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Chengcheng An

Henan Provincial Center for Disease Control and Prevention

Bohao Wang

Children's Hospital Affiliated of Zhengzhou University

Yi Li

Henan Provincial Center for Disease Control and Prevention

Dongxiao Li

Henan Provincial Center for Disease Control and Prevention

Xiao Hu

Henan Provincial Center for Disease Control and Prevention

Lin Zhu

Henan Provincial Center for Disease Control and Prevention

Yafei Li

Henan Provincial Center for Disease Control and Prevention

Ying Ye

Henan Provincial Center for Disease Control and Prevention

Jingjing Wang

Children's Hospital Affiliated of Zhengzhou University

Wencong Wang

Children's Hospital Affiliated of Zhengzhou University

Ping Li

Children's Hospital Affiliated of Zhengzhou University

Xinggong Zhen

Children's Hospital Affiliated of Zhengzhou University

Xueyong Huang

hxyzzu@163.com

Henan Provincial Center for Disease Control and Prevention

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Respiratory Pathogen Spectrum and Co-infection Networks in Pediatric Influenza-Like Illness in Henan Province, China

Authors

Chengcheng An^{12†}, Bohao Wang^{3†}, Yi Li¹², Dongxiao Li¹², Xiao Hu¹², Lin Zhu¹², Yafei Li¹², Ying Ye¹², Jingjing Wang³, Wencong Wang³, Ping Li³, Xinggang Zhen^{3*}, Xueyong Huang^{1 2*}

Affiliations

¹ Henan Province Center for Disease Control and Prevention, Zhengzhou, Henan, China

² Henan Provincial Key Laboratory of Infectious Disease Pathogens, Zhengzhou, Henan, China

³ Children's Hospital Affiliated of Zhengzhou University, Zhengzhou, Henan, China

***Corresponding Authors**

Xinggang Zhen, Email: zxgzx@126.com

Xueyong Huang, Email: hxyzzu@163.com

† These authors contributed equally to this work and share first authorship.

Keywords

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Abstract

Background

Acute respiratory infections pose a substantial global health burden, particularly among children. Influenza-like illness (ILI) is a common syndrome with complex and insufficiently characterized etiology, especially in central China. This study aimed to delineate the respiratory pathogen spectrum, age-specific dynamics, temporal succession, and co-infection patterns in pediatric ILI.

Methods

A total of 154 pediatric ILI outpatients were enrolled at Henan Children's Hospital during the 2025–2026 epidemic season. Respiratory specimens were analyzed using a 132-target multiple nucleotide polymorphism marker method with next-generation sequencing. Pathogen detection rates, viral and bacterial loads, age-stratified distributions, temporal trends across three epidemic phases (early, peak, late), and inter-pathogen co-occurrence networks were evaluated. Hierarchical clustering identified distinct co-infection clusters.

Results

Overall pathogen detection rate was 72.1%, with mixed infections in 57.8% of cases. *Neisseria meningitidis* (Nm, 36.4%) and *Haemophilus influenzae* (Hi, 33.1%) were most prevalent. Hi increased with age (0% to 51.2%, $p < 0.001$); human herpesvirus 5 predominated in infants <12 months and declined ($p < 0.001$); Nm showed a U-shaped distribution ($p = 0.002$). The epidemic season had a three-phase succession: bacteria-precede (Oct–Nov) led by Nm, virus-polarize (Dec–Jan) dominated by influenza A virus, and niche-replacement (Feb–Mar) with increased human parainfluenza virus 3 ($p = 0.003$). Respiratory syncytial virus load peaked during the peak phase ($p = 0.027$), and Nm load correlated negatively with age ($r = -0.349$, $p = 0.019$). Network analysis identified Nm and Hi as central hubs, positively correlated with *Streptococcus pneumoniae* and each other, with strong intra-subtype correlation between influenza A subtypes. Hierarchical clustering revealed three co-infection patterns: virus-dominated, bacteria-dominated, and high-colonization background.

Conclusions

This study reveals a complex respiratory pathogen landscape in pediatric ILI, with high mixed infection rates and dynamic age- and time-dependent patterns. These findings underscore the importance of pathogen interactions and microecological contexts, providing insights for clinical diagnosis and public health strategies.

List of abbreviations

Abbreviation	Full Form
ARI	Acute respiratory infection
cDNA	Complementary DNA
EBV	Epstein-Barr virus
FDR	False discovery rate
FluA H3	Influenza A virus subtype H3
FluA N2	Influenza A virus subtype N2
HHV-5	Human herpesvirus 5
HHV-7	Human herpesvirus 7
Hi	<i>Haemophilus influenzae</i>
HPIV-3	Human parainfluenza virus 3
HRV	Human rhinovirus
IFN	Interferon
IFV	Influenza virus
ILI	Influenza-like illness
IQR	Interquartile range
<i>M. catarrhalis</i>	<i>Moraxella catarrhalis</i>
mNGS	Metagenomic next-generation sequencing
MNP	Multiple nucleotide polymorphism
<i>Neisseria</i>	<i>Neisseria</i> species
Nm	<i>Neisseria meningitidis</i>
PCA	Principal component analysis
PCR	Polymerase chain reaction
PLS-DA	Partial least squares discriminant analysis
RSV	Respiratory syncytial virus
RT-PCR	Real-time polymerase chain reaction
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SNR	Signal-to-noise ratio
Spn	<i>Streptococcus pneumoniae</i>
WHO	World Health Organization

1. Introduction

Acute respiratory infections (ARIs) represent a significant global health burden, characterized by high morbidity and mortality, particularly among vulnerable populations such as young children, the elderly, and immunocompromised individuals (1). Influenza-like illness (ILI), a common clinical syndrome of ARIs, is defined by the acute onset of fever ($\geq 38^{\circ}\text{C}$) accompanied by a cough or sore throat (2, 3). The etiology of ILI is complex, involving a wide spectrum of pathogens, including influenza viruses (IFV), respiratory syncytial virus (RSV), human rhinovirus (HRV), *Streptococcus pneumoniae* (Spn), and *Haemophilus influenzae* (Hi), among others (4, 5). The prevalence and spectrum of these pathogens exhibit considerable variation across different geographical regions, seasons, and demographic groups, underscoring the necessity for continuous and localized surveillance (4, 6). In China, while some multi-pathogen surveillance programs have been initiated, comprehensive data on the pathogen spectrum of ILI in children from Henan province, a region with a large population, remains limited. This knowledge gap hinders the development of region-specific diagnostic and therapeutic strategies.

Accurate and rapid identification of causative agents is paramount for effective clinical management, guiding appropriate therapeutic strategies, and curbing the misuse of antibiotics (7). It also facilitates the implementation of timely public health interventions to contain pathogen transmission (7, 8). Traditional diagnostic methods, such as real-time polymerase chain reaction (RT-PCR), are widely used but are often limited to detecting a predefined, narrow panel of pathogens (9). Metagenomic next-generation sequencing (mNGS) offers a culture-independent and comprehensive approach for pathogen detection (10, 11). However, its application in routine surveillance is constrained by high costs, long turnaround times, and challenges in achieving accurate quantification (12, 13). The multiple nucleotide polymorphism (MNP) marker method, which integrates ultra-multiplex PCR with high-throughput sequencing, has emerged as a promising alternative. It combines broad pathogen coverage with high throughput, accurate quantification, and cost-effectiveness, making it highly suitable for large-scale epidemiological studies (14).

Respiratory co-infections are a common and complex phenomenon, influenced by a dynamic interplay of host, pathogen, and environmental factors (15, 16). Understanding the patterns of microbial interaction, such as co-occurrence or mutual exclusion, is crucial for deciphering the disruptions in respiratory microecology during infection (8, 15). However, the specific networks of interaction between various respiratory pathogens in pediatric ILI cases are not well-characterized.

Therefore, this study aimed to systematically characterize the respiratory microecology in children with ILI in Henan province, China. Utilizing MNP-based targeted sequencing, we analyzed respiratory samples collected during the 2025-2026 epidemic season to delineate the

pathogen spectrum and its age-specific distribution, investigate temporal dynamics throughout the season, and explore co-occurrence networks and distinct co-infection clusters. Our findings provide valuable epidemiological data and novel insights into pathogen interactions, which could inform future strategies for the prevention and control of ARIs in this region.

