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ORIGINAL RESEARCH

Clinical Impact of Polymicrobial Interactions in Human-Metapneumovirus-Infected Children: A Targeted Next-Generation Sequencing Based Retrospective Study

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Purpose: *Human metapneumovirus* (*hMPV*) is a significant pathogen of acute respiratory infection in children. While previous studies have relied on conventional diagnostics that underestimate complex polymicrobial interactions, this study aimed to utilize targeted next-generation sequencing (tNGS) to comprehensively profile the dynamics of its co-colonization or co-infection pathogen spectrum and their impact on disease severity, thereby informing clinical risk stratification.

Patients and Methods: This retrospective study enrolled 526 hospitalized children with *hMPV* infection. Targeted next-generation sequencing (tNGS) was used to comprehensively analyze the co-colonization or co-infection spectrum stratified by age, gender, sample origin, immune deficiency, and immunization status. Multivariable logistic regression was employed to assess independent associations with host factors and severe clinical outcomes (ICU [intensive care unit] admission, mechanical ventilation, septic shock, pneumonia on imaging).

Results: The median age of the inpatients was 36 months, and 15.6% required ICU admission. The overall prevalence of co-detection among the cohort was 100% since we identified at least one pathogen in the existing results from tNGS. The co-colonization or co-infection pathogen spectrum demonstrated significant age specificity, temporal dynamics, and sample type dependency. Multivariable analysis identified several pathogens independently associated with severe outcomes. Specifically, opportunistic and atypical pathogens (such as *Cytomegalovirus* [prevalence: 7.22%], *Herpes Simplex Virus type 1* [1.71%], *Klebsiella aerogenes* [0.76%], and *Tropheryma whipplei* [1.90%]) were strong risk factors for ICU admission, invasive ventilation, and septic shock (adjusted Odds Ratios [aOR] ranging from 4.57 to 28.69). Conversely, *Fusobacterium nucleatum* (25.67%) consistently exhibited a protective effect (eg, aOR=0.43 for ICU admission). *Mycoplasma pneumoniae* (12.93%) was an independent risk factor for pneumonia on imaging (aOR=2.25), while *Human RSV A* (1.14%) showed a significant protective association (aOR=0.09).

Conclusion: Despite limitations including a single-center retrospective design and the absence of an hMPV-negative control group, the co-colonization or co-infection pathogen spectrum in children with *hMPV* infection is complex and closely related to host characteristics. Early identification of high-risk co-pathogens (eg, CMV or *K. aerogenes*) via tNGS should trigger intensified clinical monitoring. Future prospective controlled studies are needed to validate these findings and confirm causality.

Keywords: human metapneumovirus, pediatrics, co-colonizations, co-infection, targeted NGS diagnostics, bacterial-viral interactions

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Introduction

Human metapneumovirus (hMPV) ranks among the most clinically significant pediatric respiratory pathogens globally, causing substantial morbidity in infants and young children.[1] First identified in 2001, this *Pneumoviridae* family member exhibits seasonal circulation patterns that peak in late winter and spring, often overlapping with respiratory syncytial virus (RSV).[2] Seroprevalence studies confirm near-universal exposure by age 5, yet reinfections occur throughout childhood due to incomplete immunity.[3] hMPV accounts for 1.1–86% of infection prevalence,[4] a broad range that strongly depends on the specific pediatric age group, geographical region, and local seasonal dynamics, with infants <1 year bearing the highest burden of severe disease including bronchiolitis, pneumonia, and respiratory failure.[5–8]

Critical gaps persist in understanding polymicrobial interactions during pediatric hMPV infections. Conventional diagnostics significantly underestimate co-colonization, which studies suggest occur in 30% of pediatric cases.[9] Conventional culture-based diagnostics, primarily focused on single-pathogen detection, significantly underestimate the true pediatric respiratory "pathogenome" and fail to capture complex polymicrobial interactions—including virus-virus, virus-bacteria, and virus-fungi co-detections.[9–11] Furthermore, defining the precise pathogen ecology requires a rigorous distinction between asymptomatic mucosal co-colonization (eg, baseline carriage of high-prevalence upper airway commensals) and true co-infection (where pathogen detection strongly correlates with lower respiratory tract invasion or severe clinical phenotypes).[12,13] This crucial distinction is often missing in prior syndromic surveillance,[14] leading to a knowledge deficit that impedes optimal management: unnecessary antibiotics are often prescribed empirically, while genuine, high-risk secondary infections may be missed.[14–17]

Targeted next-generation sequencing (tNGS) offers transformative advantages by simultaneously detecting pathogens with high sensitivity from minimal pediatric sample volumes.[18,19] Unlike conventional methods, tNGS simultaneously detects bacterial, viral, and fungal pathogens with high sensitivity from minimal sample volumes—critical for pediatric applications.[20] Its multiplex capacity identifies expected co-pathogens (eg, *Haemophilus influenzae*, RSV), unexpected associations (eg, *Fusobacterium*), and low-abundance pathogens often missed by singleplex assays.[21,22] Furthermore, tNGS reveals strain-level variations and antimicrobial resistance markers, providing unprecedented resolution of the respiratory "pathogenome" during hMPV infection.[23]

Despite previous syndromic surveillance, critical gaps persist in understanding the comprehensive polymicrobial interactions and their direct clinical consequences during pediatric hMPV infections using unbiased molecular approaches. This study addresses fundamental pediatric knowledge gaps regarding complex polymicrobial interactions —including virus-virus, virus-bacteria, and virus-fungi co-detections—by analyzing tNGS data from a large cohort of children and adolescents with laboratory-confirmed hMPV infection. The novelty of this study lies in uniquely utilizing unbiased tNGS across a large pediatric cohort to not only comprehensively map these complex polymicrobial interactions but also to explicitly delineate their direct clinical consequences. Our objectives were threefold: (1) Comprehensively characterize the spectrum of tNGS-detected co-pathogens across pediatric subgroups (age, gender, sample origin, immune deficiency, immunization status); (2) Identify temporal trends of hMPV infection cases; and (3) Determine whether specific pathogens can be used for clinical outcome predictors in hospitals. By delineating co-colonization and clinical impact in pediatric hMPV, this research aims to inform precision diagnostics, targeted therapeutic strategies, and preventive interventions for high-risk subgroups.

