

RESEARCH ARTICLE

Application of a Super-Multiplex Microfluidic qPCR System for Detection of Respiratory Pathogens in Patients With Influenza-Like Illness

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ABSTRACT

Respiratory tract infections (RTIs) pose a major diagnostic challenge, complicated by multiple microbial etiologies. Conventional low-throughput methods may underestimate the prevalence and impact of co-infections. This study aimed to evaluate a novel super-multiplex microfluidic qPCR system for comprehensive 49 target pathogens screening and co-infection analysis in patients with influenza-like illness (ILI). Throat swab samples ($n = 288$) were collected from febrile patients in Beijing during the spring of 2025. All samples were tested using the super-multiplex microfluidic qPCR chip, with a subset ($n = 96$) validated by conventional qPCR to determine analytical sensitivity, specificity, and Cohen's kappa. The assay demonstrated an overall pathogen detection rate of 66.32%. Influenza A virus (IAV) was the most prevalent pathogen, identified in 50.00% of all samples and constituting 70.69% of single infections, followed by SARS-CoV-2 (14.24%) and Epstein–Barr virus (EBV, 11.81%). Co-infections were frequent (39.27% of positive cases), predominantly dual (23.04%) and triple (10.47%) infections. Notably, IAV was implicated in 84.0% of co-infections. The microfluidic qPCR showed high concordance with conventional qPCR (sensitivity 91.07%, specificity 92.50%, $\kappa = 0.83$, indicating almost perfect agreement). This study reveals a complex respiratory pathogen landscape dominated by IAV and marked by frequent polymicrobial co-infections. The super-multiplex microfluidic qPCR chip is a reliable, high-throughput platform that effectively addresses the need for improved detection of co-infections, offering valuable insights for both clinical decision-making and public health surveillance.

Abbreviations: *A. baumannii*, *Acinetobacter baumannii*; *A. faecalis*, *Alcaligenes faecalis*; *B. pertussis*, *Bordetella pertussis*; *C. albicans*, *Candida albicans*; *C. diphtheriae*, *Corynebacterium diphtheriae*; *C. lusitaniae*, *Clavispora lusitaniae*; *C. pneumoniae*, *Chlamydia pneumoniae*; DENV, dengue virus; EBV, Epstein–Barr virus; EV, enterovirus; *E. cloacae* complex, *Enterobacter cloacae* complex; GAS, Group A *Streptococcus* (*Streptococcus pyogenes*); HAdV, human adenovirus; HBoV, human bocavirus; HC, healthy control; HCoV, human coronavirus; HCoV-HKU1, human coronavirus HKU1; HCoV-NL63, human coronavirus NL63; HCoV-OC43, human coronavirus OC43; HMPV, human metapneumovirus; HPBV, human picobirnavirus; HPIV, human parainfluenza virus; HPIV-1, human parainfluenza virus 1; HRV, human rhinovirus; HRV-A, human rhinovirus A; HSV-1, herpes simplex virus 1; *H. influenzae*, *Haemophilus influenzae*; IAV, influenza A virus; IBV, influenza B virus; JEV, Japanese encephalitis virus; *K. pneumoniae*, *Klebsiella pneumoniae*; *L. pneumophila*, *Legionella pneumophila*; MERS-CoV, Middle East Respiratory Syndrome Coronavirus; MeV, measles virus; MuV, mumps virus; *M. catarrhalis*, *Moraxella catarrhalis*; *M. pneumoniae*, *Mycoplasma pneumoniae*; *M. tuberculosis*, *Mycobacterium tuberculosis*; PeV, Parechovirus; *P. aeruginosa*, *Pseudomonas aeruginosa*; *P. jirovecii*, *Pneumocystis jirovecii*; *P. mirabilis*, *Proteus mirabilis*; RSV, respiratory syncytial virus; SARS-CoV, severe acute respiratory syndrome coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SFTSV, severe fever with thrombocytopenia syndrome virus; *S. aureus*, *Staphylococcus aureus*; *S. maltophilia*, *Stenotrophomonas maltophilia*; *S. pneumoniae*, *Streptococcus pneumoniae*; VACV, vaccinia virus; VZV, Varicella–Zoster virus; WUPyV, WU polyomavirus; ZIKV, Zika virus.

Yuan Huang and Yanhong Gao contributed equally to this study.

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1 | Research Background

Respiratory tract infections (RTIs) pose a major challenge for human health, which are caused by pathogens including viruses, bacteria, fungi, and atypical organisms. The viral pathogens include influenza virus, coronavirus, respiratory syncytial virus (RSV), and adenovirus (ADV). Bacterial pathogens include *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. Atypical pathogens include *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* [1]. These pathogens are primarily transmitted via droplets, aerosols, and contact. Clinical manifestations encompass fever, cough, nasal congestion, and fatigue, with severe cases potentially progressing to pneumonia or death [2].

RTIs rank among the leading causes of global morbidity and mortality from infectious diseases [3–7]. These infections pose a greater threat to children, the elderly, and immunocompromised individuals, and impose a substantial socioeconomic burden [5]. In recent years, the incidence of respiratory co-infections has shown an increasing trend. A large-scale study in China revealed that viral-bacterial (V–B) co-infections were the most common (11.40%), followed by viral-viral (V–V) (7.43%) and bacterial-bacterial (B–B) (3.63%) co-infections, with significantly higher rates observed in children and the elderly compared to adults [5]. Co-infections not only exacerbate disease severity, increase ICU admission rates and mortality, but also promote the development of antimicrobial resistance, thereby complicating treatment and elevating healthcare costs. However, systematic and in-depth research on respiratory pathogen co-infections remains limited, primarily due to the lack of simple, rapid technical methods and diagnostic reagents.

Targeted next-generation sequencing (tNGS) and metagenomic next-generation sequencing (mNGS) enable high-throughput detection of target pathogens and discovery of novel ones, but they require skilled personnel, which are costly, time-consuming, and are less suitable for rapid targeted screening of clinical samples, hindering their routine clinical application [8]. Conventional quantitative polymerase chain reaction (qPCR), recognized as the gold standard for respiratory pathogen diagnosis due to its high sensitivity and specificity [9–11], however, qPCR is typically limited to detecting one to three specific pathogens per reaction, making it impractical for rapid screening of dozens of respiratory pathogens. Although qPCR coupled with melting curve analysis can detect six to nine pathogens in a single reaction, the analysis is complex, and the amplification process is lengthy [12, 13]. Conventional diagnostic strategies, both low-plex qPCR and NGS, often fail to fully capture the spectrum of co-circulating pathogens, which may lead to an underestimation of the prevalence and clinical impact of mixed infections.

Microfluidic chip-based qPCR technology represents a high-throughput nucleic acid detection method capable of screening dozens of pathogen targets in a single reaction. This technology demonstrates advantages in speed and efficiency, showing particular promise for screening co-infections. However, the currently reported microfluidic qPCR platforms for respiratory pathogen detection are mostly limited in their multiplexing

capacity, typically targeting fewer than 21 pathogens [14–19]. While these systems offer improvements in automation and speed, their restricted target coverage may overlook less common or emerging pathogens, and are insufficient for comprehensively mapping complex co-infection patterns, especially in regions with diverse pathogen circulation. Consequently, there remains a significant unmet need for a higher-plex detection platform that can provide a more panoramic view of the respiratory pathogen landscape.

This study employs a novel high-throughput microfluidic qPCR chip capable of simultaneously detecting 50 target genes associated with respiratory pathogens, namely a super-multiplex microfluidic qPCR chip, which significantly expands the detectable spectrum compared to existing systems. We aim to perform comprehensive pathogen detection and identification of the suspected respiratory infection cases during the spring of 2025, to investigate the associated pathogen spectrum and co-infection status. Additionally, we will evaluate the detection accuracy of this super-multiplex microfluidic qPCR chip method. The findings are expected to provide a new technological tool with enhanced coverage and epidemiological insights for the precise prevention and control of respiratory infections.