2. Materials and Methods

2.1 Study Population and Sample Collection

This study was conducted at Henan Children's Hospital (Zhengzhou, China) during a complete influenza-like illness (ILI) epidemic season from October 2025 to March 2026. ILI cases were defined as outpatients or emergency department patients presenting with acute onset of fever (tympanic temperature $\geq 38^{\circ}\text{C}$) accompanied by either cough or sore throat, according to the World Health Organization (WHO) case definition for ILI surveillance. Respiratory specimens, including throat swabs, nasal swabs, or nasopharyngeal swabs, were collected from eligible patients within 3 days of symptom onset. All specimens were immediately placed in viral transport medium, transported to the laboratory on ice, and stored at -80°C until further processing. A total of 154 pediatric outpatients meeting the ILI case definition were enrolled in this study. Demographic information (age, sex, residence) and clinical data (date of symptom onset, date of sample collection) were recorded for each participant. This study was reviewed and approved by the Medical Ethics Committee of Henan Provincial Center for Disease Control and Prevention (Approval No.: 2025-KY-032-01).

2.2 MNP-Based Targeted Library Preparation and Sequencing

Follow the instructions of the DNA/RNA virus nucleic acid extraction kit (Xi'an Tianlong Technology Co., Ltd., T332) and use the Tianlong nucleic acid extraction instrument GeneRotex 96 to extract total nucleic acid from respiratory specimens. For detection of both DNA and RNA pathogens, complementary DNA (cDNA) was first synthesized. Library preparation was performed using the "Respiratory Pathogen 132-plex MNP Library Preparation Kit" (Wuhan Zhongwei Gene Technology Co., Ltd., Wuhan, China) according to the manufacturer's instructions. This kit is based on the MNP marker method (GB/T 41895-2022), which employs ultra-multiplex PCR to enrich specific MNP marker sites, followed by high-throughput sequencing. The workflow comprised two rounds of PCR: first-round target enrichment and second-round addition of sequencing adapters and dual-index barcodes. Final libraries were quantified using a Qubit fluorometer and sequenced on an Illumina MiSeq platform with paired-end 150 bp reads.

2.3 Bioinformatics Analysis

Raw sequencing data were processed using a standardized bioinformatics pipeline. Low-quality reads and adapter sequences were trimmed, and the remaining high-quality reads were aligned against to a reference database containing the target pathogen genomes.

Pathogen detection was determined according to the criteria established in the Chinese national standard GB/T 41895–2022 (MNP marker method). Specifically, a pathogen was considered detected if: (1) the number of detected MNP marker loci was ≥ 3 ; and (2) the signal-to-noise ratio (SNR), calculated as the ratio of the target pathogen signal index (S_{Ti}) to the noise index (P_{Ri}), was ≥ 10 . The signal index S_{Ti} was defined as the ratio of the number of sequencing reads mapped to the i -th target pathogen (n_{Ti}) to the number of reads mapped to the internal control DNA (N_T) in the test sample. The noise index P_{Ri} was defined as the ratio of the number of reads mapped to the i -th target pathogen in the negative control (n_{Ri}) to the number of reads mapped to the internal control DNA in the negative control (N_R). A detected MNP marker locus was defined as having ≥ 5 raw sequencing reads (before normalization) and ≥ 1 normalized read (after normalizing to 10^6 internal control reads).

For downstream analysis, a binary matrix was constructed, where a '1' indicated the presence and a '0' indicated the absence of a specific pathogen in a given sample. Based on this matrix, we calculated the Jaccard similarity coefficient to evaluate the co-occurrence of pathogen pairs. Spearman's rank correlation was used to assess the association between pathogen load (represented by read counts) and continuous variables like age. Furthermore, hierarchical clustering was performed to identify patterns of co-infection, and Principal Component Analysis (PCA) was used to visualize the overall distribution of samples based on their pathogen profiles.

2.4 Statistical Analysis

All statistical analyses were conducted using R software (version 4.6.1). Categorical variables, such as pathogen detection rates across different groups, were compared using the Chi-square test or Fisher's exact test, as appropriate. Continuous variables were presented as median and interquartile range (IQR). The strength of the association between pathogen co-occurrence was measured by the Jaccard similarity coefficient. Correlations between pathogen load and patient age were determined using Spearman's rank correlation test. A two-sided p -value of less than 0.05 was considered statistically significant. For multiple comparisons, the Benjamini-Hochberg procedure was applied to control the false discovery rate (FDR).

3. Results

3.1 Demographic Characteristics and Overall Pathogen Detection

A total of 154 pediatric patients with influenza-like illness were enrolled in this study,

comprising 84 males (54.5%) and 70 females (45.5%), with no statistically significant difference in sex distribution ($\chi^2 = 1.273$, $P = 0.259$) (Table 1). The median age was 45.5 months (interquartile range [IQR]: 21.2–72.8). Children aged <12 months accounted for only 9.1% of the cohort, whereas those aged ≥ 12 months constituted the vast majority (90.9%). The distribution of participants across the four predefined age groups was significantly imbalanced ($\chi^2 = 21.636$, $P < 0.001$). However, when the youngest group (<12 months) was excluded, the remaining three groups (12–35 months, 36–71 months, and ≥ 72 months) showed no statistically significant difference in sample size distribution ($\chi^2 = 0.700$, $P = 0.705$). Samples were collected across three epidemic phases: early (October–November, 33.1%), mid (December–January, 40.3%), and late (February–March, 26.6%), with no significant differences in sample distribution among the three periods ($\chi^2 = 4.299$, $P = 0.117$).

The overall pathogen detection profile is illustrated in Figure 1. Among the 154 samples, pathogens were detected in 72.1% of cases, while 27.9% tested negative for all targeted agents. Notably, mixed infections were highly prevalent, constituting 57.8% of the total cohort, whereas single infections accounted for only 14.3%. As shown in Figure 1B, Nm exhibited the highest individual detection rate at 36.4%, followed closely by Hi at 33.1% and FluA N2 at 30.5%. Other frequently detected pathogens included FluA H3 (27.9%), Spn (26.6%), and M. catarrhalis (24.0%). In contrast, viruses such as Rhinovirus (4.5%) and EBV (3.9%) showed relatively lower detection rates within this population.

Table 1. Demographic and Clinical Characteristics of Pediatric Patients with Influenza-Like Illness (N=154)

Characteristics	Total (N=154)	χ^2	P
Age (months), median (IQR)	45.5 (21.2, 72.8)		
Gender, n (%)			
Female	70 (45.5%)	1.273	0.259
Male	84 (54.5%)		
Age group, n (%)			
<12 m	14 (9.1%)	21.636	<0.001
12–35 m	46 (29.9%)	0.700	0.705
36–71 m	51 (33.1%)		
≥72 m	43 (27.9%)		
Sample collection period, n (%)			
Early (Oct-Nov)	51 (33.1%)	4.299	0.117
Mid (Dec-Jan)	62 (40.3%)		
Late (Feb-Mar)	41 (26.6%)		

Note: Data are presented as median (interquartartile range [IQR]) or number (percentage). Chi-square tests were used to compare categorical variables. Abbreviations: ILI, influenza-like illness; m, months.

