via cytokine-storm mechanisms and accelerate progression to severe disease^[19]. Strengthening real-time surveillance of co-infections and establishing co-infection-oriented management

strategies could therefore play an essential role in reducing severe outcomes^[20].

Patients with severe influenza A(H1N1)pdm09 pneumonia were characterized by older age, higher BMI, greater smoking exposure, and a higher burden of comorbidities, including hypertension and diabetes, compared with those with mild disease. These findings are consistent with previous epidemiological studies of seasonal influenza A(H1N1)pdm09^[21,22], reinforcing that advanced age and underlying comorbidities are major contributors to severe disease progression. Severe cases also presented more frequently with cough, dyspnea, and respiratory distress—symptoms associated with lower respiratory tract involvement, which is more likely to evolve into severe pneumonia^[23,24]. These insights can inform targeted prevention strategies, including prioritizing high-risk groups, reinforcing symptom-based surveillance, and enabling early antiviral intervention to reduce severe outcomes.

Laboratory data showed significant differences between groups in neutrophil count (NEUT) and lymphocyte count (LYMPH). Severe pneumonia was accompanied by a pronounced neutrophil–lymphocyte imbalance, characterized by neutrophilia and lymphopenia, reflecting an exaggerated inflammatory response and impaired adaptive immunity. Unlike reports from the early 2009 H1N1 pandemic in Mexico, where lymphopenia was prominent without major neutrophil changes^[21], our study found group differences in several white cell parameters. Reductions in lymphocytes and eosinophils can stimulate pro-inflammatory cytokines (IL-1 β , IL-6, IL-8, TNF- α , TGF-1 β) and suppress IL-10, thereby amplifying systemic inflammation^[25]. Markers of liver and kidney injury also differed significantly, indicating more severe organ dysfunction in the severe pneumonia group. Elevated erythrocyte sedimentation rate (ESR) further suggests heightened inflammatory response. During pneumonia, inflammatory cytokines stimulate hepatic production of acute-phase proteins such as fibrinogen, a

key driver of ESR elevation that promotes red blood cell aggregation and accelerates sedimentation, forming a vicious cycle that exacerbates disease severity^[26]. These findings enhance the understanding of H1N1 pathogenesis and support early risk stratification and personalized treatment.

Risk factor analysis using two-stage logistic regression identified hypertension, diabetes, cough, dyspnea, BMI, age, and smoking as significant predictors in univariate models, with BMI, age, and several respiratory symptoms (cough, dyspnea/respiratory distress, headache, nasal congestion/rhinorrhea) remaining significant independent factors after multivariable adjustment. High BMI may impair respiratory mechanics by reducing lung volume and increasing ventilatory load^[27], contributing to hypoxemia and impaired pathogen clearance, thereby increasing the risk of respiratory failure. Obesity is also associated with chronic low-grade inflammation due to adipose-derived cytokines (TNF- α , IL-6, IL-1 β)^[28], which may potentiate cytokine storm responses during infection. Additionally, impaired neutrophil and macrophage function in obesity can delay pathogen clearance^[29]. Advanced age further contributes to immunosenescence, characterized by reduced naïve T-cell numbers, diminished memory T-cell responsiveness, and decreased B-cell diversity^[30], along with structural lung aging and reduced ventilatory reserve^[31]. Diminished alveolar regenerative capacity and decreased surfactant production in older adults may delay recovery and worsen lung injury^[32]. These mechanisms collectively heighten susceptibility to severe pneumonia.

This study has several limitations. First, inclusion of a healthy control group would have enabled clearer differentiation of host-related factors. Second, severe pneumonia cases were highly concentrated in older adults, which may limit generalizability. Third, the study did not include several immunological biomarkers, such as serum amyloid A

or cytokines, nor detailed data on chronic respiratory diseases, restricting deeper mechanistic exploration. Fourth, as a single-center study, the findings may be subject to selection bias and limited external validity. Future multicenter studies with standardized data collection and broader population coverage are warranted to validate these findings and enhance their generalizability.