2 | Materials and Methods

2.1 | Sample Description and Source

This study collected 288 throat swab samples from febrile patients between February and March 2025 at The First Medical Center of the Chinese PLA General Hospital (Beijing). The cohort comprised 167 males and 121 females. Age distribution was as follows: infants and preschool children (0–5 years), 9 cases; school-age children and adolescents (6–17 years), 23 cases; adults (18–59 years), 161 cases; and elderly individuals (≥ 60 years), 95 cases. All 288 throat swab samples were aliquoted into sterile cryovials and stored at -80°C for 12 weeks (from collection to extraction), and underwent only a single freeze-thaw cycle before nucleic acid extraction. Subsequent nucleic acid extraction was performed on all samples using the MagaPlus Virus DNA/RNA Purification Kit III.

2.2 | Experimental Design

To achieve efficient and accurate screening and identification of respiratory pathogens, the detection strategy for the collected 288 throat swab samples from patients with respiratory infections was as follows: First, nucleic acids were extracted from all of the samples, then 10 samples were mixed in one pool to construct 29 mixed pools. High-throughput sequencing technology was employed for preliminary screening of these pooled samples to determine the spectrum of infecting pathogens and to verify whether all detected positive respiratory pathogens were covered by the targets of the microfluidic chip.

Subsequently, all individual samples underwent 50-plex respiratory pathogen microfluidic chip detection to precisely identify

the pathogen(s) present in each sample. This was followed by analysis of the pathogen spectrum and co-infection for the febrile cases. In parallel, a subset of samples was tested and identified using commercially available conventional qPCR reagents. The results from the microfluidic chip detection were compared with those from qPCR for consistency (Kappa analysis) to evaluate the performance of the microfluidic qPCR technology for rapid screening of respiratory infection pathogen spectra (Figure 1).

2.3 | Nucleic Acid Extraction

Viral nucleic acid extraction in this study was performed using the MagaPlus Virus DNA/RNA Purification Kit III (China, Hangzhou Bioer Technology Co. Ltd., Catalog No. BSC86T1E/BSC8651E) according to the manufacturer's instructions. Briefly, the reagent plate was equilibrated to room temperature, inverted to mix, centrifuged to remove droplets from the wells, and the aluminum foil seal was peeled off while confirming the position of the magnetic beads. Subsequently, 200 μ L of each sample was added to the corresponding well of a 96-well nucleic acid extraction plate. The plate was then placed into a nucleic acid extraction and purification instrument, the magnetic rod sleeve was installed, and the automated protocol was initiated. The process included viral lysis (80°C), binding of nucleic acids to magnetic beads, three wash steps, and elution (80°C). Finally, the eluate (50 μ L) was collected into nuclease-free microcentrifuge tubes. The extracted nucleic acids were either used immediately in downstream assays or stored at -80°C for future use.

2.4 | NGS

The high-throughput sequencing workflow employed in this study was as follows: ribosomal RNA was depleted using the Hongweitesi Pathogen Enrichment rRNA Depletion Kit (China, HWKF-UAN036). The RNA was fragmented to approximately 280 bp, and then reverse-transcribed into cDNA using the miScript II RT Kit (Cat. No. 218161, QIAGEN). Library construction was carried out using the Hongweitesi Pathogen Metagenomic Detection Kit (RNA) (HWKF-MN0012), involving steps such as cDNA end repair, adapter ligation, and PCR amplification. The concentration of the constructed libraries was measured using a Qubit fluorometric quantification system (Thermo Fisher Scientific), and the library fragment size distribution was assessed using a Qseq. 100 system (Hangzhou Houze Biotechnology Co. Ltd.). Libraries meeting the expected fragment size criteria were accurately quantified using the KAPA Library Quantification Kit (Roche). Qualified libraries were sequenced on a Salus Pro sequencing platform (Sai Lu) using a paired-end 150 bp (PE150) strategy.

Raw sequencing data underwent quality control using fastp (v0.23.1) to remove adapter sequences and low-quality reads, resulting in high-quality clean data. Clean data were aligned to the human reference genome using HISAT2 (v2.1.0), and sequences aligning to the host genome were removed. The de-hosted data were then subjected to taxonomic classification using Kraken2 (v2.0.9-beta) against the RefSeq database, and the relative abundance of species was calculated using Bracken. For sequences related to viruses, de novo assembly was

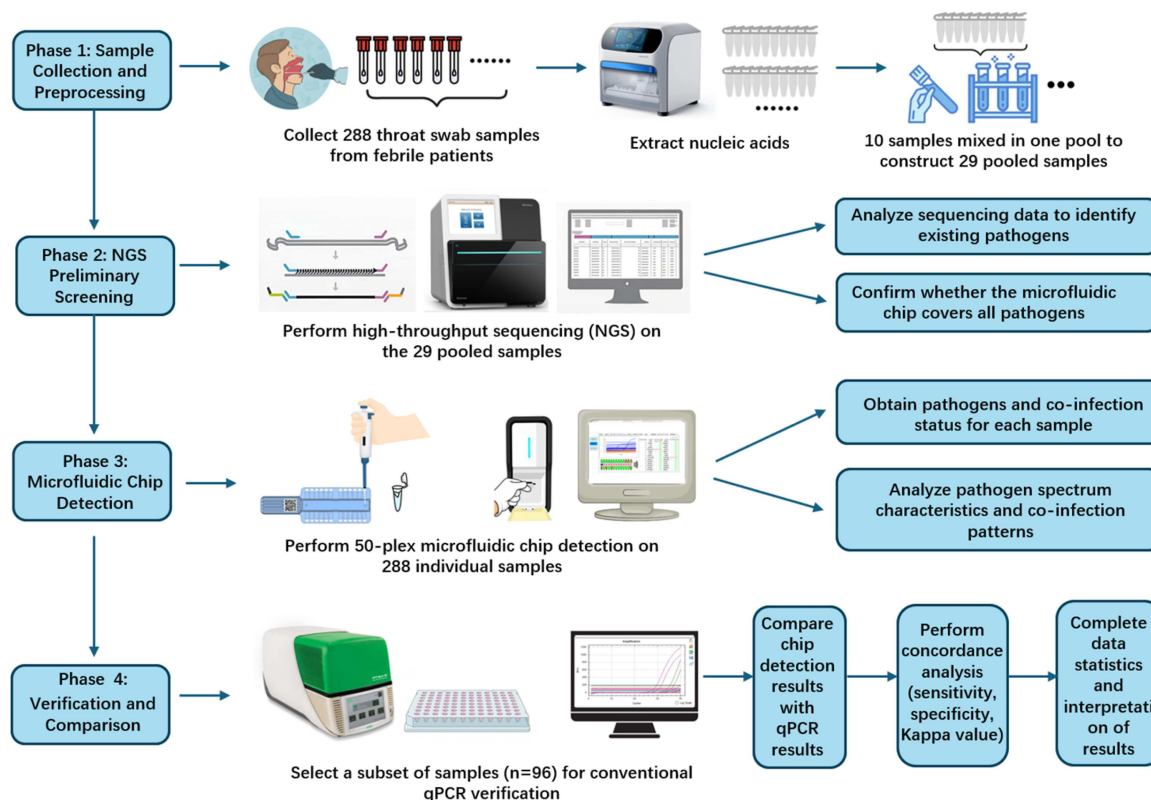


FIGURE 1 | Overview of the study workflow. A total of 288 throat swab samples were collected, pooled into 29 groups for NGS screening, and then individually tested using a 50-plex microfluidic qPCR chip. Results were compared with conventional qPCR for validation.

performed using MEGAHIT (v1.2.9) to generate contigs. Contigs were aligned to the NT database using BLAST (v2.10.1+) for taxonomic confirmation. Based on the BLAST results, a specific species reference genome was selected. The original clean reads were then mapped back to this reference genome using BWA (v0.7.17-r1188) with a matching threshold of ≥ 50 bp. Using these mapped reads as input, assembly was performed again using SPAdes (v3.15.3) to generate scaffolds. Reads were once more mapped back to the assembled scaffolds using BWA, and the assembly results were corrected based on sequencing depth to ultimately obtain complete sequences.