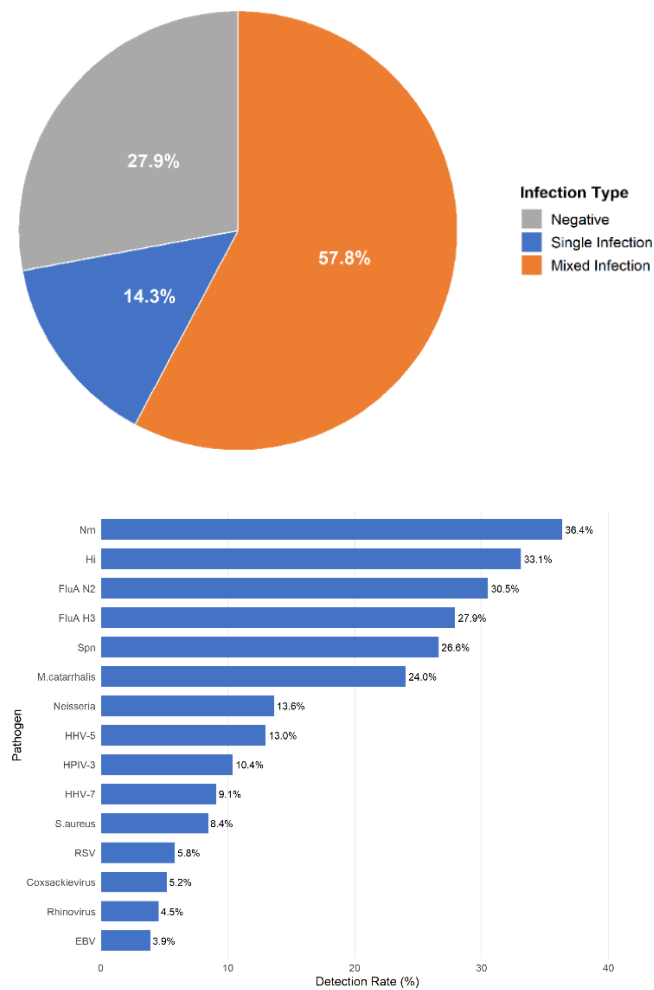


Figure 1. Overview of infection types and pathogen detection rates.

(A) Distribution of infection types among the 154 pediatric patients, categorized into Negative, Single Infection, and Mixed Infection. Data are presented as the number of cases (percentage, %). (B) Detection rates of the top 15 pathogens identified in the study cohort. Pathogens are ranked in descending order of detection rate. Only the 15 most frequently detected pathogens are displayed. Data are presented as the number of positive cases (detection rate, %). Abbreviations: Nm, *Neisseria meningitidis*; Hi, *Haemophilus influenzae*; Spn, *Streptococcus pneumoniae*; M.cat, *Moraxella catarrhalis*; HHV - 5, human herpesvirus 5; HPIV - 3, human parainfluenza virus 3; HHV - 7, human herpesvirus 7; RSV, respiratory syncytial virus; EBV, Epstein - Barr virus.

3.2 Age-Related Differences in Pathogen Detection Rates and Viral Loads

To investigate the relationship between pathogen prevalence and age, we stratified the 154 pediatric patients with ILI into four age groups: <12 months, 12–35 months, 36–71 months, and ≥ 72 months. We then analyzed the detection rates of the top 15 pathogens (Table 2, Figure 2, Figure 3).

Among these pathogens, distinct age-specific patterns emerged. Hi and Spn showed increasing detection rates with advancing age. Hi was rarely detected in the youngest group (<12 m, 0%) but became increasingly prevalent, reaching 51.2% in children aged ≥ 72 m ($\chi^2 = 16.34$, $P < 0.001$). Similarly, Spn detection rose from 14.3% (<12 m) to a peak of 39.2% (36–71 m) before declining to 18.6% (≥ 72 m) ($\chi^2 = 6.82$, $P = 0.078$). In contrast, Nm exhibited a U-shaped pattern, with the highest detection rate in the 12–35 m group (50.0%) and lower rates in the other groups ($\chi^2 = 14.97$, $P = 0.002$). FluA subtypes also demonstrated age-dependent trends. FluA N2 detection increased progressively with age, from 21.4% (<12 m) to 46.5% (≥ 72 m) ($\chi^2 = 11.00$, $P = 0.012$). FluA H3 showed a different trajectory, peaking at 41.9% in the oldest group (≥ 72 m) after increasing from 21.4% (<12 m) to 29.4% (36–71 m) ($\chi^2 = 8.19$, $P = 0.042$). Notably, some pathogens exhibited preferential detection in specific age strata. HHV-5 was predominantly detected in the youngest age group (<12 m, 50.0%) and its prevalence declined sharply with age ($\chi^2 = 25.34$, $P < 0.001$). Conversely, RSV showed a marked increase in detection from infancy to early childhood ($\chi^2 = 9.62$, $P = 0.014$). Other pathogens, such as HPIV-3 and Coxsackievirus, did not show significant variations across the four age groups ($P > 0.05$).

The relationship between pathogen load (log₁₀ copies/mL) and continuous age is depicted in Figure 3. Among the analyzed pathogens, only Nm displayed a statistically significant negative correlation with age ($r = -0.349$, $p = 0.019$), indicating that younger patients tended to carry higher bacterial loads. For other pathogens, such as FluA N2 ($r = -0.152$, $p = 0.309$) and HHV-5 ($r = -0.315$, $p = 0.252$), although negative trends were observed, they did not achieve statistical significance. Similarly, no significant correlations were found for FluA H3, Hi, Spn, or the other viral agents examined, suggesting that while detection frequency varies by age, the magnitude of pathogen shedding remains relatively stable across different pediatric age groups for most agents.

Table 2. Detection Rates of Top 15 Pathogens by Age Group

Pathogen	<12 m (n=14)	12–35 m (n=46)	36–71 m (n=51)	≥72 m (n=43)	χ^2	P
Nm	1 (7.1%)	23 (50.0%)	23 (45.1%)	9 (20.9%)	14.97	0.002
Hi	0 (0.0%)	10 (21.7%)	19 (37.3%)	22 (51.2%)	16.34	<0.001
FluA N2	3 (21.4%)	7 (15.2%)	17 (33.3%)	20 (46.5%)	11.00	0.012
FluA H3	3 (21.4%)	7 (15.2%)	15 (29.4%)	18 (41.9%)	8.19	0.042
Spn	2 (14.3%)	11 (23.9%)	20 (39.2%)	8 (18.6%)	6.82	0.078
M.catarrhalis	2 (14.3%)	9 (19.6%)	19 (37.3%)	7 (16.3%)	7.53	0.057
Neisseria	0 (0.0%)	7 (15.2%)	6 (11.8%)	8 (18.6%)	3.36	0.339
HHV-5	7 (50.0%)	9 (19.6%)	3 (5.9%)	1 (2.3%)	25.34	<0.001
HPIV-3	3 (21.4%)	7 (15.2%)	5 (9.8%)	1 (2.3%)	6.01	0.111
HHV-7	0 (0.0%)	1 (2.2%)	6 (11.8%)	7 (16.3%)	7.19	0.066
S.aureus	3 (21.4%)	4 (8.7%)	4 (7.8%)	2 (4.7%)	3.88	0.275
RSV	1 (7.1%)	0 (0.0%)	7 (13.7%)	1 (2.3%)	9.62	0.014
Coxsackievirus	0 (0.0%)	4 (8.7%)	3 (5.9%)	1 (2.3%)	2.68	0.572
Rhinovirus	1 (7.1%)	2 (4.3%)	0 (0.0%)	4 (9.3%)	4.89	0.099
EBV	0 (0.0%)	3 (6.5%)	3 (5.9%)	0 (0.0%)	3.70	0.381

Data are presented as number of positive cases (percentage). The denominator for percentage calculation is the total number of subjects in each age group. Intersubgroup comparisons were performed using Fisher's exact test when the expected frequency in any cell was less than 1; otherwise, Pearson's chi-square test was used.

Abbreviations: m, months; n, number of subjects.