Materials and Methods

Study Design and Patient Selection

This retrospective study enrolled 526 consecutive hospitalized children with laboratory-confirmed hMPV infection diagnosed between January 2022 and April 2025. Patients were identified through the institutional microbiology database of Fujian Children's Hospital. Inclusion criteria required: 1) Positive hMPV detection via PCR in respiratory specimens (NPS, BALF, or sputum); 2) Availability of concomitant tNGS results, and 3) Complete demographic and clinical metadata. Exclusion criteria eliminated: 1) Patients with incomplete clinical records, 2) Loss of concomitant tNGS results (Figure 1). The BALF procedure was conducted in 93 patients based on predefined clinical scenarios, including: (1) evaluation of pulmonary infiltrates in patients with oncology/hematology/immune deficiency; (2) severe or refractory

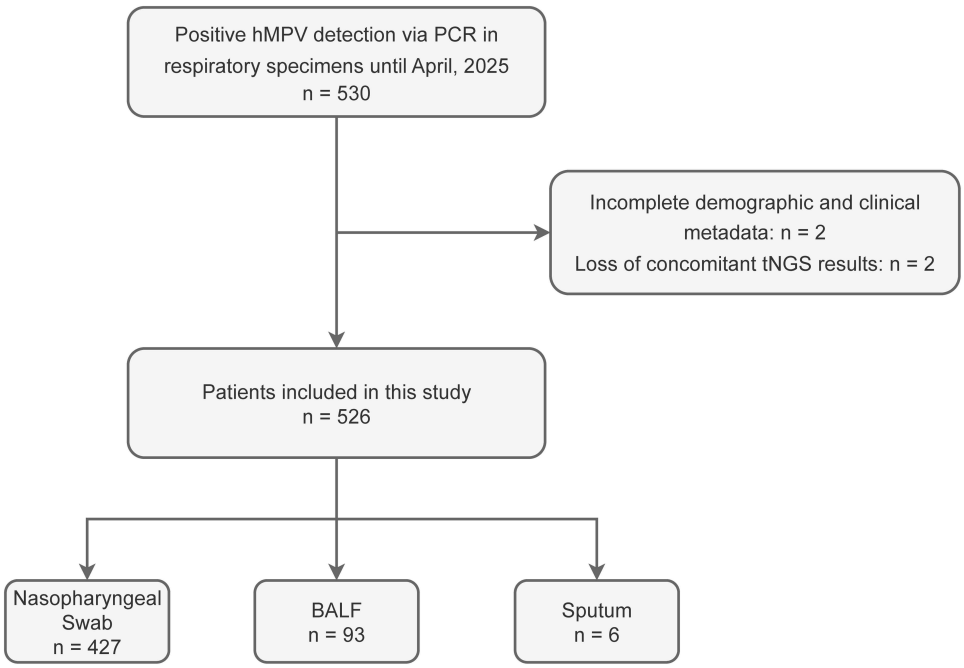

Figure 1 Flowchart illustrating patient selection for the human metapneumovirus (hMPV) study. Among 530 respiratory specimens positive for hMPV by targeted Next-Generation Sequencing (tNGS) until April 2025, patients were excluded for incomplete demographic/clinical metadata (n=2) and loss of concomitant tNGS results (n=2). A total of 526 pediatric patients were included, distributed by specimen type: Nasopharyngeal Swab (n=427, 81.18%), Bronchoalveolar Lavage Fluid (BALF) (n=93, 17.68%), and Sputum (n=6, 1.14%).

community-acquired pneumonia unresponsive to empiric therapy; (3) suspicion of atypical or chronic infections (eg, tuberculosis, fungal infection); (4) suspected ventilator-associated pneumonia (VAP); and (5) the diagnostic work-up of unexplained diffuse parenchymal lung disease to exclude infection. Final cohort stratification followed predefined categories: age groups (Infant: <12 months, Toddler: 12–36 months, Preschool: 37–72 months, School-Age: 72–144 months, Adolescent: >144 months), and infection status (clinically diagnosed concurrent infection requiring antimicrobial therapy).

Sample Collection

Respiratory specimens, including nasopharyngeal swabs (NPS), bronchoalveolar lavage fluid (BALF), and sputum, were collected under strict aseptic protocols by trained clinical personnel. All specimens were maintained at room temperature and transported to the molecular diagnostics laboratory within 2 hours of collection for subsequent nucleic acid extraction. Highly detailed technical descriptions of the specific collection procedures for each sample type are provided in the Supplementary Methods.

tNGS Methodology and Pathogen Detection

Clinical specimens were sent to Kingmed Diagnostics Group Co., Ltd., Guangzhou, China, for tNGS sequencing using a commercial toolkit. For tNGS, the panel was designed for detecting 153 causative agents of acute lower respiratory tract infections. This comprehensive coverage encompassed bacteria (Gram-positive/Gram-negative, acid-fast bacilli), respiratory viruses (enveloped/non-enveloped DNA/RNA viruses), fungi (yeasts, molds, *Pneumocystis*), and atypical pathogens—defined as fastidious organisms refractory to conventional culture that are epidemiologically linked to atypical pneumonia (*Mycoplasma pneumoniae, Chlamydia pneumoniae, Legionella pneumophila, Coxiella burnetii*, and related species). Given that the panel is strictly optimized for acute lower respiratory tract infections in a predominantly immunocompetent, non-endemic pediatric population, parasitic agents were excluded from the targeted spectrum. Pathogen selection adhered to *World Health Organization (WHO) Acute Lower Respiratory Infection (ALRI) Etiology Guidelines* and pediatric epidemiological priorities.[24] In brief, the clinical specimens were diluted 1:1 with 0.1

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M DTT before nucleic acid extraction using the MagPure Pathogen DNA/RNA Kit B on a KingFisher™ Flex system. Nuclease-free water served as the negative control. Multiplex PCR pre-amplification and library preparation were performed with the RP100 kit (KingCreate). Libraries were quantified using Qubit™ (≥0.5 ng/mL) and analyzed with Qsep100 before sequencing on the KM MiniSeq Dx-CN platform. Data processing involved quality control with fastp v0.20.1, reference-based assembly with Bowtie2 v2.4.1 (very-sensitive mode), and pathogen detection defined as RPhK ≥10. This threshold was selected to optimize the balance between sensitivity and specificity for respiratory pathogens, minimizing the detection of transient background noise. Final interpretation was conducted jointly by interpreters and bioinformaticians.