2.5 | Microfluidic qPCR Detection

Microfluidic chip-based qPCR is a high-throughput nucleic acid detection technology. The chip is designed with a sample loading well and dozens of reaction wells, interconnected via microfluidic channels. Primers, probes, and the necessary enzymatic mix for different target pathogens are pre-dried in their respective reaction wells, with each well constituting an independent qPCR detection system. For virus detection, the nucleic acid sample is injected into the loading well and sequentially flows through the microfluidic channels into each reaction well, enabling the screening of dozens of pathogen targets in a single run.

The microfluidic qPCR chip used in this study targets 50 important respiratory pathogen genes (for the list of target pathogens, see Table 2). It was manufactured by Tianjin Zhishan Biotechnology Co. Ltd. Among the primers and probes for the 50 targets, 38 were based on sequences reported in the literature [20–31] and were synthesized by TopBioligand Biotechnology (Shanghai) Co. Ltd. (Table S1); the remaining 12 were designed and provided by Tianjin Zhishan Biotechnology Co. Ltd. (Product ID: 100041-50), and their sequences are not disclosed due to commercial confidentiality. During chip fabrication, primers and probes for the 50 pathogens, along with the PCR enzyme mix, were precisely dispensed in pairwise combinations into 26 independent reaction chambers (each with a volume of 6.5 μ L). This was followed by sealing, film lamination, and low-temperature drying. The manufactured chips can be stored stably at room temperature for up to 6 months. A schematic diagram of the microfluidic chip detection principle is shown in Figure 2.

For detection, 200 μ L of the nucleic acid sample was added to the chip's loading well. After sealing, the chip was shaken for 2 min using a compatible ultrasonic mixer for the reaction mixed. Subsequently, the chip was placed in a chip-based PCR instrument for detection. The thermal cycling protocol was as follows: reverse transcription at 50°C for 10 min, initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s

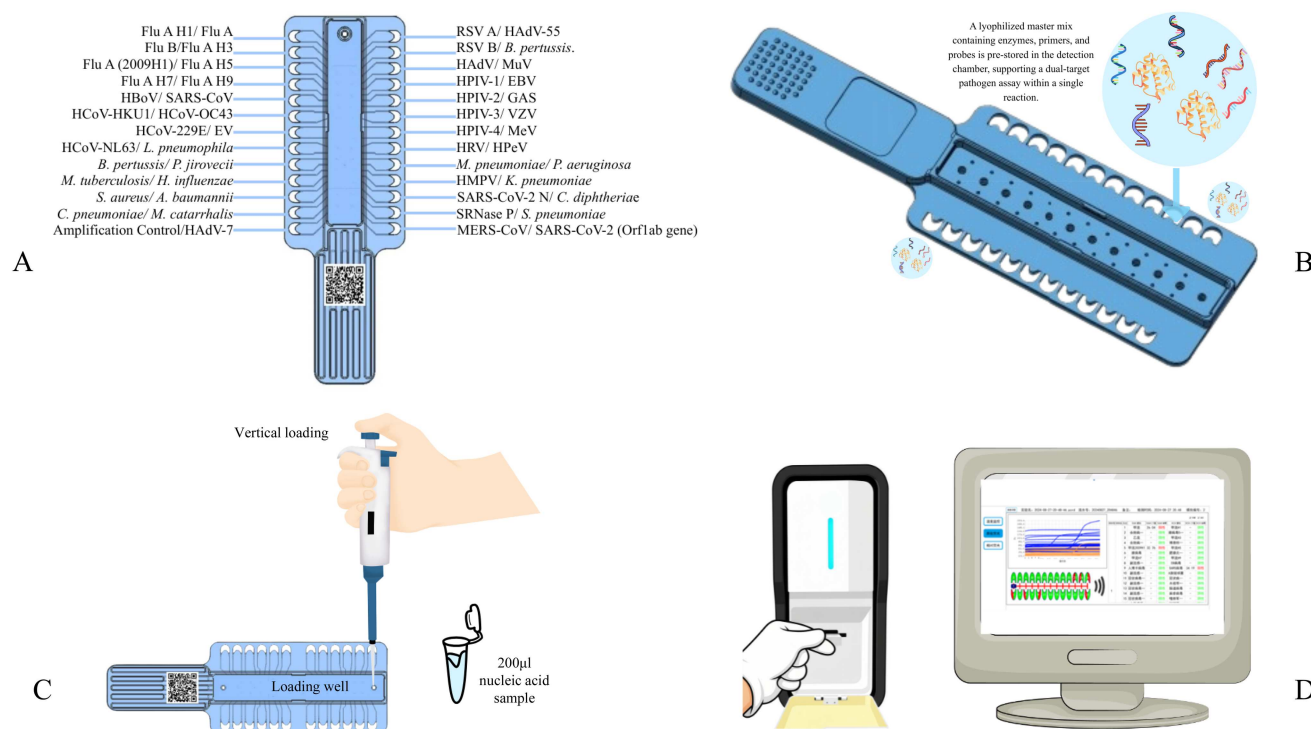


FIGURE 2 | Schematic of the 50-plex microfluidic qPCR chip for respiratory pathogen detection. (A) Layout of the 26 independent reaction wells pre-loaded with lyophilized primers, probes, and enzyme master mix for detecting 50 respiratory pathogen targets. Representative target abbreviations are labeled (e.g., Ph.A.H1/Ph.A: influenza A virus H1 and pan-influenza A; RSV A/HAdV: respiratory syncytial virus type A and human adenovirus; SARS-CoV-2N/SARS-CoV-2 Orf1ab: SARS-CoV-2 nucleocapsid and Orf1ab genes; MERS-CoV/HAdV-7: Middle East respiratory syndrome coronavirus and adenovirus type 7). (B–D) Workflow of chip-based detection. (B) A lyophilized master mix containing enzymes, primers, and probes is pre-dried in the detection chamber, supporting a dual-target pathogen assay within a single reaction; (C) 200 μ L of extracted nucleic acid is loaded into the sample inlet, and the sample is distributed through microfluidic channels into each reaction well; and (D) simultaneous multiplex qPCR amplification is performed in a chip-compatible thermal cycler. The chip enables one-step, high-throughput screening of 50 pathogen targets in a single run.

and 60°C for 30 s, with FAM/ROX fluorescence signals acquired during the 60°C step.

For detection, the entire 50 µL of eluted nucleic acid was pre-mixed with 150 µL of nuclease-free water to obtain a total volume of 200 µL, and the entire mixture was then loaded into the chip's loading well in a single step (the minimum loading volume required by the chip is 200 µL to ensure proper distribution through the microfluidic channels). After sealing, the chip was shaken for 2 min using a compatible ultrasonic mixer to mix the reaction components. Subsequently, the chip was placed in a chip-based PCR instrument for detection. The thermal cycling protocol was as follows: reverse transcription at 50°C for 10 min, initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s, with FAM/ROX fluorescence signals acquired during the 60°C step.

For result interpretation, a human-derived sample was considered positive only if the internal control Ct value was ≤ 35 and the target Ct value was ≤ 37 . For nonhuman derived samples, positivity was determined based solely on the target Ct value.

2.6 | qPCR Detection

For a subset of the nucleic acid samples, we performed detection using commercial qPCR detection kits to compare the result consistency with the microfluidic chip assay. The reagents, purchased from Beijing Maixiwei Biotechnology Co. Ltd., included: a 14-Plex Respiratory Pathogen Nucleic Acid Detection Kit (covering SARS-CoV-2, Influenza A/B viruses, RSV, Adenovirus, Human Metapneumovirus, Rhinovirus, Parainfluenza Virus, Bocavirus, Enterovirus, Coronavirus, *M. pneumoniae*, *C. pneumoniae*, and *S. pneumoniae*); a 5-Plex Respiratory Pathogenic Bacteria Nucleic Acid Detection Kit (including *H. influenzae*, *S. pneumoniae*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*); an Epstein-Barr Virus Nucleic Acid Detection Kit (Fluorescence PCR); and a *M. catarrhalis* Nucleic Acid Detection Kit (Fluorescence PCR).