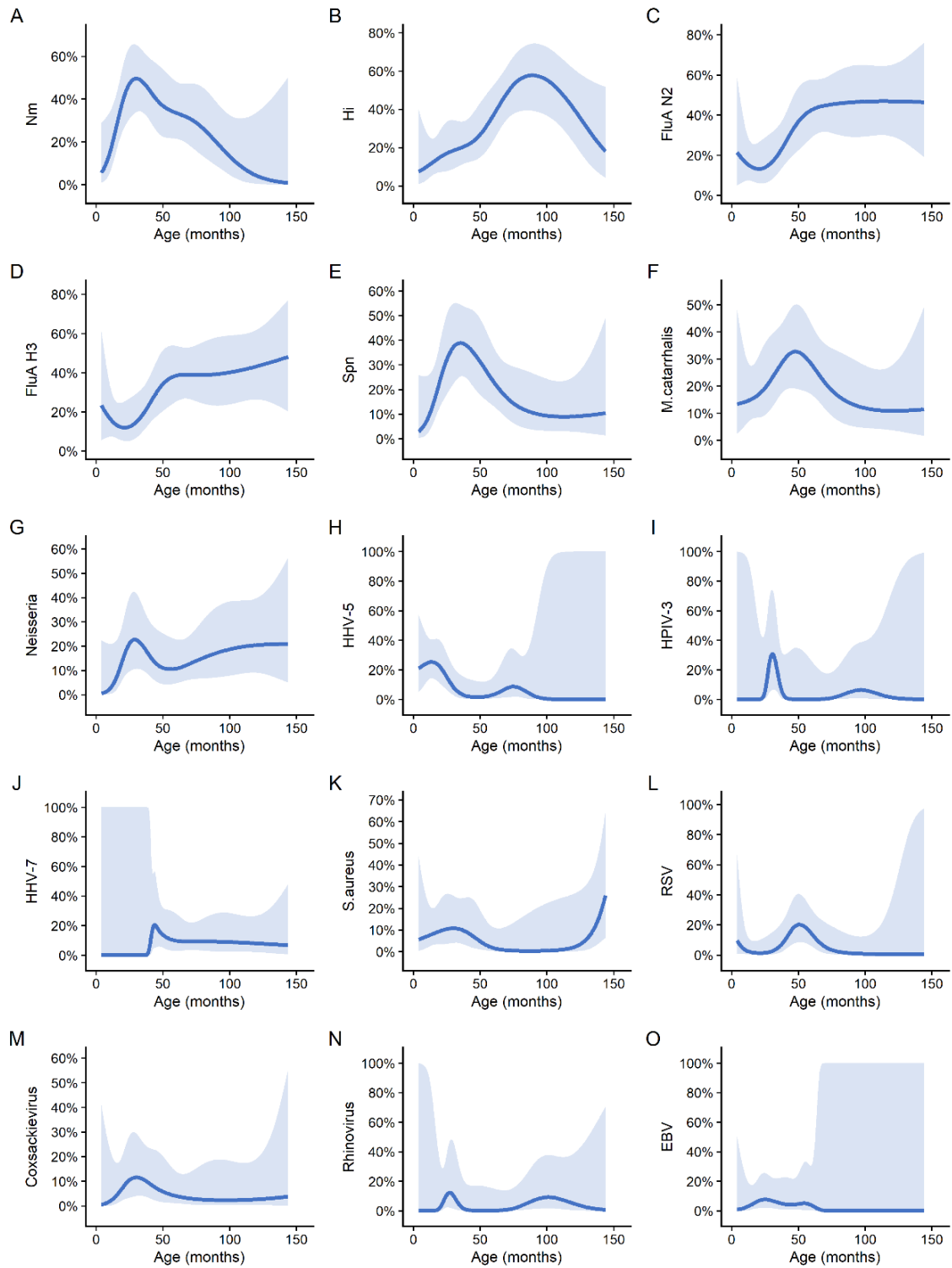


Figure 2. Age-specific positivity rates of the top 15 pathogens.

Note: The line plots illustrate the smoothed detection rates (%) of each pathogen across different ages (months). The shaded areas represent the 95% confidence intervals.

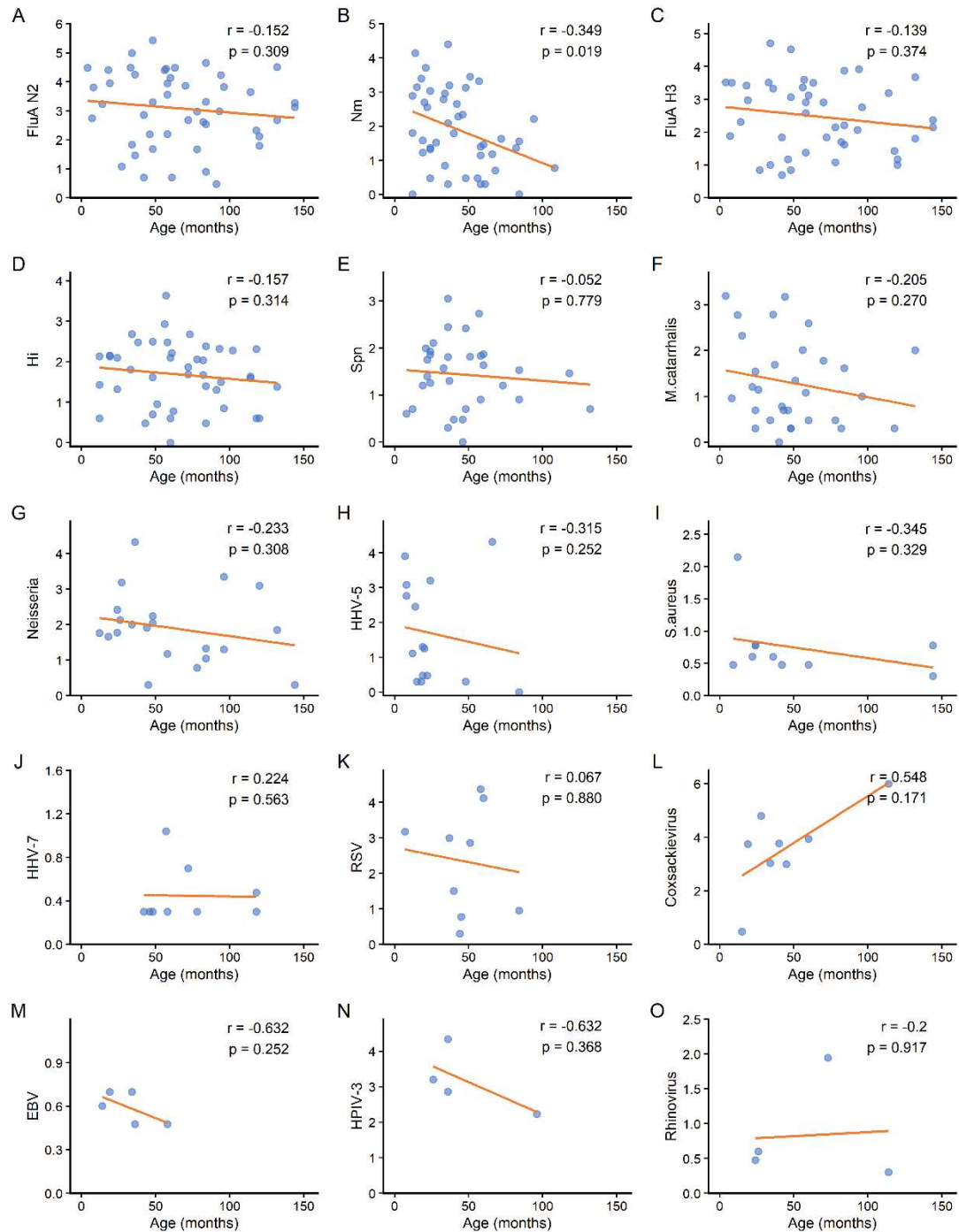


Figure 3. Correlation between pathogen load and patient age.

Note: Scatter plots show the relationship between log₁₀ pathogen load (copies/mL) and age (months) for the top 15 pathogens. The orange line represents the linear regression fit. Spearman correlation coefficients (r) and p-values are displayed for each pathogen.