Clinical Data Abstraction and Variable Definition

Standardized electronic health record (EHR) abstraction captured demographic, clinical, and laboratory variables using the Research Electronic Data Capture (REDCap) system. Collected parameters included: 1) Age at diagnosis; 2) Biological sex (male or female); 3) Sample type and collection date; 4) Underlying comorbidities ascertained from physician diagnoses (such as oncology/hematology disorders, immune deficiency (congenital or acquired),[25] and allergies (including food, drug, or respiratory allergies), immunization status (defined as having completed the age-appropriate routine vaccination schedule according to national guidelines: https://en.chinacdc.cn/health_topics/immunization/202203/t20220302_257317.html); 5) Indicators of clinical severity, including admission to the intensive care unit (ICU), need for non-invasive (continuous positive airway pressure or bilevel positive airway pressure) or invasive ventilation (endotracheal intubation and mechanical ventilation), and occurrence of septic shock[26] based on standard clinical criteria reflecting profound circulatory and cellular/metabolic dysfunction; 5) The pneumonia on imaging criterion required the presence of a new and persistent pulmonary infiltrate, consolidation, or cavitation on a chest X-ray or CT scan, interpreted by a staff radiologist. Data abstraction was performed by two independent clinicians with >95% inter-rater reliability (Cohen's κ=0.87) for key variables. Any discrepancies were resolved through consensus or by adjudication from a third senior pediatric intensivist.

Operational Definitions of Pathogen Detection

Given the high sensitivity of tNGS in detecting nucleic acids regardless of organism viability, we established strict operational definitions for this study to differentiate between background mucosal carriage and true pathogenic involvement. Co-colonization was defined as the tNGS identification of typically commensal organisms (eg, *Streptococcus pneumoniae, Fusobacterium nucleatum, Haemophilus influenzae*) primarily from upper respiratory tract specimens (NPS) without direct proportional correlation to severe lower respiratory tract clinical manifestations. This term acknowledges the presence of the organism's nucleic acid without presuming active tissue invasion. Probable active co-infection was defined as the tNGS detection of a secondary pathogen that meets one or both of the following criteria: (1) Originating from a lower respiratory tract enrichment sample (eg, bronchoalveolar lavage fluid [BALF]); or (2) Demonstrating a strong, statistically significant independent association with severe clinical phenotypes (eg, ICU admission, mechanical ventilation, or septic shock) after adjusting for confounders, particularly when involving recognized opportunistic, multidrug-resistant, or atypical pathogens (eg, *Cytomegalovirus, Klebsiella aerogenes, Tropheryma whipplei*).

Statistical Analysis

Analyses utilized R v4.3.1 with significance defined as $p<0.05$ (two-tailed). Continuous variables (eg, age) were reported as median with 1st-3rd quartiles (Q1-Q3) due to non-normal distribution (Shapiro–Wilk test, $P<0.01$). Categorical variables (pathogen detection rates) were expressed as counts (percentages). Stratified analyses across age groups, gender, sample types, infection status, and calendar years employed Pearson's χ^2-tests for >5 expected counts per cell; Fisher's exact tests were substituted for sparse data. To identify independent risk factors associated with severe clinical outcomes, a two-step logistic regression analysis approach was employed. Prior to multivariable modeling, multicollinearity among candidate pathogens was assessed using the Variance Inflation Factor (VIF) to ensure the stability of the regression estimates. Firstly, univariable logistic regression analyses were performed for all candidate variables

(including various microbial pathogens) to assess their initial association strength with each outcome. At this screening stage, a relatively liberal inclusion criterion ($P < 0.1$) was set to minimize the risk of omitting potentially important variables (Type II error). All variables with a P-value less than 0.1 in the univariable analysis were subsequently entered into a multivariable logistic regression model to adjust for potential confounders. In the multivariable model, a more stringent significance level ($P < 0.05$) was used to determine statistical significance, thereby identifying variables independently associated with the outcome.

Result

Baseline Characteristics of the Study Cohort

Before presenting the detailed stratified data, it is crucial to clarify the distinction between asymptomatic colonization and probable active infection in the context of tNGS diagnostics. Because tNGS detects microbial nucleic acids regardless of viability or pathogenic activity, the high prevalence of certain organisms—such as *Streptococcus pneumoniae, Haemophilus influenzae*, and *Fusobacterium nucleatum*—particularly in upper respiratory tract samples (eg, NPS), likely reflects background mucosal colonization rather than definitive infection. Consequently, to maintain scientific rigor when interpreting these high-prevalence organisms, we predominantly utilize the terms "co-detection" or "co-colonization" throughout the results. The term "probable infection" is strictly reserved for scenarios where pathogen detection strongly correlates with severe clinical phenotypes or originates from lower respiratory tract enrichments (eg, BALF).

Table 1 details the baseline characteristics of the 526 patients with hMPV infection included in this study. The cohort exhibited a median age of 36.0 months (Q1-Q3: 12.0–48.0) with a male predominance (58.94%, n=310). The most common sample type was NPS (81.18%, n=427), followed by BALF (17.68%, n=93) and sputum (1.14%, n=6).

Table 1 Characteristics of Patients with Human Metapneumovirus Infection

Variables	Total (n = 526)
Age (months), M (Q_1, Q_3)	36.00 (12.00, 48.00)
Age, n (%)	
Infant: <12 months	110 (20.91)
Toddler: 12–36 months	146 (27.76)
Preschool: 36–72 months	195 (37.07)
School Age: 72–144 months	71 (13.50)
Adolescent: >144 months	4 (0.76)
Gender, n (%)	
Female	216 (41.06)
Male	310 (58.94)
Oncology/hematology, n (%)	7 (1.33)
Immunizations Completed, n (%)	461 (87.64)
Immune Deficiency, n (%)	25 (4.75)
Clinical severity, n (%)	
Intensive care unit admission	82 (15.59)
Noninvasive ventilation	68 (12.93)
Invasive ventilation	52 (9.89)
Septic shock	38 (7.22)
Pneumonia on imaging	413 (78.52)
Sample type, n (%)	
BALF	93 (17.68)
Nasopharyngeal Swab	427 (81.18)
Sputum	6 (1.14)

(*Continued*)

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Table 1 (Continued).