The reaction mixture preparation and amplification protocols were performed strictly according to the manufacturer's instructions. Amplification was carried out using a Bio-Rad CFX Opus 96 Real-Time PCR System. Detection results were interpreted based on the amplification curves and Ct values. A sample was judged positive for a specific pathogen if the corresponding Ct value was < 37 and a distinct amplification curve was observed.

2.7 | Statistical Analysis

All statistical analyses in this study were performed using SPSS software (version 27.0; IBM Corp., Armonk, NY, USA). Categorical data are presented as numbers (*n*) and percentages (%). First, descriptive statistical methods were used to analyze the detection distribution of each pathogen. To evaluate the diagnostic performance of the 50-plex microfluidic qPCR chip, we calculated its overall sensitivity and specificity, using the results from the traditional single-target qPCR as the reference

standard. The agreement between the results of the two methods was assessed using the Kappa test. The interpretation of Kappa values was as follows: ≤ 0.20 indicated "slight agreement"; 0.21–0.40, "fair agreement"; 0.41–0.60, "moderate agreement"; 0.61–0.80, "substantial agreement"; and > 0.81 , "almost perfect agreement". All statistical tests were two-sided, and a *p* value < 0.05 was considered statistically significant. Finally, the degree of agreement and its clinical relevance were comprehensively judged by combining the agreement coefficient with clinical practice.

2.8 | Ethics Approval and Participant Consent

The study protocol and data collection methods adhered to standard operating procedures and were approved by the Ethics Committee of the Academy of Military Medical Sciences (Approval No. AF/SC-08/02.461). Informed consent was obtained from all participants. As for the child participants, verbal consent was obtained from the parent/guardian.

3 | Results

3.1 | Pathogen Spectrum Identification

3.1.1 | NGS Identified 14 Respiratory Pathogens in the Samples

For the collected clinical samples, nucleic acids were pooled by every 10 samples into one group, resulting in the construction of 29 pooled samples for deep sequencing. The sequencing results identified a total of 24 distinct pathogens, comprising 13 viruses, 9 bacteria, and 2 fungi (Table 1).

Among them, 10 pathogens are not considered typical respiratory infection agents: Severe fever with thrombocytopenia syndrome virus, which is a tick-borne pathogen primarily causing systemic infection with hemorrhagic tendencies; Human picobirnavirus and WU polyomavirus; the fungi *Clavispora lusitaniae*; Herpes simplex virus type 1; *Stenotrophomonas maltophilia*; *Alcaligenes faecalis*; *Candida albicans*; *Enterobacter cloacae* complex; and *Proteus mirabilis*. These are mostly opportunistic pathogens with a weak etiological association with acute respiratory infections.

Consequently, the preliminary NGS identified 14 pathogens relevant to respiratory infections, including 9 viruses and 5 bacteria (Table 1). Upon comparison with the target pathogen list of the multiplex microfluidic chip, it was confirmed that the 50-plex respiratory pathogen microfluidic chip covers all 14 of these positively identified pathogens, enabling subsequent sample detection and identification.

3.1.2 | The Microfluidic qPCR Assay Enables Sensitive Detection of 50 Target Pathogens

The multiplex chip is pre-loaded with amplification primers and probes for 50 target genes. Among these, SARS-CoV-2 corresponds to two sets of primers and probes, while each of the other targets is represented by one set, enabling the detection of 49 species/types/subtypes of respiratory pathogens, encompassing 35 viruses and 14 bacteria. Notably, the assay not only

TABLE 1 | Pathogens identified by next-generation sequencing (NGS) in 29 pooled respiratory samples.

Pathogen	Reads	p/29	Catalog
SARS-CoV-2	36 027	4	Virus
IAV	6307	10	Virus
HRV-A	839	2	Virus
HCoV-HKU1	415	1	Virus
HPIV-1	405	2	Virus
RSV	309	4	Virus
EBV	167	5	Virus
HCoV-NL63	105	2	Virus
HCoV-OC43	42	1	Virus
<i>P. aeruginosa</i>	56 813	3	Bacteria
<i>A. baumannii</i>	31 281	4	Bacteria
<i>H. influenzae</i>	10 797	5	Bacteria
<i>K. pneumoniae</i>	2711	2	Bacteria
<i>M. catarrhalis</i>	1813	2	Bacteria
*HSV-1	11 690	2	Virus
*HPBV	100	2	Virus
*WUPyV	84	1	Virus
*Bunyavirus	55	1	Virus
* <i>A. faecalis</i>	36 924	1	Bacteria
* <i>S. maltophilia</i>	7043	3	Bacteria
* <i>P. mirabilis</i>	1445	2	Bacteria
* <i>E. cloacae</i> complex	1373	2	Bacteria
* <i>C. albicans</i>	184	6	Fungi
* <i>C. lusitaniae</i>	57	1	Fungi

Note: NGS was performed on 29 pooled samples (10 samples per pool). Reads indicate the total number of sequencing reads mapped to the pathogen. *p/29* represents the number of pooled samples in which the pathogen was detected out of 29 pools. Asterisks (*) denote pathogens not typically associated with acute respiratory tract infections.

Abbreviations: *A. baumannii*, *Acinetobacter baumannii*; *A. faecalis*, *Alcaligenes faecalis*; *C. albicans*, *Candida albicans*; *C. lusitaniae*, *Clavispora lusitaniae*; *E. cloacae* complex, *Enterobacter cloacae* complex; EBV, Epstein-Barr virus; *H. influenzae*, *Haemophilus influenzae*; HCoV-HKU1, human coronavirus HKU1; HCoV-NL63, human coronavirus NL63; HCoV-OC43, human coronavirus OC43; HPBV, human picobirnavirus; HPIV-1, human parainfluenza virus 1; HRV-A, human rhinovirus A; HSV-1, herpes simplex virus 1; IAV, influenza A virus; *K. pneumoniae*, *Klebsiella pneumoniae*; *M. catarrhalis*, *Moraxella catarrhalis*; *P. aeruginosa*, *Pseudomonas aeruginosa*; *P. mirabilis*, *Proteus mirabilis*; RSV, respiratory syncytial virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; *S. maltophilia*, *Stenotrophomonas maltophilia*; WUPyV, WU polyomavirus.

identifies IAV but also allows for subtype discrimination (H1N1, 2009 H1, H3N2, H5, H7N9, and H9). It also provides typing for Adenovirus (types 7 and 55), Parainfluenza virus (types 1–4), RSV (types A and B), and Coronaviruses (HKU1, OC43, 229E, and NL63).

To determine the analytical sensitivity of the microfluidic chip-based qPCR assay for all covered pathogens, we first performed testing using quantified nucleic acids from viral cultures or recombinant plasmids containing the target genes. The results, as shown in Table 2, demonstrated excellent analytical sensitivity for all 49 pathogens (50 target genes). The limit of detection (LoD) for most viruses fell within the range of 10^2 to

10^4 copies/mL (Table 2), indicating the chip's capability to meet clinical screening requirements. Specifically, the LoDs for several targets, including Human bocavirus, coronaviruses NL63/HKU1, and SARS virus, reached 3.0×10^3 copies/mL. For common clinical bacterial pathogens, the LoDs were 2.0×10^3 copies/mL for *A. baumannii* and 3.9×10^3 copies/mL for *K. pneumoniae*. The assay also maintained good detection capability for fastidious bacteria such as *Bordetella pertussis* (7.4×10^2 copies/mL) and *Staphylococcus aureus* (5.3×10^2 copies/mL). The LoDs for IAV subtypes H1N1, H3N2, and H7N9 ranged between 4.9×10^3 and 9.2×10^3 copies/mL.