3.3 Temporal Dynamics of Pathogen Positivity and Viral Load Across Epidemic Phases

To characterize the temporal distribution of the top 15 pathogens, we stratified the 154 pediatric ILI patients into three epidemic phases: early (October–November), peak (December–January), and late (February–March), based on the established seasonal influenza surveillance framework for northern China (17, 18), where the influenza activity peak predominantly occurs in December–January, and analyzed their positivity rates (Table 3). Among these pathogens, Nm exhibited a significantly higher positivity rate in the early phase (45.1%) than in the late phase (19.5%) ($\chi^2 = 7.13$, $p = 0.028$). FluA demonstrated a marked seasonal pattern: both FluA N2 and FluA H3 peaked during the peak phase (53.2% and 50.0%, respectively) and were undetected in the late phase (FluA N2: $\chi^2 = 33.31$, $p < 0.001$; FluA H3: $\chi^2 = 31.39$, $p < 0.001$). Similarly, M.catarrhalis showed a significant decline from the early phase (31.4%) to the late phase (9.8%) ($\chi^2 = 6.47$, $p = 0.039$), while Neisseria decreased significantly from 23.5% in the early phase to 0% in the late phase ($\chi^2 = 10.75$, $p = 0.005$). Conversely, HPIV-3 prevalence increased significantly toward the end of the season, rising from 5.9% in the early phase to 24.4% in the late phase ($\chi^2 = 11.8$, $p = 0.003$). Coxsackievirus also exhibited significant temporal variation, with detection rates dropping from 11.8% in the early phase to 0% in the late phase ($\chi^2 = 7.2$, $p = 0.027$). No statistically significant differences were observed for Hi, Spn, HHV-5, HHV-7, S.aureus, RSV, Rhinovirus, or EBV across the three phases (all $p > 0.05$).

To investigate whether pathogen abundance varied temporally among positive cases, we compared the log10-transformed copy numbers across the epidemic phases (Figure 4). For the majority of pathogens, including Nm, Hi, FluA-N2, FluA-H3, Spn, M. catarrhalis, Neisseria, HHV-5, HPIV-3, HHV-7, S. aureus, Coxsackievirus, Rhinovirus, and EBV, no significant differences in viral or bacterial loads were observed between the early, peak, and late periods. However, RSV load exhibited a distinct temporal dynamic; the median log10 copy number was significantly higher during the peak epidemic period compared to the other phases ($p = 0.027$), suggesting an increase in viral replication intensity during the height of the season despite stable overall positivity rates.

To further evaluate the overall structural shifts in the pathogen community composition over time, we performed Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) (Figure 5). The PCA plot revealed that samples from the early epidemic phase (blue circles) exhibited greater dispersion along PC1 compared to the more tightly clustered peak (orange triangles) and late (grey diamonds) phase samples, although substantial overlap remained among all groups. To enhance the separation of these temporal groups, PLS-DA was applied. The PLS-DA score plot demonstrated a clearer distinction between the epidemic phases, with the early phase samples separating distinctly

from the peak and late phases along X-variate 1. The peak and late phases formed relatively distinct clusters along X-variate 2, indicating that the overall pathogen profile undergoes significant compositional changes as the epidemic season progresses.

Table 3. Positivity Rates of Top 15 Pathogens Across Epidemic Phases (Early, Peak, Late)

Pathogen	Early (n=51)	Peak (n=62)	Late (n=41)	χ^2	P
Nm	23 (45.1%)	25 (40.3%)	8 (19.5%)	7.13	0.028
Hi	14 (27.5%)	25 (40.3%)	12 (29.3%)	2.47	0.291
FluA N2	14 (27.5%)	33 (53.2%)	0 (0.0%)	33.31	<0.001
FluA H3	12 (23.5%)	31 (50.0%)	0 (0.0%)	31.39	<0.001
Spn	16 (31.4%)	18 (29.0%)	7 (17.1%)	2.69	0.261
M.catarrhalis	16 (31.4%)	17 (27.4%)	4 (9.8%)	6.47	0.039
Neisseria	12 (23.5%)	9 (14.5%)	0 (0.0%)	10.75	0.005
HHV-5	6 (11.8%)	10 (16.1%)	4 (9.8%)	0.99	0.610
HPIV-3	3 (5.9%)	3 (4.8%)	10 (24.4%)	11.8	0.003
HHV-7	6 (11.8%)	4 (6.5%)	4 (9.8%)	0.99	0.611
S.aureus	4 (7.8%)	6 (9.7%)	3 (7.3%)	0.21	0.899
RSV	5 (9.8%)	4 (6.5%)	0 (0.0%)	4.04	0.133
Coxsackievirus	6 (11.8%)	2 (3.2%)	0 (0.0%)	7.2	0.027
Rhinovirus	2 (3.9%)	3 (4.8%)	2 (4.9%)	0.07	0.966
EBV	2 (3.9%)	3 (4.8%)	1 (2.4%)	0.38	0.827

Data are presented as number of positive cases (percentage). Early, peak, and late epidemic periods correspond to October–November, December–January, and February–March, respectively. Intersubgroup comparisons were performed using Pearson’s chi - square test (or Fisher’s exact test when expected frequencies < 1). A two - tailed *P* value < 0.05 was considered statistically significant.

Abbreviations: Early, October–November; Peak, December–January; Late, February–March; m, months; n, number of subjects.

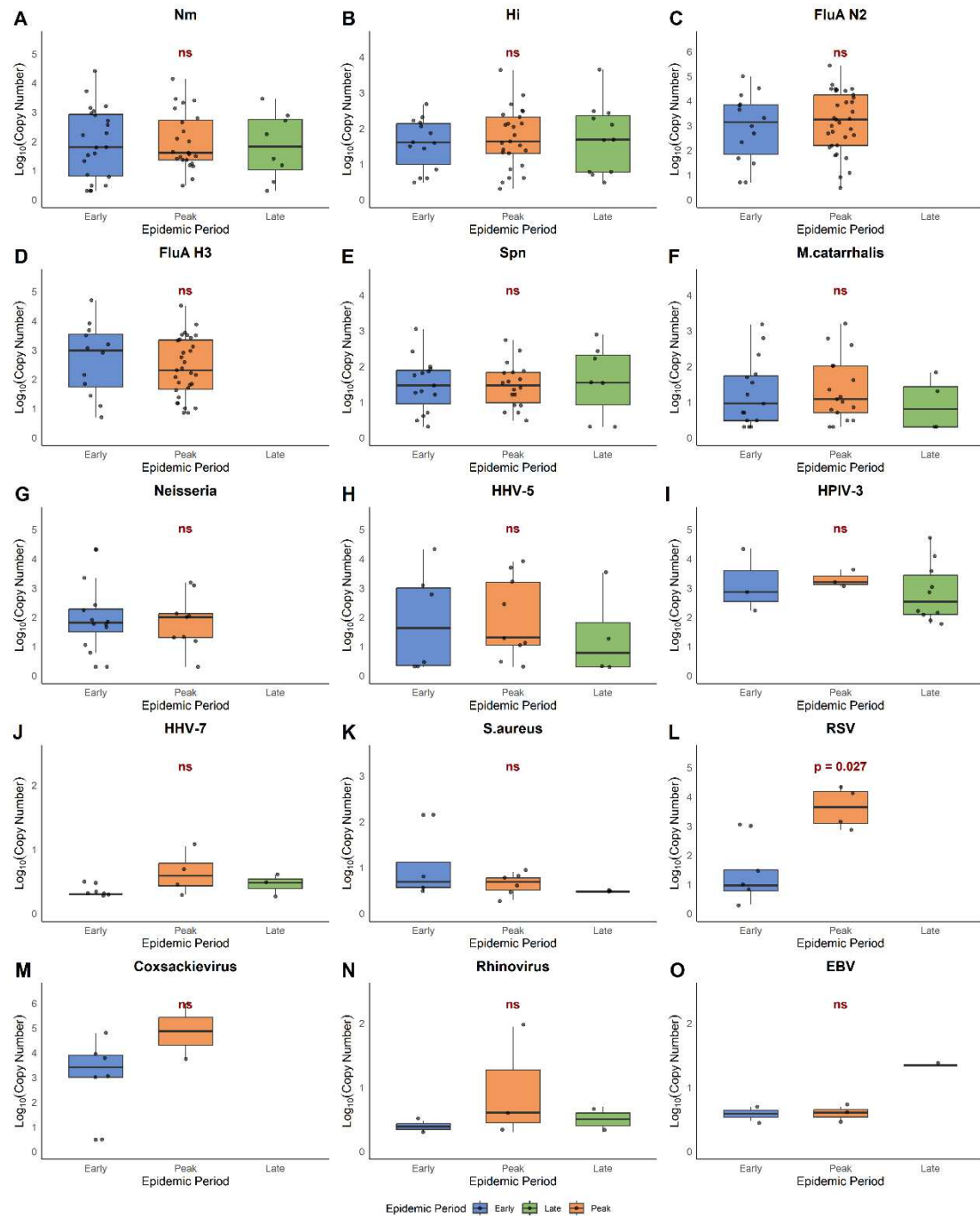


Figure 4. Comparison of pathogen loads (log₁₀ copy number) across epidemic phases. Box plots illustrate the distribution of log₁₀-transformed copy numbers for each of the top 15 pathogens during the early, peak, and late epidemic periods. Horizontal lines within boxes represent medians. Statistical significance was assessed using the Kruskal-Wallis test. "ns" indicates no significant difference ($p \geq 0.05$)