Variables	Total (n = 526)
Year, n (%)	
2022	43 (8.17)
2023	148 (28.14)
2024	248 (47.15)
2025	87 (16.54)

Notes: M, median; Q1, first quartile; Q3, third quartile; BALF, bronchoalveolar lavage fluid.

Regarding underlying conditions, the vast majority of patients (87.6%) had completed their routine immunizations, while the prevalence of oncology/hematology disorders and immune deficiency was low, at 1.3% and 4.8%, respectively. For clinical outcomes, 15.6% (82/526) of patients required admission to the ICU. Non-invasive and invasive ventilation were administered to 12.93% and 9.89% of patients, respectively. Septic shock occurred in 7.2% (38/526) of the cohort. And 78.52% of patients had pneumonia confirmed by radiological evidence. Collectively, these clinical indicators reflect a cohort with a substantial burden of severe disease.

tNGS Pathogen Patters Stratified by Age, Gender, Sample and Year

The analysis of tNGS results stratified by age revealed distinct patterns of pathogen co-detection among pediatric patients with hMPV infection (Figure 2A). Supplementary Table 1 revealed several statistically significant ($P < 0.05$) age-stratified co-detection patterns among the 526 patients with hMPV infection. *Cytomegalovirus* co-detection was most prevalent in infants (22.73%, 25/110), showing a significant decrease with increasing age ($P < 0.001$). In contrast, the detection rate of *Fusobacterium nucleatum* increased with age, from 2.73% (3/110) in infants to 50.00% (2/4) in adolescents ($P < 0.001$). *Streptococcus pneumoniae* was most frequently co-detected in preschool-age children (42.05%, 82/195, $P < 0.001$), while *Pneumocystis jirovecii* was exclusively identified in infants (6.36%, 7/110, $P = 0.001$). *Klebsiella pneumoniae*co-detection was notably higher in infants (9.09%, 10/110) and school-age children (8.45%, 6/71) compared to other groups ($P < 0.001$). *Streptococcus intermedius* was predominantly found in preschool children (13.33%, 26/195, $P < 0.001$). Epstein-Barr virus (EBV, $P = 0.008$) and *Mycoplasma pneumoniae* ($P = 0.016$) also demonstrated significant age-related variations, with detection rates generally increasing with age. Moreover, analysis of pathogen co-detection stratified by sex revealed that *Fusobacterium nucleatum was* significantly more prevalent in females (31.48%, 68/216) compared to males (21.61%, 67/310, $P = 0.011$) (Figure 2A and Supplementary Table 2). Conversely, co-detection of the *Streptococcus mitis group* was significantly higher in males (9.68%, 30/310) than in females (4.63%, 10/216, $P = 0.032$). For all other pathogens, no statistically significant differences in detection rates were observed between female and male patients.

The analysis of pathogen co-detection stratified by sample type (BALF, nasopharyngeal swab, sputum) revealed distinct profiles (Figure 2A and Supplementary Table 3). *Fusobacterium nucleatum* was significantly more prevalent in nasopharyngeal swabs (30.21%, 129/427) compared to BALF samples (6.45%, 6/93) ($P < 0.001$). Conversely, several pathogens demonstrated a significantly higher detection rate in BALF samples, including Human herpesvirus 5 (also known as *Cytomegalovirus*, CMV) (6.45% in BALF vs. 0.00% in swabs, $P < 0.001$), *Tropheryma whipplei* (7.53% vs. 0.70%, $P < 0.001$), *Pneumocystis jirovecii* (6.45% vs. 0.47%, $P = 0.001$), *Staphylococcus aureus* (21.51% vs. 9.13%, $P = 0.002$), and *Candida albicans* (16.13% vs. 5.85%, P=0.003). Notably, *Klebsiella pneumoniae* was not detected in any BALF samples but had a 5.62% detection rate in nasopharyngeal swabs ($P = 0.046$). For the majority of pathogens, no statistically significant differences in detection rates were observed across the different sample types.

The analysis of pathogen co-detection across the study years (2022–2025) revealed significant temporal variations for multiple pathogens (Figure 2B and Supplementary Table 4). A strikingly high prevalence of the *Streptococcus mitis* group was observed exclusively in 2025 (45.98%, 40/87), while it was absent in the preceding years ($P < 0.001$). *Fusobacterium nucleatum* exhibited its highest detection rate in 2023 (36.49%, 54/148), which subsequently