To thoroughly evaluate specificity, nucleic acids from nine target pathogens, five unrelated pathogens, and two healthy control (HC) throat swabs were tested using the microfluidic qPCR assay. The unrelated pathogens included severe fever with thrombocytopenia syndrome virus (SFTSV), dengue virus (DENV), Japanese encephalitis virus (JEV), Zika virus (ZIKV), and vaccinia virus (VACV). As shown in Figure 3, each target pathogen nucleic acid produced specific amplification signals exclusively in its corresponding detection channel, with no nonspecific amplification curves observed in the remaining 49 channels. All results for the five unrelated pathogens and the two HC samples were negative, demonstrating no cross-reactivity. This confirms the absence of cross-reactivity among the detection wells in the multiplex chip-based qPCR assay, indicating its high specificity and suitability for the accurate identification of multiple pathogens in clinical samples.

3.1.3 | Microfluidic qPCR Detection Identified IAV, SARS-CoV-2, and EB Virus as the Predominant Pathogens in the Current Respiratory Infections

The results from the multiplex chip qPCR assay on 288 throat swab samples from febrile patients showed that, among the tested samples, 191 were positive for respiratory pathogens and 97 were negative, yielding a positivity rate of 66.32%. A total of 19 different pathogens were detected by the microfluidic chip. Besides encompassing all 14 respiratory infection-related target pathogens identified by the initial NGS screening, the chip additionally detected five other pathogens: Adenovirus (5.56%), *S. pneumoniae* (3.47%), *M. pneumoniae* (0.35%), Parainfluenza virus type II (0.35%), and Parainfluenza virus type III (0.35%). This discrepancy is primarily attributed to the distinct detection characteristics of the two technologies: mNGS, as an untargeted screening method, has its detection capability constrained by sequencing depth and high host background noise, making it prone to missing low-load pathogens. In contrast, targeted microfluidic qPCR, leveraging its specific amplification power, offers higher sensitivity for low-abundance nucleic acids.

Among the positive pathogens, IAV had the highest infection rate, with a positive detection rate of 50.00%, constituting the absolute majority of infections. The detected subtypes included 2009 H1 and H3. SARS-CoV-2 and Epstein-Barr virus (EBV) ranked second and third, with detection rates of 14.24% and 11.81%, respectively, together forming the main causative pathogens of the current respiratory infections (Table 3, Figure 4A).

TABLE 2 | Target pathogens and limits of detection (LoDs) for the 50-plex microfluidic qPCR chip.

No.	Position (well, channel)	Target pathogen (abbreviation)	LoD (copies/mL)
1	Well 1, FAM	IAV	6.6×10^3
2	Well 1, ROX	IAV (H1N1)	4.9×10^3
3	Well 2, FAM	RSV-A	3.16×10^3
4	Well 2, ROX	HAdV-55	3.0×10^3
5	Well 3, FAM	IBV	4.9×10^3
6	Well 3, ROX	IAV (H3N2)	7.0×10^3
7	Well 4, FAM	RSV-B	6.8×10^3
8	Well 4, ROX	<i>B. pertussis</i>	3.0×10^3
9	Well 5, FAM	IAV (2009 H1N1)	3.0×10^3
10	Well 5, ROX	IAV (H5)	3.0×10^3
11	Well 6, FAM	HAdV	9.0×10^3
12	Well 6, ROX	MuV	3.0×10^3
13	Well 7, FAM	IAV (H7N9)	9.2×10^3
14	Well 7, ROX	IAV (H9)	3.0×10^3
15	Well 8, FAM	HPIV-1	2.69×10^3
16	Well 8, ROX	EBV	3.0×10^3
17	Well 9, FAM	HBoV	3.0×10^3
18	Well 9, FAM	SARS-CoV	3.0×10^3
19	Well 10, FAM	HPIV-2	1.0×10^3
20	Well 10, ROX	GAS	3.4×10^3
21	Well 11, FAM	HCoV-HKU1	3.0×10^3
22	Well 11, ROX	HCoV-OC43	3.7×10^3
23	Well 12, FAM	HPIV-3	1.78×10^2
24	Well 12, ROX	VZV	3.0×10^3
25	Well 13, FAM	HCoV-229E	9.8×10^3
26	Well 13, ROX	EV	3.0×10^3
27	Well 14, FAM	HPIV-4	7.3×10^3
28	Well 14, ROX	MeV	6.8×10^3
29	Well 15, FAM	HCoV-NL63	3.0×10^3
30	Well 15, ROX	<i>L. pneumophila</i>	3.0×10^3
31	Well 16, FAM	HRV	2.5×10^2
32	Well 16, ROX	PeV	3.0×10^3
33	Well 17, FAM	<i>B. pertussis</i>	7.4×10^2
34	Well 17, ROX	<i>P. jirovecii</i>	3.0×10^3
35	Well 18, FAM	<i>M. pneumoniae</i>	1.0×10^2
36	Well 18, ROX	<i>P. aeruginosa</i>	6.5×10^3
37	Well 19, FAM	<i>M. tuberculosis</i>	3.0×10^3
38	Well 19, ROX	<i>H. influenzae</i>	3.0×10^3
39	Well 20, FAM	HMPV	4.2×10^3
40	Well 20, ROX	<i>K. pneumoniae</i>	3.9×10^3
41	Well 21, FAM	<i>S. aureus</i>	5.3×10^2
42	Well 21, ROX	<i>A. baumannii</i>	2.0×10^3
43	Well 22, FAM	SARS-CoV-2 (N gene)	5.0×10^3
44	Well 22, ROX	<i>C. diphtheriae</i>	3.0×10^3
45	Well 23, FAM	<i>C. pneumoniae</i>	3.0×10^3
46	Well 23, ROX	<i>M. catarrhalis</i>	3.0×10^3

(Continues)

TABLE 2 | (Continued)

No.	Position (well, channel)	Target pathogen (abbreviation)	LoD (copies/mL)
47	Well 24, ROX	<i>S. pneumoniae</i>	3.0×10^3
48	Well 25, ROX	HAdV-7	9.0×10^3
49	Well 26, FAM	MERS-CoV	3.1×10^3
50	Well 26, ROX	SARS-CoV-2 (Orf1ab gene)	5.0×10^3

Note: LOD is expressed as copies/mL. Each target was tested using quantified viral nucleic acids or recombinant plasmids.

Abbreviations: *A. baumannii*, *Acinetobacter baumannii*; *B. pertussis*, *Bordetella pertussis*; *C. diphtheriae*, *Corynebacterium diphtheriae*; *C. pneumoniae*, *Chlamydia pneumoniae*; EBV, Epstein-Barr virus; EV, enterovirus; GAS, Group A *Streptococcus* (*Streptococcus pyogenes*); *H. influenzae*, *Haemophilus influenzae*; HAdV, human adenovirus; HBoV, human bocavirus; HCoV, human coronavirus; HMPV, human metapneumovirus; HPIV, human parainfluenza virus; HRV, human rhinovirus; IAV, influenza A virus; IBV, influenza B virus; *K. pneumoniae*, *Klebsiella pneumoniae*; *L. pneumophila*, *Legionella pneumophila*; MeV, measles virus; MERS-CoV, Middle East respiratory syndrome coronavirus; *M. catarrhalis*, *Moraxella catarrhalis*; *M. pneumoniae*, *Mycoplasma pneumoniae*; *M. tuberculosis*, *Mycobacterium tuberculosis*; MuV, mumps virus; *P. aeruginosa*, *Pseudomonas aeruginosa*; PeV, parechovirus; *P. jirovecii*, *Pneumocystis jirovecii*; RSV, respiratory syncytial virus; SARS-CoV, severe acute respiratory syndrome coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; *S. aureus*, *Staphylococcus aureus*; *S. pneumoniae*, *Streptococcus pneumoniae*; VZV, varicella-zoster virus.