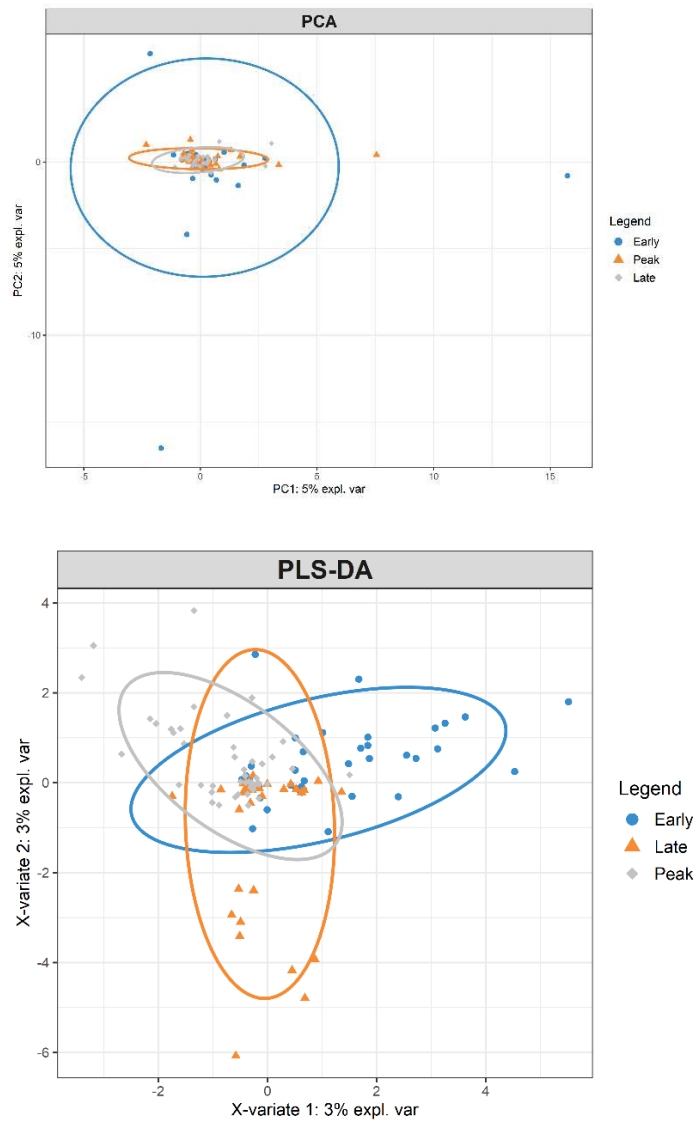


Figure 5. Multivariate analysis of pathogen profiles across epidemic phases.

(A) Principal component analysis (PCA) score plot: samples are colored by epidemic period (Early = blue circles, Peak = orange triangles, Late = gray diamonds). **(B)** Partial least squares discriminant analysis (PLS - DA) score plot: samples are similarly colored. Ellipses represent 95% confidence intervals for each period.

3.4 Pathogen Interaction Networks and Co-occurrence Patterns

To elucidate the potential interactions among the top 15 pathogens, we performed a correlation analysis based on Jaccard similarity coefficients and constructed a co-occurrence network (Figure 6). The heatmap (Figure 6A) illustrates the pairwise correlation strengths, revealing distinct clustering patterns among specific pathogen groups. Notably, FluA exhibited a strong positive correlation with each other (FluA N2 vs. FluA H3, coefficient = 0.91). Furthermore, Spn showed moderate positive correlations with both Nm (0.31) and Hi (0.24), suggesting potential co-colonization or shared ecological niches. In contrast, most viral pathogens, such as Rhinovirus and EBV, displayed weak to negligible correlations with other agents in the panel.

The network visualization (Figure 6B) further maps these associations, where nodes represent individual pathogens and edges indicate significant co-occurrence relationships. Red lines denote positive correlations, while blue lines represent negative correlations. The network topology highlights Nm and Hi as central hub nodes with high connectivity, linking various bacterial and viral species. Specifically, a dense cluster of positive interactions (red edges) is observed among the respiratory bacteria Nm, Spn, and Hi, as well as between the two FluA strains. Conversely, widespread negative correlations (blue edges) are evident between Nm and several other pathogens, including HPIV-3 and HHV-7, indicating potential competitive exclusion or temporal separation in their prevalence. This complex web of interactions underscores the non-random distribution of pathogens within the host population during the epidemic season.

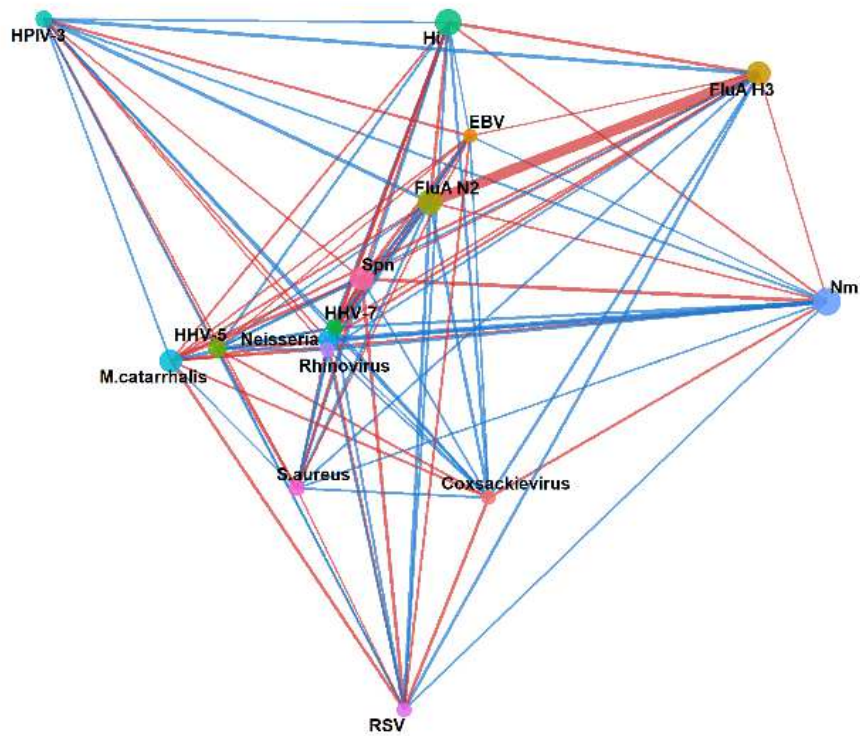
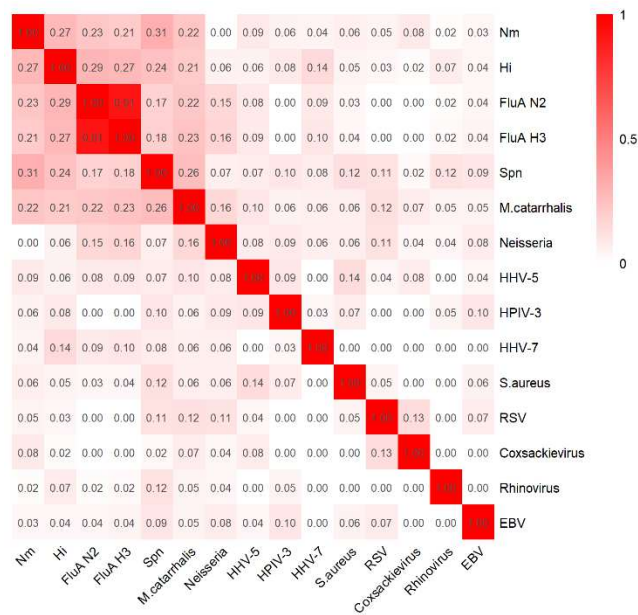


Figure 6. Pathogen interaction networks and co-occurrence patterns.