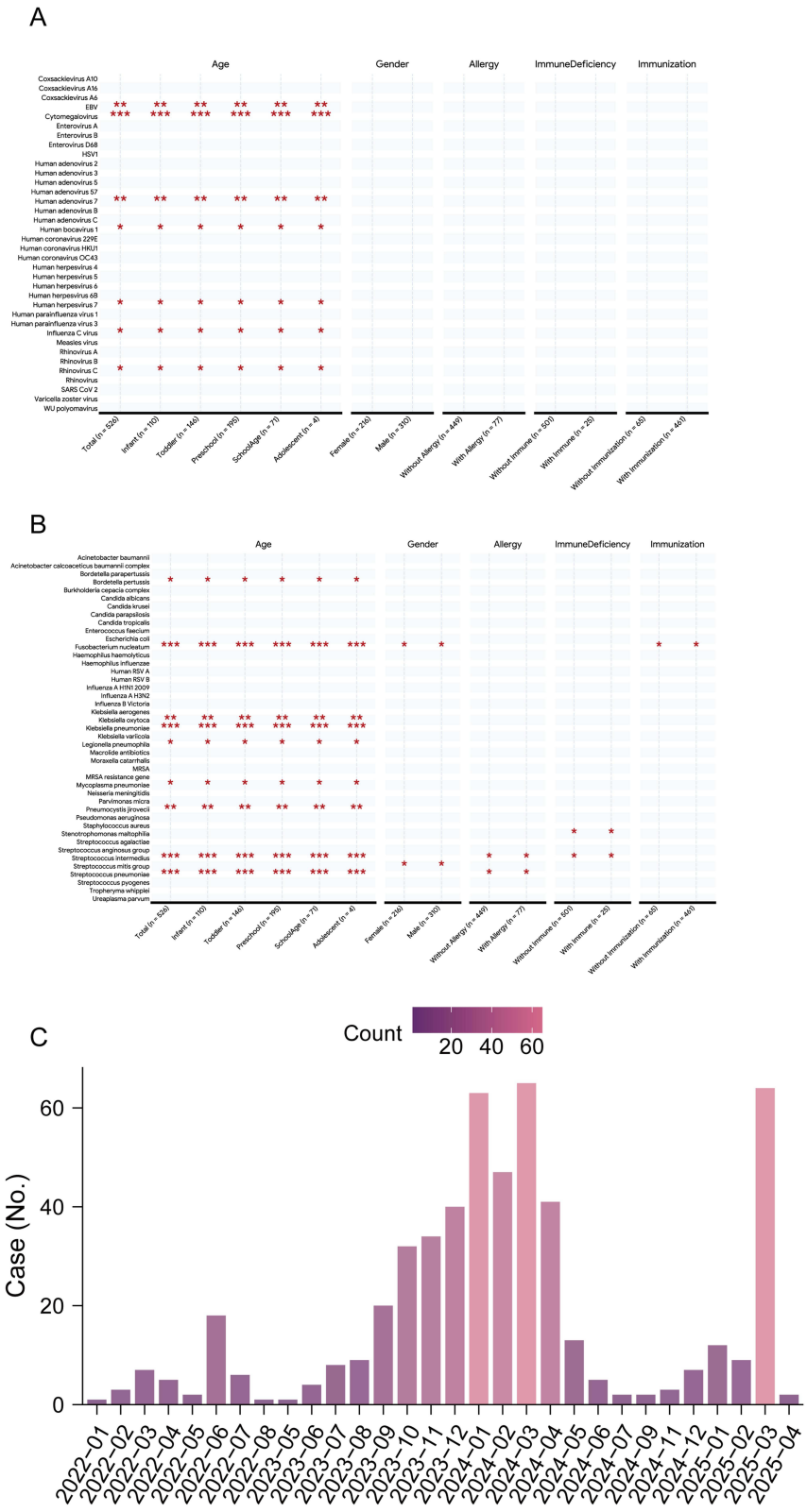

Figure 2 Epidemiological characteristics and pathogen pattern associations in patients with Human Metapneumovirus (hMPV) infection. Heatmap displaying the statistical associations (P values) between the detection of various co-colonizing organisms (rows) including viruses (**A**) and bacteria and fungi (**B**), and host characteristics including age group, sex, immune deficiency status, and immunization status (columns). The sample size (n) for each group is indicated below the column labels. Significance levels: *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001. Results without asterisks (blank) are not significant. This analysis highlights significant age-specific associations for multiple pathogens. (**C**) Bar graph illustrating the year distribution of the 526 enrolled hMPV-positive cases.

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decreased in 2024 (29.84%, 74/248) and further in 2025 (8.05%, 7/87) (P < 0.001). Conversely, *Moraxella catarrhalis* showed a marked increase in prevalence in 2025 (33.33%, 29/87) compared to earlier years (P < 0.001). Several pathogens, including CMV, *Haemophilus haemolyticus*, and *Pneumocystis jirovecii*, were detected almost exclusively in 2022, with rates of 16.28%, 6.98%, and 13.95%, respectively (P < 0.001 for all). *Haemophilus influenzae*was most prevalent in 2024 (52.82%, 131/248), whereas *Mycoplasma pneumoniae*, which was present from 2022 to 2024 (peaking at 23.26% in 2022), was not detected in 2025 (P < 0.001). For the majority of pathogens, no statistically significant temporal variations were observed.

tNGS Pathogen Patterns Related to Immune Status

Analysis of pathogen co-detection stratified by immune status revealed that *Streptococcus intermedius* was detected at a significantly higher rate in patients with immune deficiency (20.00%, 5/25) compared to those without (6.79%, 34/501, P = 0.038) (Figure 2A and Supplementary Table 5). Additionally, *Stenotrophomonas maltophilia* was detected exclusively in the immunodeficient group (4.00%, 1/25, P = 0.048). For the vast majority of pathogens, bacteria, viruses, and fungi analyzed, including common pathogens such as *Haemophilus influenzae, Streptococcus pneumoniae, Moraxella catarrhalis, Fusobacterium nucleatum, Mycoplasma pneumoniae*, and *Staphylococcus aureus*, no statistically significant differences in detection rates were observed between patients with and without immune deficiency. Analysis of pathogen detection stratified by vaccination status revealed that *Fusobacterium nucleatum* was detected at a significantly higher rate in patients with complete immunization (27.33%, 126/461) compared to those without (13.85%, 9/65, P = 0.020) (Figure 2A and Supplementary Table 6). In contrast, a higher detection rate of *Candida albicans* was observed in the non-fully immunized group (13.85%, 9/65) compared to the fully immunized group (6.94%, 32/461), although this difference showed a trend towards significance without reaching the conventional threshold (P = 0.052). Similarly, *Pneumocystis jirovecii* was detected more frequently in the non-fully immunized group (4.62%, 3/65 vs. 1.08%, 5/461, P = 0.064). For the vast majority of other pathogens, bacteria, and viruses analyzed, including *Haemophilus influenzae, Streptococcus pneumoniae*, and *Mycoplasma pneumoniae*, no statistically significant differences in detection rates were observed between the two groups.