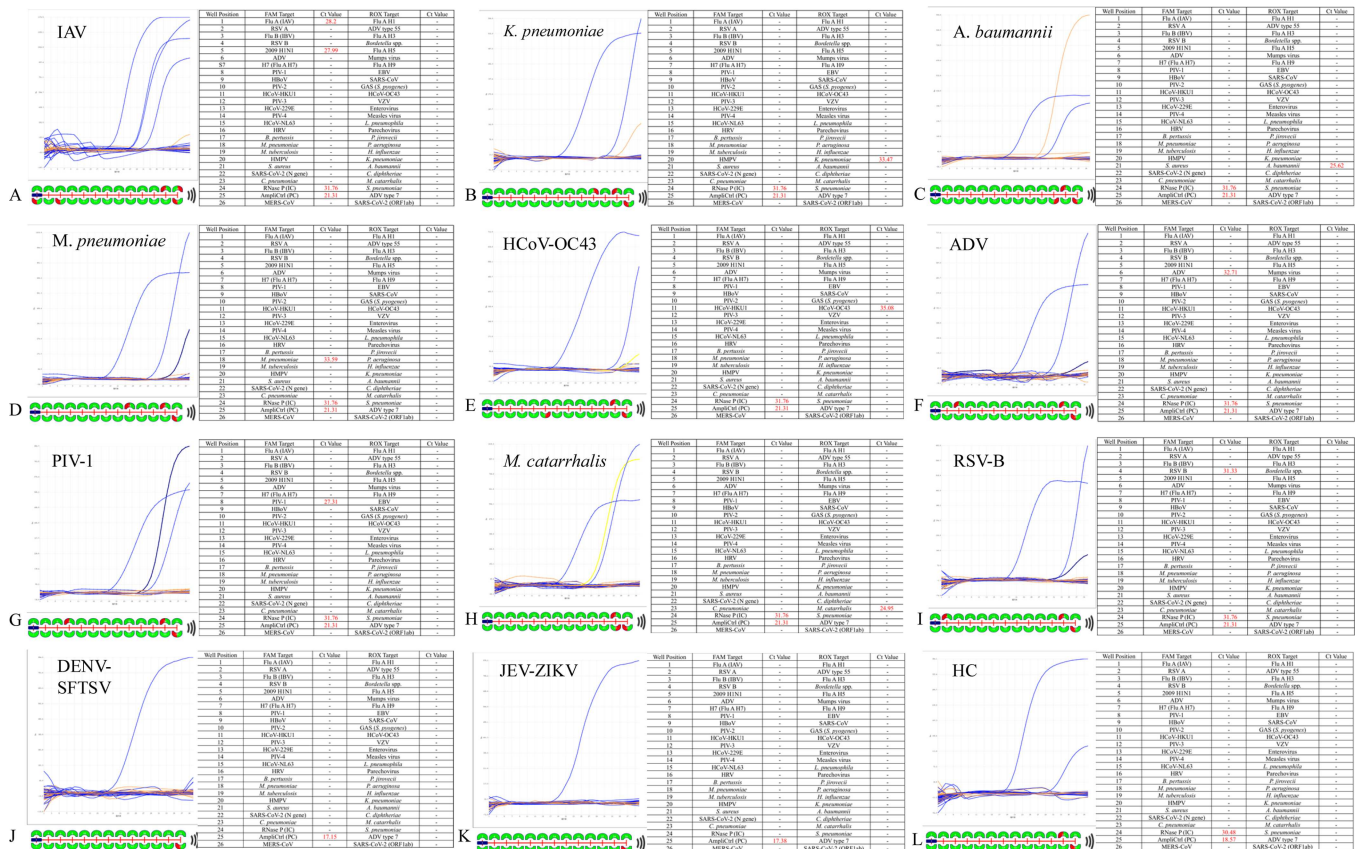


FIGURE 3 | Specificity validation of the 50-plex microfluidic respiratory chip. The chip was challenged with a panel of respiratory pathogens, non-respiratory viruses (severe fever with thrombocytopenia syndrome virus, SFTSV; dengue virus, DENV; Japanese encephalitis virus, JEV; and Zika virus, ZIKV), and a healthy control (HC) to assess detection specificity. Signal intensities (in Arbitrary Units, AU) for each target are shown, with data representing the mean $\pm$ standard deviation from three independent experiments. Signals from all non-target organisms, including SFTSV, DENV, JEV, ZIKV, and the HC sample, remained at baseline levels (~ 0.0000 AU), demonstrating negligible cross-reactivity. The chip maintains high specificity and low background noise in a multiplexed format, confirming its robust performance for reliable respiratory pathogen identification.

The detection rates of all other pathogens were below 6%. Among these, Adenovirus (5.90%), *A. baumannii* (3.82%), and *S. pneumoniae* (3.47%) were relatively common. Detection rates for RSV, *P. aeruginosa*, *H. influenzae*, and *M. catarrhalis* ranged between 1.74% and 3.47%. These results delineate the current epidemiological landscape of respiratory pathogens, revealing a complex spectrum characterized by the dominance of IAV alongside the co-existence of multiple viruses and bacteria.

3.2 | Co-Infection Analysis

Among the 191 febrile patients positive for respiratory pathogens, 116 cases (60.73%) were single-pathogen infections, while 75 cases (39.27%) were mixed-pathogen co-infections. The infection patterns among the 75 co-infection samples were predominantly dual and triple infections. Specifically, dual infections accounted for 44 cases, representing 23.04% (44/191)

TABLE 3 | Frequency of target pathogens detected by microfluidic qPCR in 288 clinical samples.

Pathogen	Count	Percentage
IAV	144	50.00%
SARS-CoV-2	41	14.24%
EBV	34	11.81%
AdV	16	5.56%
<i>A. baumannii</i>	11	3.82%
<i>K. pneumoniae</i>	10	3.47%
<i>S. pneumoniae</i>	9	3.13%
RSV	8	2.78%
<i>P. aeruginosa</i>	8	2.78%
<i>H. influenzae</i>	7	2.43%
<i>M. catarrhalis</i>	5	1.74%
PIV-I	4	1.39%
RV	3	1.04%
HCoV-NL63	2	0.69%
HCoV-OC43	2	0.69%
PIV-II	1	0.35%
PIV-III	1	0.35%
HCoV-229E	1	0.35%
<i>M. pneumoniae</i>	1	0.35%
Negative	97	33.68%

Note: Data are presented as the number (Count) and percentage of samples positive for each pathogen out of 288 total clinical samples tested. Percentages are rounded to two decimal places. Neg indicates samples in which no target pathogen was detected.

Abbreviations: *A. baumannii*, *Acinetobacter baumannii*; EBV, Epstein-Barr virus; *H. influenzae*, *Haemophilus influenzae*; HAdV, human adenovirus; HCoV, human coronavirus (includes subtypes OC43, NL63, and 229E); HPIV, human parainfluenza virus; HRV, human rhinovirus; IAV, influenza A virus; *K. pneumoniae*, *Klebsiella pneumoniae*; *M. catarrhalis*, *Moraxella catarrhalis*; *M. pneumoniae*, *Mycoplasma pneumoniae*; *P. aeruginosa*, *Pseudomonas aeruginosa*; RSV, respiratory syncytial virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; *S. pneumoniae*, *Streptococcus pneumoniae*.

of all positive cases and 58.67% (44/75) of co-infections. Triple infections accounted for 20 cases (10.47% of positives, 26.67% of co-infections). Quadruple infections accounted for 11 cases (5.76% of positives, 14.67% of co-infections) (Figure 4B).

Sixteen pathogens were identified in single-pathogen infections: IAV, EBV, SARS-CoV-2, *K. pneumoniae*, *S. pneumoniae*, *H. influenzae*, *P. aeruginosa*, *M. catarrhalis*, Parainfluenza virus types I, II, and III, RSV, Coronavirus OC43, Adenovirus, *M. pneumoniae*, and *A. baumannii*. Among these, IAV (subtypes 2009 H1 and H3), EBV, and SARS-CoV-2 remained the top three pathogens. IAV constituted 70.69% (82/116) of single infections, followed by EBV at 6.90% (8/116) and SARS-CoV-2 at 4.31% (5/116) (Figure 4C).