(A) Heatmap of pairwise Jaccard similarity coefficients among the top 15 pathogens. The color intensity represents the strength of the correlation, ranging from 0 (white) to 1 (dark red). **(B)** Network visualization of pathogen co-occurrence. Nodes represent pathogens, and edges represent correlations. Red edges indicate positive correlations, and blue edges indicate negative correlations. Node size reflect relative abundance or centrality.

3.5 Clustering of Co-infection Patterns

To systematically categorize the complex co-infection profiles observed in the study population, we performed hierarchical clustering analysis on the pathogen detection data (Figure 7). This analysis revealed distinct infection patterns among the samples, which could be broadly classified into three primary clusters based on their pathogen composition and load intensity.

The first major cluster, characterized as a "virus-dominated" pattern, is located at the top of the heatmap. This group comprises a large number of samples exhibiting high viral loads, predominantly driven by FluA (FluA N2 and FluA H3) and Nm. The intense red coloration in this section indicates a high prevalence and concentration of these specific pathogens, suggesting a period or subgroup heavily impacted by acute viral respiratory infections often accompanied by Nm colonization.

The second distinct cluster represents a "bacteria-dominated" or mixed bacterial pattern, situated in the middle section of the dendrogram. Samples in this group show significant positivity for Hi, Spn, and M.catarrhalis. Unlike the virus-dominated cluster, these samples display lower intensities for influenza viruses but maintain consistent detection rates for these common respiratory bacteria, indicating a different etiological profile potentially related to bacterial superinfection or primary bacterial respiratory disease.

The third cluster, identified as a "high-colonization background" type, is found in the lower portion of the heatmap. These samples are characterized by a broader but generally lower-intensity distribution of various pathogens, including Rhinovirus, RSV, HHV-5, HHV-7, and EBV, alongside sporadic bacterial detections. The more diffuse and lighter red signals suggest a state of multiple low-level colonizations or mild co-infections rather than a single dominant pathogenic driver, reflecting the complex baseline microbial landscape in the studied cohort.

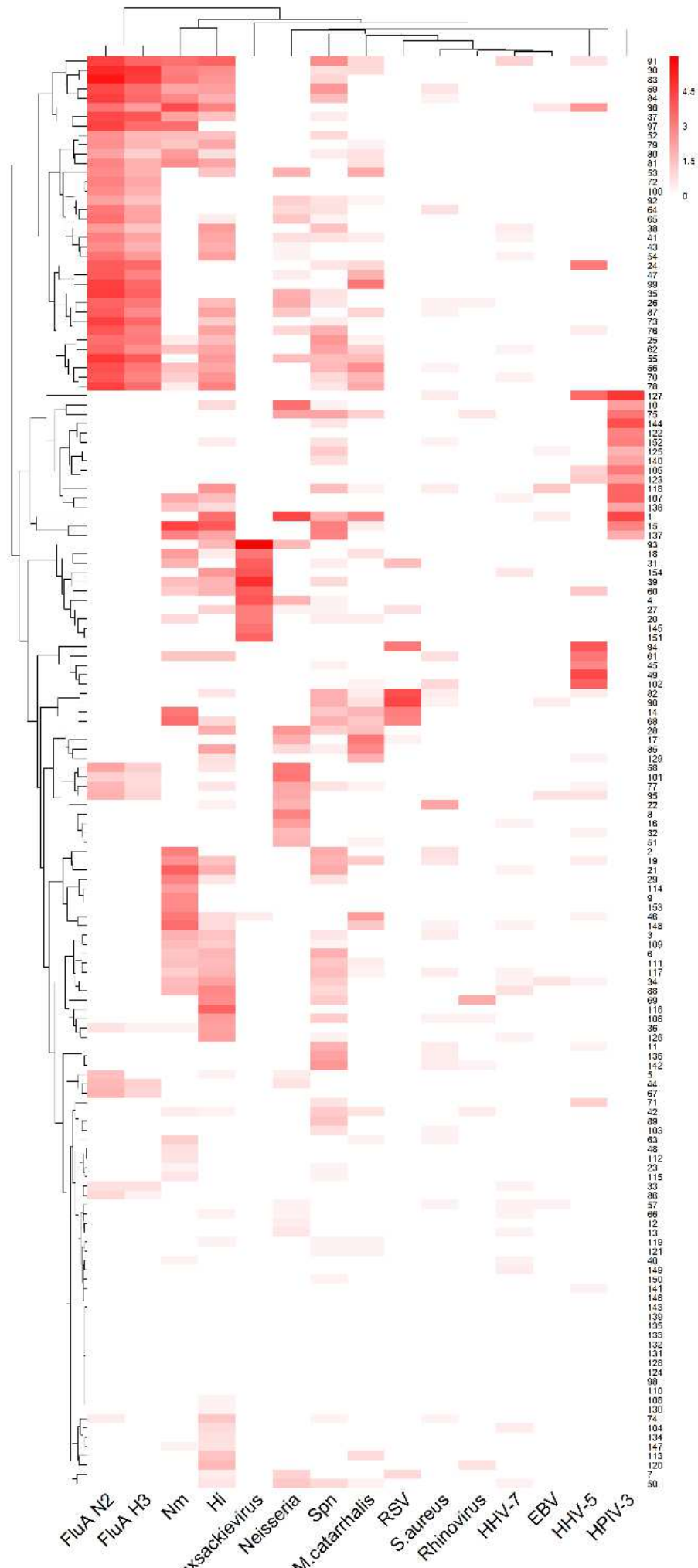


Figure 7. Hierarchical clustering heatmap of co-infection patterns among the top 15 pathogens.

Rows represent individual samples (labeled by ID numbers on the right), and columns represent the detected pathogens. The color intensity ranges from white (negative/low) to dark red (positive/high load), indicating the presence and relative abundance of each pathogen. The dendrograms on the top and left sides illustrate the similarity relationships between pathogens and samples, respectively, revealing three distinct clustering patterns: virus-dominated, bacteria-dominated, and high-colonization background types.

4. Discussion

This study is the first to employ MNP-based targeted sequencing technology to systematically delineate the respiratory microbiome panorama of pediatric ILI patients in Henan Province during the 2025–2026 epidemic season. Through highly sensitive multiplex detection, we not only uncovered the complex pathogen spectrum and high prevalence of mixed infections in this population, but also thoroughly analyzed age-related differences in pathogen distribution, temporal succession dynamics, and intricate inter-pathogen interaction networks. These findings provide novel insights into the epidemiological characteristics and ecological relationships of acute respiratory infections in children, and offer important data to support disease prevention and control strategies in this region.

The study cohort exhibited balanced sex and period distributions (all $P > 0.05$), minimizing the potential for sex- or period-related confounding. However, the overrepresentation of children aged ≥ 12 months (90.9%) relative to infants < 12 months (9.1%) introduces age as a potential confounder. Notably, maternal vaccination during pregnancy or lactation can induce elevated and sustained antibody levels in human milk—particularly against influenza, and the independent protective contribution of vaccine-induced human milk antibodies remains poorly characterized(19). The underrepresentation of the < 12 -month stratum in our cohort thus limits the generalizability of age-stratified findings. Future studies should prioritize infant enrollment and collect breastfeeding history alongside maternal vaccination records to disentangle the interplay between passive immunity and direct pathogen challenge in early infancy.