Association Between Clinical Outcomes and tNGS Pathogen Detection

As detailed in Figure 3 and Supplementary Table 7, multivariable logistic regression identified several independent predictors for ICU admission. Opportunistic and atypical pathogens, including *Candida albicans*, Herpes Simplex Virus Type 1 (HSV-1), CMV, *Klebsiella aerogenes*, and *Tropheryma whipplei*, were strongly associated with an increased risk of ICU admission. Notably, *K. aerogenes* and CMV exhibited the most pronounced risk profiles among the cohort. Conversely, the co-colonization of *Fusobacterium nucleatum*—an anaerobic bacterium typically recognized as a pro-inflammatory agent in other anatomical sites (such as the oral cavity and gastrointestinal tract)—consistently demonstrated a significant protective association against ICU admission in this specific respiratory context, retaining its significance even after adjusting for confounding variables (aOR: 0.43, 95% CI: 0.21–0.88, P = 0.021).

Regarding respiratory support (Figure 3, Supplementary Tables 8 and 9), distinct pathogen profiles emerged. For invasive mechanical ventilation, *Bordetella pertussis*, HSV-1, CMV, and *Klebsiella aerogenes* were confirmed as independent risk factors in the multivariate model. For non-invasive ventilation, the viral pathogens HSV-1 and CMV remained the strongest independent predictors of requirement. Similar to the ICU admission trends, *Fusobacterium nucleatum* maintained a significant protective association against the need for invasive ventilation, further highlighting its unique inverse relationship with disease severity.

The incidence of septic shock was independently driven by specific bacterial agents (Figure 3 and Supplementary Table 10). Specifically, *Klebsiella aerogenes* and *Tropheryma whipplei* were identified as significant independent risk factors, with *K. aerogenes* conferring a markedly high adjusted risk. It is important to note that the extremely wide confidence intervals observed for certain rare pathogens (eg, *Klebsiella aerogenes*) necessitate cautious interpretation of the exact effect sizes, as they reflect the statistical limitations associated with small sample sizes in these specific subgroups.

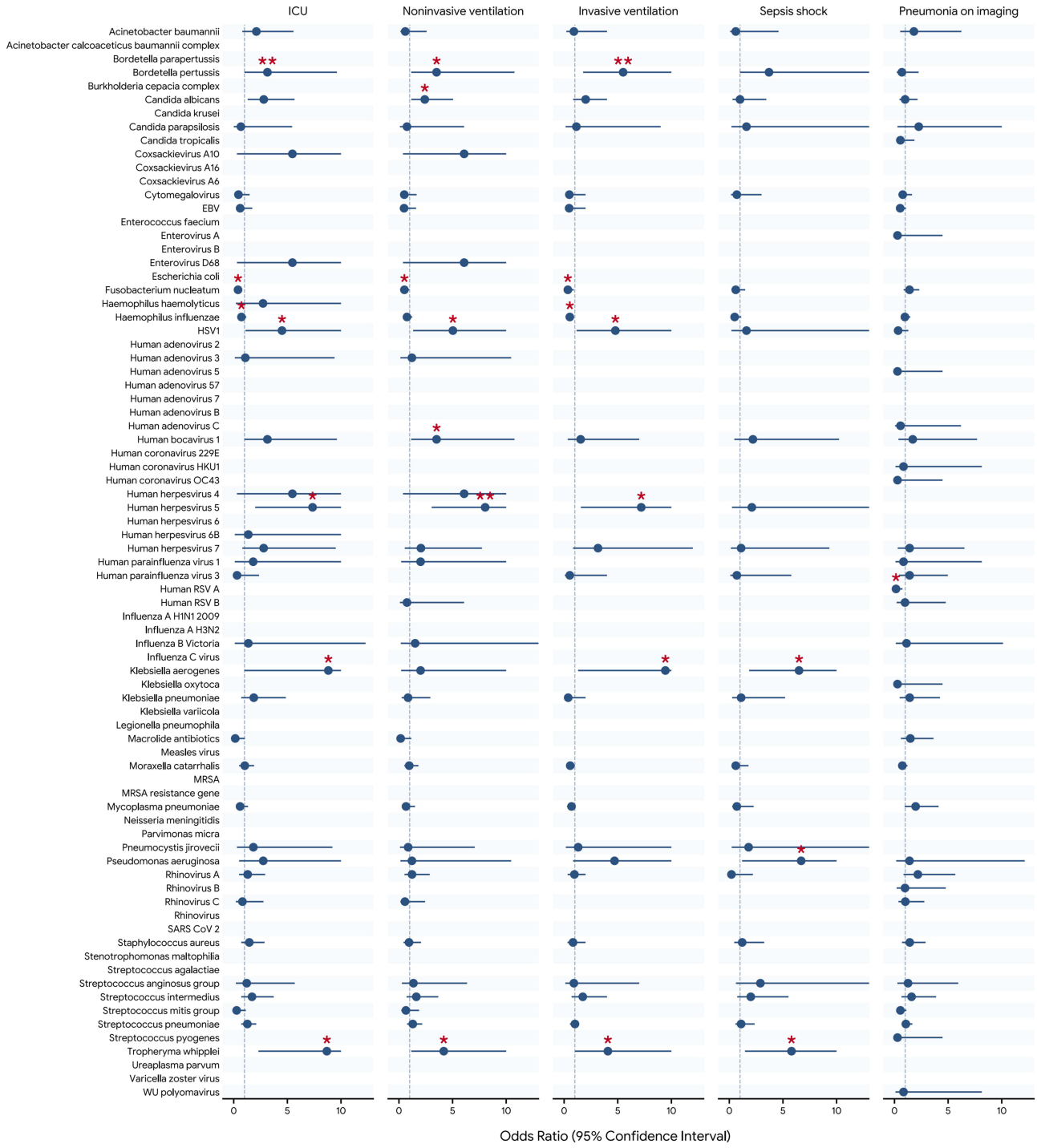

Figure 3 Forest plot showing the associations between specific pathogens and different severe clinical phenotypes. This figure presents the odds ratios (ORs) with 95% confidence intervals (CIs) derived from multivariable logistic regression analyses, illustrating the strength of association between specific pathogens and five severe clinical phenotypes. The x-axis represents the OR values, and the y-axis lists the analyzed pathogens. The five clinical phenotypes (ICU [intensive care unit] admission, Non-invasive ventilation, Invasive mechanical ventilation, Septic shock, Pneumonia on imaging) are represented by five vertically arranged columns. Each point indicates the OR, and the horizontal bars represent the 95% CI. Significance levels: *p-value < 0.05, **p-value < 0.01. Results without asterisks (blank) are not significant.