In conclusion, this study provides a systematic characterization of the clinical features and risk factors of severe pneumonia in influenza A(H1N1)pdm09 patients. Key determinants of severity include advanced age, elevated BMI, and lower respiratory tract symptoms, together with marked neutrophil–lymphocyte imbalance, organ dysfunction, and region-specific co-infection profiles. Future research should pursue multi-omics analysis of host–pathogen interactions, validation of risk predictors through multi-center cohorts, and integration of biomarker-based dynamic warning systems. These efforts will support the transition from reactive treatment to precision prevention in influenza management.

Conclusions

This study identified distinct co-infection patterns among influenza A(H1N1)pdm09 patients, with severe pneumonia cases predominantly exhibiting fungal co-infection—particularly *Candida albicans*—whereas mild cases were mainly associated with bacterial co-infection, led by *Haemophilus influenzae*. These findings suggest that disease progression is influenced by exacerbated immunosuppression and increasing diversity of co-infecting pathogens. Abnormal BMI and advanced age were strongly associated with progression to severe pneumonia. Laboratory indicators further revealed pronounced neutrophil–lymphocyte imbalance in severe cases, accompanied by significant alterations in multiple markers of liver and kidney injury (UREA,

CREN, eGFR, UA, ALT, AST, GGT), as well as markedly elevated ESR. By leveraging tNGS technology, this study expanded detectable pathogen coverage and elucidated a more comprehensive co-infection pattern, enabling detailed characterization of clinical features and risk factors. These findings provide evidence to inform optimization of clinical management strategies for H1N1. Early intervention should be prioritized for high-risk individuals—particularly older adults presenting with lower respiratory tract symptoms—and empirical antimicrobial regimens should be tailored according to local pathogen distribution. From a public health perspective, our findings advocate for a precision prevention framework. This should integrate risk-scoring systems that incorporate factors like age, BMI, and lower respiratory symptoms to identify high-risk individuals. A tripartite strategy—enhancing vaccination coverage, ensuring early antiviral treatment for targeted groups, and implementing stringent surveillance and management of secondary infections—could facilitate a paradigm shift from passive treatment to proactive prevention, potentially reducing the incidence of severe influenza and its associated healthcare burden.

List of abbreviations

ALB	albumin
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BASO	basophil count
BMI	body mass index
CAP	community-acquired pneumonia
CI	confidence interval
CREN	creatinine
EBV	Epstein-Barr virus

eGFR	estimated glomerular filtration rate
EOS	eosinophil count
ESR	erythrocyte sedimentation rate
GGT	gamma-glutamyl transferase
IL	interleukin
IQR	interquartile range
LYMPH	lymphocyte count
mNGS	metagenomic next-generation sequencing
MONO	monocyte count
NEUT	neutrophil count
NGS	next-generation sequencing
NMPA	National Medical Products Administration
OR	odds ratio
PCR	polymerase chain reaction
RR	respiratory rate
tNGS	targeted next-generation sequencing
TGF	transforming growth factor
TNF	tumor necrosis factor
UA	uric acid
UREA	urea
WBC	white blood cell count
WHO	World Health Organization

501 **Declarations**

502 **Ethics approval and consent to participate**

503 This retrospective study was approved by the Ethics Committee of Panyu Central

Hospital of Guangzhou Medical University. Given the retrospective nature of the study and the use of anonymized data, the requirement for informed consent was waived. ES-2025-009-01

Clinical trial number: not applicable.

Consent for publication

Not applicable. This manuscript does not contain any individual person's data.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Jingyi Liang, Zifeng Yang, Zhiqi Zeng contributed to the conceptualization, methodology, and supervision of the study. Zhihao Lie handled data collection. Zhihao Lie and Yinghong Hu performed experiment and data analysis. Jiaqi Chen, Zeqi Lai, Zixin Fang, Huizhong Liu wrote the original draft. Yinghong Hu, Zeqi Lai and Jiaqi Chen, Zhiqi Zeng provided review and editing. All authors approved the final manuscript.

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