Pathogens involved in co-infections encompassed 16 of the positive pathogens detected in this study (excluding *M. pneumoniae* and Parainfluenza virus types II and III), indicating the complexity of pathogen co-circulation. Although the combinations of co-infecting pathogens were diverse (e.g., IAV with SARS-CoV-2; IAV with Adenovirus; EBV with *K. pneumoniae* and *A. baumannii*), IAV was present in a striking 84.0% (63/75)

of co-infection samples, spanning all dual infections and some triple and quadruple infections. The pathogens co-infecting with IAV were diverse, including various viruses and bacteria such as SARS-CoV-2, EBV, Adenovirus, RSV, and *K. pneumoniae*. This further indicates that during the spring of 2025, IAV was not only the most prevalent pathogen but also possessed a strong capacity for co-circulation with other pathogens (Table S3).

Notably, SARS-CoV-2 was found to co-occur with IAV in the vast majority of instances. The ratio of single SARS-CoV-2 infections to SARS-CoV-2/IAV co-infections was 1:6.6 (5 cases vs. 33 cases). This finding holds significant implications for surveillance and clinical practice during periods of concurrent influenza and COVID-19 circulation.

Among the 75 co-infection cases, V-V co-infections accounted for 34 cases (45.33%), V-B co-infections for 8 cases (10.67%), viral-viral-bacterial (V-V-B) co-infections for 6 cases (8.00%), and B-B co-infections for 2 cases (2.67%). Overall, bacterial pathogens were involved in 27 co-infection cases, representing 36.00% of all co-infections.

The most frequent V-B pairings were IAV with *A. baumannii* ($n = 6$, including triple/quadruple infections), IAV with *K. pneumoniae* ($n = 5$), and IAV with *S. pneumoniae* ($n = 3$). EBV was also frequently co-detected with bacteria, particularly with *A. baumannii* ($n = 7$), *K. pneumoniae* ($n = 4$), and *P. aeruginosa* ($n = 2$). Notably, the detections of *A. baumannii* (10/11, 90.91%), *P. aeruginosa* (6/8, 75.00%), and *K. pneumoniae* (7/10, 70.00%) predominantly occurred in the context of co-infection, indicating that bacterial detection in this cohort was closely associated with polymicrobial co-infections rather than single-pathogen infections.

These findings suggest that primary viral infections, particularly IAV infection, may create conditions conducive to secondary bacterial colonization or infection, leading to opportunistic bacterial infections represented by *A. baumannii*, *K. pneumoniae*, and *S. pneumoniae*. This observation highlights the clinical challenge posed by V-B co-infections, which may require broader antimicrobial coverage and more intensive monitoring, thereby increasing the complexity of clinical management (Figure 5, Table S3).

3.3 | Concordance Analysis Between Microfluidic Chip Detection and Conventional qPCR

To further evaluate the performance of the multiplex microfluidic chip, we used commercially available conventional qPCR detection kits for respiratory pathogens to test nucleic acids from a subset of respiratory swab samples, aiming to determine the concordance of the results. A total of 96 samples were selected, including 39 samples identified as negative by the chip and 57 samples identified as positive. The positive samples comprised both single-pathogen and mixed-pathogen infections, and the involved pathogens covered all 15 pathogens detected as positive by the chip. The Ct values from the chip PCR for these samples ranged from 25 to 35, encompassing both low and high concentrations.

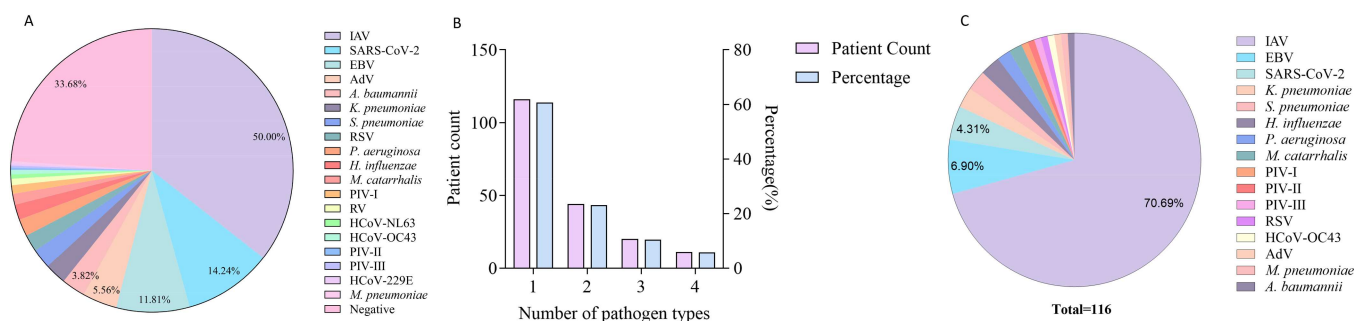


FIGURE 4 | Pathogen spectrum and co-infection patterns among febrile patients with respiratory infections. (A) Distribution of detected respiratory pathogens in all samples ($N = 288$). Influenza A virus (IAV, 50.0%), SARS-CoV-2 (14.2%), and EBV (11.8%) were the most prevalent. The overall positivity rate was 66.3% (191/288). (B) Case count and proportion by infection type ($N = 191$). The bar graph further details the distribution of single, dual, triple, and quadruple infections. (C) Pathogen composition in single-infection cases ($N = 116$). IAV dominated (70.7%), followed by EBV (6.9%) and SARS-CoV-2 (4.3%); all other pathogens individually represented $< 3.5\%$.

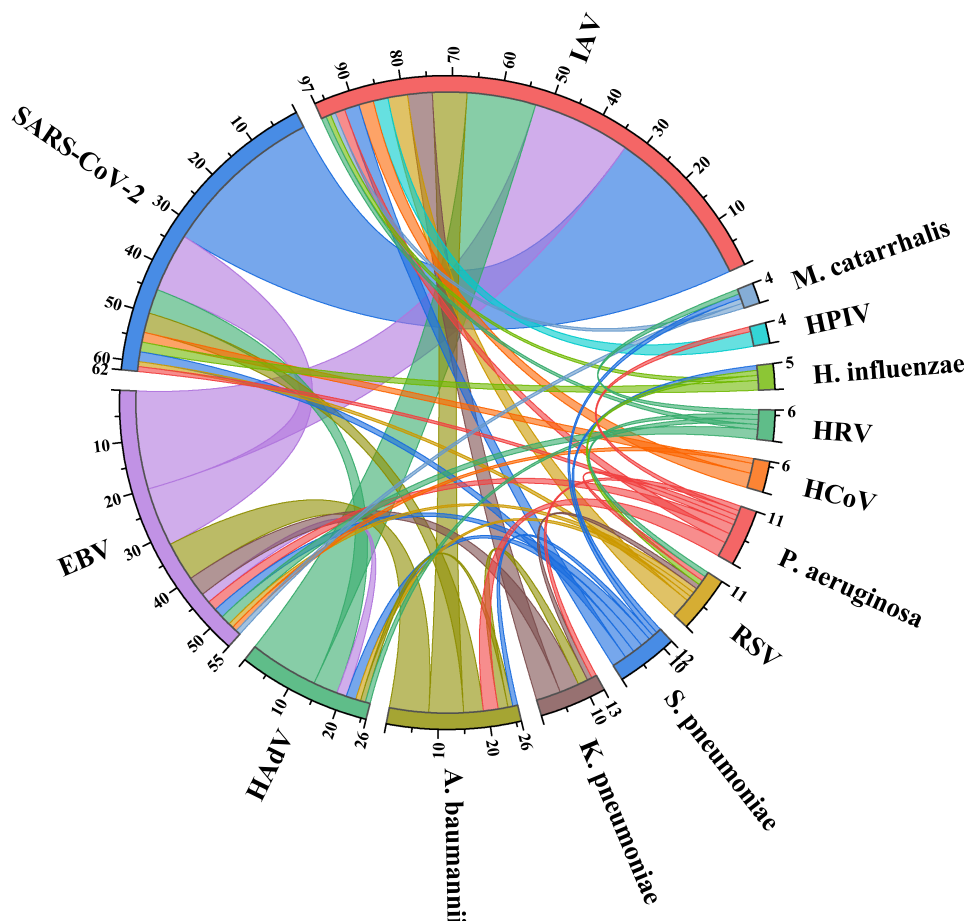


FIGURE 5 | Key pathogens frequently identified in co-infection patterns. Common agents included *Moraxella catarrhalis*, human parainfluenza virus (HPIV), *Haemophilus influenzae*, human rhinovirus (HRV), human coronavirus (HCoV), *Pseudomonas aeruginosa*, respiratory syncytial virus (RSV), *Streptococcus pneumoniae*, and *Klebsiella pneumoniae*.