Finally, regression analysis for pneumonia on imaging (Supplementary Table 11) revealed that while *Mycoplasma pneumoniae* acted as an independent risk factor for radiological consolidation, the co-detection of Human RSV A demonstrated a strong and significant protective association.

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Discussion

The primary objective of this study was to elucidate the clinical impact of the respiratory "pathogenome" in hMPV-infected children using tNGS. Our principal findings demonstrate that pediatric hMPV infections are frequently characterized by complex, host-specific polymicrobial co-detections. Crucially, we identified specific opportunistic pathogens that independently drive severe clinical trajectories—such as *Cytomegalovirus* (CMV) and *Klebsiella aerogenes*—while others, notably *Fusobacterium nucleatum* and RSV A, exhibited unexpected protective associations against critical illness. These findings underscore that the clinical severity of hMPV is not solely dictated by the viral load or intrinsic viral virulence, but is profoundly modulated by the concurrent microbial landscape of the respiratory tract.

While previous culture-based epidemiological studies have well-established the prevalence of common co-infecting bacteria (eg, *Streptococcus pneumoniae*) in viral pneumonias, our tNGS approach challenges several traditional clinical assumptions through critical comparison. Unlike conventional syndromic testing, which often targets a narrow, pre-defined panel and relies on viable organisms, our unbiased profiling revealed that atypical and opportunistic pathogens (eg, *Tropheryma whipplei* and CMV) play a disproportionately massive role in driving critical illness (such as ICU admission and septic shock) in hMPV-infected children. This critical divergence from older literature suggests that historical mortality or severity rates attributed directly to hMPV may have been significantly confounded by these undetected, high-risk co-invaders.

The most striking observation is the profound age-dependent stratification of co-pathogens, confirming and significantly extending prior syndromic surveillance reports.[27] The high burden of *Streptococcus pneumoniae* in preschool and school-age children aligns with known peaks in nasopharyngeal colonization,[28,29] suggesting hMPV infection may disrupt mucosal barriers or impair bacterial clearance mechanisms specific to this developmental stage. Conversely, the dominance of CMV in infants represents a critical and underappreciated finding. The high colonization rate of distinct *Fusobacterium nucleatum* in preschoolers and school-age children has not been previously highlighted in hMPV literatures. This anaerobic bacterium,[30] typically associated with periodontal disease and colorectal cancer, is increasingly linked to respiratory infections in children with viral co-triggers.[31] Its abundance likely reflects viral-induced dysbiosis facilitating anaerobic niche expansion in the inflamed oropharynx and tonsillar crypts, anatomical sites undergoing significant immunological maturation during these years. Similarly, the elevated *Klebsiella pneumoniae* in infants and *Pneumocystis jirovecii* exclusively in this group underscores their unique susceptibility to opportunistic pathogens, potentially linked to immature phagocytic function and lower levels of surfactant proteins.[32,33] *Mycoplasma pneumoniae*'s age-progressive increase in school-age children mirrors its typical epidemiological spread within close-contact settings like schools, amplified by hMPV-induced mucosal damage.[34] While most pathogens showed minimal gender disparity, the higher *Fusobacterium nucleatum* prevalence in females warrants investigation. These subtle differences suggest host factors beyond age significantly shape the co-colonization landscape.

Our data compellingly demonstrate that sample origin profoundly biases tNGS pathogen profiles, a critical consideration for clinical interpretation and research comparability. BALF samples were uniquely enriched for opportunistic pathogens including *Pneumocystis jirovecii*, CMV, and *Tropheryma whipplei*. This likely reflects the sampling of lower airway/alveolar compartments where these pathogens reside, bypassing upper airway colonization flora.[35] Conversely, *Klebsiella pneumoniae* detection was exclusive to NPS, suggesting robust upper respiratory tract colonization suppressed in the lower airways or masked by other flora in BALF.[36] The higher *Candida albicansin* BALF may indicate genuine infection or reflect oropharyngeal contamination during sampling – a known limitation of bronchoscopy. These findings challenge the assumption that NPS suffices for comprehensive co-colonization screening in severe hMPV cases, advocating for lower respiratory sampling when opportunistic infections are clinically suspected, particularly in immunocompromised hosts.

Significant year-on-year fluctuations underscore the dynamic nature of respiratory pathogen interactions. The surge in *Haemophilus influenzae* in 2024 coincided with a notable decline in *Streptococcus pneumoniae*, suggesting competitive exclusion or shifts in serotype prevalence following pandemic disruptions. The dramatic emergence of *Streptococcus mitis* group in 2025 replacing earlier *Fusobacterium nucleatum* dominance is unprecedented and alarming. This α-hemolytic streptococcus, often dismissed as commensal, is increasingly associated with bacteremia and endocarditis in

vulnerable hosts.[37] Its abrupt dominance could reflect antigenic shifts in circulating hMPV strains altering mucosal adherence sites, widespread antibiotic selection pressure favoring low-virulence colonizers, or the introduction of a novel, highly transmissible lineage within the population – urgent areas for genomic surveillance. The early prominence of CMV and *Pneumocystis jirovecii* might represent pandemic-era "immunity debt" effects,[38] where reduced viral exposures temporarily increased susceptibility to opportunistic reactivation, subsequently normalizing.

The higher detection rate of *Streptococcus intermedius* among immunodeficient patients suggests an increased susceptibility to opportunistic pathogens in this population, which may reflect impaired mucosal immunity or altered oral-pulmonary microbial dynamics. The exclusive detection of *Stenotrophomonas maltophilia* in the immunodeficient group further supports this notion, as this organism is a well-recognized opportunistic pathogen frequently associated with immunocompromised hosts. Regarding vaccination status, the higher prevalence of *Fusobacterium nucleatum* in fully immunized individuals is unexpected and may reflect indirect ecological effects of vaccination on airway microbiota composition rather than a causal relationship. Conversely, the increased detection of *Candida albicans* and *Pneumocystis jirovecii* in non-fully immunized patients, though not statistically robust, suggests a trend toward greater fungal susceptibility in individuals with incomplete immunization. Together, these findings highlight the complex interplay between host immunity, vaccination, and respiratory microbial ecology.