Our results showed that the overall pathogen detection rate among 154 pediatric ILI cases reached 72.1%, with a mixed infection rate as high as 57.8%. This proportion of co-infections is markedly higher than the 11% reported in routine medical service populations (20), highlighting the ubiquity and complexity of pathogen co-infections in children. Among the detected pathogens, Nm (36.4%) and Hi (33.1%) exhibited the highest detection rates, followed by FluA and Spn. Notably, the high detection rates of bacterial pathogens such as Nm and Hi may reflect their high carriage status in the pediatric upper respiratory tract (21), and the high sensitivity of the MNP marker method enables simultaneous capture of signals from both colonizing and pathogenic bacteria. Therefore, the pathogen spectrum revealed here should be interpreted as an ecological snapshot of the respiratory microbiome rather than a definitive etiological assignment. This pathogen spectrum characterized by concurrent viral-bacterial presence and high prevalence of multiple infections suggests that the possibility of synergistic effects among multiple pathogens should be considered in the diagnosis and treatment of acute respiratory infections in children, which is of great significance for guiding rational clinical medication and formulating precise public health intervention strategies.

Age-stratified analysis revealed a close association between pathogen infection and the maturation of the immune system as well as exposure history in children. The detection rate of Hi increased monotonically from 0% in infants aged <12 months to 51.2% in children aged ≥ 72 months ($p < 0.001$). This "late onset, continuous rise" trajectory aligns well with the maturation of nasopharyngeal mucosal barrier function and the complement system in children: newborns lack specific IgG targeting the Hi capsule, and their nasopharyngeal ciliary clearance function remains immature(22), which actually restricts the initial colonization of Hi. After 12 months of age, as maternal antibodies wane (23) and group exposure increases, the window for Hi colonization opens. Conversely, HHV-5 showed a detection rate as high as 50.0% in infants <12 months, followed by a sharp decline ($p < 0.001$), typically reflecting the "infant susceptibility, older child immunity establishment" pattern of herpesviruses (24). Nm exhibited a U-shaped distribution (peaking at 50.0% in the 12–35 month group), suggesting its transmission advantage in close-contact settings such as daycare centers (25). Both FluA N2 and H3 increased significantly with age (reaching 46.5% and 41.9%, respectively), consistent with previously reported trends of increasing influenza detection with age (26), mainly attributable to expanded social circles in school-aged children and repeated susceptibility due to antigenic drift. Spn peaked at 39.2% in the 36–71 month group and then declined, corresponding precisely to the inflection point of "daycare peak plus immune establishment at school age." This inflection point carries important public health implications, suggesting that pneumococcal vaccination strategies should focus on children aged 3–6 years (21).

The entire epidemic season exhibited a distinctive three-phase succession pattern: "bacteria precede – virus polarizes – niche replacement." In the early phase (October–November), Nm led with a detection rate of 45.1%, together with *M. catarrhalis* (31.4%), forming a "bacteria first" colonization pattern, coinciding with the close-contact transmission window after school reopening in September (27). In the peak phase (December–January), FluA N2 and H3 surged to 53.2% and 50.0%, respectively, creating an overwhelming "virus-dominant" peak. This winter peak pattern is consistent with previously reported seasonal influenza dynamics (28); notably, the dominance of FluA during this period may have suppressed the concurrent transmission of competing viruses such as HPIV-3 through type I interferon-mediated innate immune activation (29). In the late phase (February–March), FluA was no longer detected ($p < 0.001$), while HPIV-3 jumped from 5.9% in the early phase to 24.4% ($p = 0.003$), exemplifying the phenomenon of "niche replacement"—when the dominant virus recedes, a subdominant virus rapidly fills the vacated ecological space (30). PCA analysis showed that early-phase samples exhibited the highest dispersion along PC1, indicating the greatest pathogen diversity, whereas mid- and late-phase samples clustered tightly. This demonstrates that the early epidemic phase is a stage of "co-accumulation" of multiple pathogens, the middle phase is a polarization stage driven

solely by FluA, and the late phase is a niche reconstruction stage, further corroborating previously reported seasonal succession of pathogens (17, 31). This three-phase model provides precise time-window guidance for seasonal prevention and control of pediatric ILI in Henan Province.

Network analysis of inter-pathogen interactions revealed complex co-occurrence and mutual exclusion relationships. Bacteria such as Nm, Spn, and Hi exhibit higher co-occurrence rates, reflecting their shared nasopharyngeal niche and tendency toward co-colonization. In contrast, co-occurrence rates between viruses were relatively low—for example, between Rhinovirus and EBV—strongly suggesting the existence of "viral interference." The underlying mechanism may be that early infection with one virus induces a broad-spectrum type I interferon (IFN) response in the host, establishing an antiviral state that inhibits the invasion and replication of other viruses (29). Additionally, we observed positive correlations between viruses and bacteria, such as FluA and Hi, which may corroborate the "ladder effect": viral infection damages the respiratory epithelium, exposing bacterial receptors and thereby promoting secondary bacterial infection (32). The quantitative capacity of the MNP method was instrumental in this regard, as it not only reveals co-infection phenomena but also helps distinguish high-load pathogenic bacteria from low-load background colonizers through load differences, providing a powerful tool for understanding the dysbiosis and reconstitution of the respiratory microbiome.

This study has several limitations. First, the sample size was relatively small ($n = 154$) and derived from a single center; thus, the generalizability of our conclusions requires validation through larger, multi-center collaborative studies. Notably, infants aged <12 months were substantially underrepresented (9.1%, 14/154), which limits the statistical power for age-stratified analyses in this critical age group and may have masked age-specific pathogen dynamics. Future studies should prioritize enrolling a larger proportion of infants, ideally with collection of breastfeeding history and maternal vaccination records, to disentangle the interplay between passive immunity and direct pathogen exposure. Second, the cross-sectional design lacks longitudinal follow-up data, preventing assessment of pathogen clearance kinetics, chronic colonization, or risk of recurrent infection. Finally, this study relied solely on nucleic acid data from respiratory specimens and did not incorporate synchronized host immune response information (e.g., cytokine levels). Future integration of host transcriptomic or proteomic data will enable a more comprehensive elucidation of infection mechanisms.

5. Conclusions

In summary, using MNP targeted sequencing technology, this study systematically depicted the respiratory microbiome panorama of pediatric ILI patients in Henan Province, revealing a high prevalence of mixed infections, prominent age and temporal distribution characteristics, and complex pathogen interaction networks. Our findings underscore the importance of considering multiple pathogens and the microbiological background in childhood respiratory infections, and provide a scientific basis for precise clinical diagnosis and treatment as well as regional public health prevention and control strategies. Future research should expand sample sizes and incorporate host immune indicators to more fully unravel the pathogenesis of respiratory infections. The results of this study offer important epidemiological evidence for developing age- and season-stratified precision prevention and control strategies for pediatric ILI.

Declarations

Abbreviations

ARI, acute respiratory infection; ILI, influenza-like illness; MNP, multiple nucleotide polymorphism; Nm, *Neisseria meningitidis*; Hi, *Haemophilus influenzae*; Spn, *Streptococcus pneumoniae*; RSV, respiratory syncytial virus; HHV-5, human herpesvirus 5; HPIV-3, human parainfluenza virus 3; mNGS, metagenomic next-generation sequencing; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; FDR, false discovery rate; SNR, signal-to-noise ratio; cDNA, complementary DNA; PCR, polymerase chain reaction; RT-PCR, real-time polymerase chain reaction; IFN, interferon; WHO, World Health Organization. (Full list available in the "List of abbreviations" section after the Abstract.)

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki and was reviewed and approved by the Medical Ethics Committee of Henan Provincial Center for Disease Control and Prevention (Approval No.: 2025-KY-032-01). Informed consent was obtained from the parents or legal guardians of all participating children.

Consent for publication

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

Availability of data and materials

The raw sequencing data generated during the current study have been deposited in the Genome Sequence Archive (GSA) at the National Genomics Data Center. The data are currently under file validation (Submission ID: subCRA079884) and will be publicly available upon completion. The raw data are also 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

CA, BW, XH, and XZ conceived and designed the study. CA, BW, YL, DL, LZ, YL, YY, JW, WW, and PL performed the experiments and data collection. CA, BW, and YL analyzed the data. CA and BW drafted the manuscript. XH and XZ critically revised the manuscript. All authors read and approved the final manuscript.

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