For the 39 chip-negative samples, conventional qPCR yielded 37 negative results and 2 positive results for Rhinovirus.

For the 57 chip-positive samples (28 single infections and 29 co-infections, including 13 dual, 11 triple, and 5 quadruple infections), conventional qPCR results were completely consistent with the chip results for 51 samples. Inconsistencies were observed in 6 samples: including 3 single-infection

samples, 2 triple-co-infection samples, and 1 quadruple-co-infection sample.

Using conventional qPCR results as the reference, the sensitivity and specificity of the chip-based qPCR were calculated to be 91.07% and 92.50%, respectively. The Kappa value for agreement between the two methods was 0.8298, indicating excellent consistency (Table 4).

TABLE 4 | Concordance analysis between microfluidic chip detection and conventional qPCR (N = 96).

	qPCR positive	qPCR negative	Total
Chip positive	TP = 51	FP = 3	54
Chip negative	FN = 5	TN = 37	42
Total	56	40	96

Note: Data are derived from 96 clinical samples tested in parallel by both methods. These values were used to calculate sensitivity (91.07%), specificity (92.50%), positive predictive value (94.44%), negative predictive value (88.10%), overall accuracy (91.67%), and Cohen's kappa coefficient ($\kappa = 0.83$, indicating almost perfect agreement). Abbreviations: FN, false negative; FP, false positive; TN, true negative; TP, true positive.

The discrepancies between the chip-based qPCR and conventional qPCR primarily involved six target pathogens: Rhinovirus, *Mycoplasma*, *H. influenzae*, Coronavirus, *K. pneumoniae*, and *A. baumannii* (see Table S4). Notably, the Ct values for most inconsistent samples were above 30, indicating low pathogen concentration. We speculated that the difference in primer/probe sequences between the two assay kits is the main reason for the inconsistent results.

4 | Discussion

As one of the most prevalent infectious diseases globally, RTIs pose ongoing challenges for clinical diagnosis and public health control due to their complex pathogen composition and diverse infection patterns [3]. This study validated a super-multiplex microfluidic qPCR chip capable of simultaneously detecting 50 target genes associated with 49 respiratory pathogens. To our knowledge, this chip represents one of the higher-throughput technical solutions among currently reported integrated respiratory pathogen detection panels. By applying it to samples from 288 febrile patients with influenza-like illness (ILI), we have not only demonstrated the robustness of this technology but also revealed more detailed characteristics of respiratory pathogen prevalence. It revealed a complex pathogen spectrum and a high proportion of co-infections among adult febrile patients, providing new evidence for improving current pathogen surveillance and clinical diagnostic strategies for respiratory infections.

From the 288 respiratory samples, a relatively high overall pathogen detection rate (66.32%) and a considerable proportion of co-infections (39.27% of positive cases) were identified. Co-infection patterns included dual (23.04%), triple (10.47%), and even quadruple (5.76%) infections—such complexity is easily overlooked by traditional low-plex or single-pathogen tests. Notably, the predominant pathogen, IAV (detection rate 50.00%), was involved in 84.0% of co-infection events, often co-detected with SARS-CoV-2, EBV, and various bacterial pathogens, reflecting the complex landscape of pathogen coexistence in current respiratory infections. In comparison, large-scale surveillance studies using conventional methods, such as the report by Liu et al., noting an 11.40% rate of V–B co-infection in community-acquired pneumonia in China [5], may somewhat underestimate mixed infections, especially in

populations like ILI patients, where V–B co-infections are relatively common.

The findings of this study offer insights for clinical practice and public health surveillance. The frequent occurrence of IAV and SARS-CoV-2 co-infection (constituting 13.95% of all co-infections) suggests that during seasons when both circulate, clinical differentiation and control strategies need to account for both pathogens. Such concurrent infections may influence disease progression and treatment response, highlighting the practical need for technological platforms capable of synchronous multi-pathogen detection [32]. Furthermore, the detection of conditional pathogens such as *A. baumannii* and *P. aeruginosa* in co-infections in this study suggests the possibility of secondary or mixed infections, warranting attention, particularly among individuals with underlying conditions or compromised immune function. This shares certain similarities with risk profiles for drug-resistant bacterial infections noted in other studies [33]. The ability of our chip to rapidly identify infection profiles, including V–B combinations (e.g., IAV co-infected with *K. pneumoniae*), can help advance antimicrobial therapy and infection control from empirical towards more precise approaches.

In terms of technical performance, the super-multiplex chip showed strong agreement with traditional single-target qPCR (the gold standard), with a sensitivity of 91.07%, specificity of 92.50%, and a substantial level of concordance ($\kappa = 0.83$), indicating its suitability for clinical screening. We also acknowledge several limitations of this technology. First, like all targeted PCR assays, its detection capability is confined by the pre-designed primers and probes, and it cannot identify pathogens or novel variants outside the panel. However, with coverage of 50 targets, the chip provides relatively comprehensive pathogen coverage in a single, rapid microfluidic format, encompassing the vast majority of common respiratory pathogens. Second, there is room for improvement in detection consistency for a few very low-load pathogens (e.g., rhinovirus), suggesting potential for further optimization of the reaction system. Third, the clinical severity of individual infections and co-infections could not be systematically assessed in this study. The enrolled cases were patients presenting with ILI at the fever clinic, and detailed clinical outcome data, including hospitalization duration and ICU admission rate, were unavailable; thus, a statistically meaningful analysis of the association between specific co-infection patterns (e.g., V–V or V–B) and disease severity could not be performed. Nevertheless, the chip achieves a good balance among throughput, detection speed, and result completeness, demonstrating considerable practical value. Future iterations could consider implementing updatable target modules, dynamically adjusted based on regional pathogen prevalence, and complemented with unbiased detection methods like mNGS when necessary to maintain the system's forward-looking capability and adaptability.

In summary, this super multiplex microfluidic qPCR chip is a high-throughput, well-consistent screening platform for respiratory pathogens, offering a feasible technological option between broad-spectrum pathogen discovery (NGS) and targeted, precise detection (single-target qPCR). Through more comprehensive pathogen spectrum analysis, this technology

reveals the complex coexistence of pathogens and the relatively high proportion of mixed infections in febrile patients. It provides a technical basis for advancing the diagnosis of respiratory infections from single-pathogen testing to comprehensive syndromic screening, contributing to support for precise clinical decision-making, rational antimicrobial use, and enhanced public health surveillance efficacy in the context of multi-pathogen co-circulation.

Author Contributions

Xiaoping Kang and Tao Jiang formulated and supervised the project. Yuan Huang, Yuchang Li, and Ganji Song performed the experiments and analyzed the data. Yanhong Gao, Sen Zhang, and Yuehong Chen collected the samples. Jing Li and Ye Feng assisted with the data analysis. Yuan Huang wrote the original draft. Xiaoping Kang revised the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All the data from the current study are shown in the manuscript and [Supporting Information](#) and are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File: jmv71072-sup-0001-supplemental_tables.docx.