A key finding of our study is the identification of independent associations between specific co-infecting pathogens and clinical severity in hMPV infection, some of which challenge conventional wisdom. Members of the Herpesviridae family (HHV-5, HSV-1) were consistently identified as strong risk factors for multiple severe outcomes,[39] aligning with the view that herpesvirus reactivation is a marker of critical illness, yet their high ORs suggest a potentially active pathogenic role in the context of hMPV infection. Similarly, *Klebsiella aerogenes* and *Tropheryma whipplei* strong risk factors for septic shock highlight the need to consider these atypical pathogens in hMPV-associated sepsis. Furthermore, the presence of several highly virulent agents as secondary co-detections significantly escalates disease severity. Although hMPV serves as the primary viral trigger, co-colonization with opportunistic and multidrug-resistant pathogens—such as *Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumannii*, and *Staphylococcus aureus*—poses profound clinical risks. Drawing parallels from recent viral pneumonia studies, such as those on SARS-CoV-2, the secondary invasion by these virulent bacteria and fungi (eg, *Candida* species) strongly correlates with fatal pneumonia, extensive tissue damage, and heightened systemic inflammatory responses.[40–46] This reinforces the necessity of early targeted detection of these specific high-risk agents to prevent rapid clinical deterioration in hMPV-infected children. The consistent protective association of *Fusobacterium nucleatum* is intriguing. Rather than implying a direct therapeutic benefit, this finding should be considered hypothesis-generating. It may reflect a less disrupted baseline microbiota or competition dynamics that limit the overgrowth of more virulent pathogens. Further functional studies and comparisons with matched hMPV-negative controls are required to determine if this represents a true protective effect or merely a marker of milder mucosal disruption. This contrasts with its known role as a pro-inflammatory agent in fields like colorectal cancer[47] but offers a novel perspective in respiratory infections. The identification of *Mycoplasma pneumoniae* as an independent risk factor for pneumonia was expected, but the protective role of RSV A was surprising, potentially related to viral interference. This complex network of associations demonstrates that the impact of co-colonizations on outcomes is highly pathogen-specific and cannot be simplistically categorized as "beneficial" or "detrimental".

Several limitations of this study should be acknowledged. First, its single-center, observational design means the patient cohort may not be fully representative of other regions or healthcare settings, limiting generalizability. Furthermore, we did not incorporate specific environmental, meteorological, or climate parameters into our analysis, which are known to significantly influence respiratory pathogen seasonality and co-infection dynamics. Also, tNGS detects pathogen nucleic acid but cannot reliably distinguish active infection, colonization, or residual DNA/RNA, potentially leading to misinterpretation of some associations. Second, tNGS detects pathogen nucleic acid but cannot reliably distinguish active infection, colonization, or residual DNA/RNA, potentially leading to misinterpretation of some associations. Third, despite multivariable adjustment, residual confounding from unmeasured or unknown factors (eg, detailed antibiotic exposure history, severity of underlying conditions) might influence the observed associations. Fourth, the sample size was relatively small for some subgroup analyses (eg, immunodeficient patients), potentially limiting

statistical power to detect significant differences and contributing to some extreme ORs with wide confidence intervals. Most critically, the retrospective design lacked an hMPV-negative control group. Consequently, we cannot definitively ascertain whether the observed microbial dynamics—particularly the counterintuitive protective association of *F. nucleatum*—are specific to the hMPV-altered airway microenvironment or merely reflect a generalizable phenomenon of less severe mucosal disruption. Furthermore, while multivariable logistic regression identified profound risk factors, the extremely wide confidence intervals for certain severe outcomes (eg, septic shock associated with *K. aerogenes*) are directly attributable to very small subgroup sizes. Therefore, the extreme adjusted odds ratios reported herein must be interpreted with strict caution and viewed as preliminary risk signals rather than definitive epidemiological estimates. Finally, tNGS detects nucleic acids without confirming viability, meaning some associations may reflect transient carriage rather than active pathogenic invasion. Finally, the observational nature precludes establishing causality, and the observed associations require functional studies to explore the underlying biological mechanisms.

Given these limitations, future research must pivot from broad descriptive profiling to highly focused investigations. First, large-scale, multi-center prospective cohorts are urgently required for external validation to overcome our sample size constraints and refine the precision of these extreme odds ratios. Second, prospective case-control studies incorporating hMPV-negative matched groups are essential to establish definitive causality between specific co-colonizers and clinical deterioration. Finally, experimental models should prioritize targeted mechanistic questions—specifically investigating how primary hMPV-induced epithelial damage and immune dysregulation structurally facilitate the secondary invasion of the high-risk opportunistic agents identified in this cohort.

Conclusion

In conclusion, moving beyond traditional syndromic surveillance, this tNGS-based study demonstrates that co-colonizing pathogens in pediatric hMPV infection exhibit specific patterns influenced by host factors and are independently associated with severe clinical outcomes. Importantly, the early identification of high-risk co-pathogens (such as CMV or *K. aerogenes*) via tNGS should trigger intensified clinical monitoring. These findings directly inform risk stratification and guide early, targeted therapeutic interventions to improve clinical outcomes in vulnerable pediatric populations. Moving forward, integrating such broad-spectrum molecular diagnostics into routine care, coupled with future prospective, multi-center studies, will be essential to translate these pathogen-specific profiles into actionable clinical guidelines.

Data Sharing Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding authors, Qiuyu Tang and Feng Cheng.

Ethics Approval and Informed Consent

Ethical approval was obtained from the Ethics Committee of Fujian Children's Hospital (No: 2025ETKLRK07013). Since this study was a retrospective observational study without any intervention measures, the Ethics Committee uniformly waived the requirement for informed consent. All patient data were kept strictly confidential, and the study was conducted in full compliance with the principles of the Declaration of Helsinki.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflict of interest.

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