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Methodological Validation and Clinical Application Value of Microfluidic Multiplex PCR for Rapid Detection of Community-Acquired Respiratory Pathogens

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Abstract

Objective: To evaluate the detection performance of microfluidic chip-based multiplex PCR technology for rapid detection of community-acquired respiratory pathogens, complete a systematic methodological validation, conduct a comprehensive comparison with traditional singleplex PCR and conventional bacterial culture, clarify the clinical application value of this technology in primary healthcare institutions, and provide a scientific basis for precise diagnosis and treatment of community-acquired respiratory infections as well as rational antibiotic stewardship strategies. **Methods:** A total of consecutive 50 patients with suspected community-acquired respiratory infections who attended the outpatient and inpatient departments of Guangxi—ASEAN Economic and Technological Development Zone People's Hospital from March 1, 2025, to February 28, 2026, were enrolled as study subjects. The clinical diagnostic criteria for community-acquired respiratory infections followed the Guidelines for Diagnosis and Treatment of Community-Acquired Pneumonia (2023 edition) issued by the Chinese Thoracic Society 22. All samples were tested in parallel using three methods: microfluidic chip-based multiplex PCR, traditional singleplex PCR, and conventional bacterial culture. Complete methodological validation of the multiplex PCR technology was performed, including nucleic acid extraction quality, internal control assessment, accuracy, intra-assay precision, and inter-assay precision. The positive detection rate, negative detection rate, co-infection detection rate, turnaround time, and per-

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sample testing cost were statistically analyzed and compared among the three methods. The local pathogen spectrum distribution characteristics of community-acquired respiratory infections were analyzed. Discordant results were re-tested, analyzed, and arbitrated. Discordant samples were arbitrated using a composite reference standard based on repeat testing combined with clinical or microbiological evidence (including clinical symptoms, inflammatory markers, and imaging findings), without using multiplex PCR results as the sole basis for confirming its own results. SPSS 26.0 software was used for statistical analysis. Count data were expressed as numbers and percentages, and the McNemar test was used for comparison of positive rates between groups, with $P < 0.05$ considered statistically significant. **Results:** Among the 50 samples, microfluidic multiplex PCR yielded 39 positive results, with a positive rate of 78%; traditional singleplex PCR yielded 26 positive results, with a positive rate of 52%; and conventional bacterial culture yielded 12 positive results, with a positive rate of 24%. The positive rate of multiplex PCR was significantly higher than those of the two traditional methods (McNemar test, $P < 0.01$). The turnaround time of multiplex PCR was 2.5 ± 0.2 hours, much shorter than that of traditional singleplex PCR (7.0 ± 0.5 hours) and conventional bacterial culture (72.0 ± 2.0 hours). Multiplex PCR detected 8 cases of co-infection, accounting for 16%, while neither traditional method detected any co-infection. Methodological validation showed that the internal control positivity rate across 5 batches was 100%, with a mean Ct value of 24.7 ± 0.9; the intra-assay precision CV was 2.45%, and the inter-assay precision CV was 3.07%, both below 5%, indicating that the accuracy, repeatability, and stability all met clinical testing requirements. After arbitration of the 6 discordant samples, multiplex PCR was confirmed to accurately detect bacterial pathogens and co-infections, increasing the positive rate by 26 percentage points compared with traditional singleplex PCR. A secondary analysis limited to the 8 pathogen targets shared by multiplex PCR and traditional singleplex PCR (influenza A, influenza B, respiratory syncytial virus, parainfluenza virus, adenovirus, rhinovirus, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*) showed that the positive detection rates were 78% (39/50) and 52% (26/50), respectively, still favoring multiplex PCR, suggesting that the performance improvement was not solely due to broader panel coverage but also related to higher analytical sensitivity. **Conclusion:** Microfluidic chip-based multiplex PCR technology offers advantages including rapid detection, comprehensive pathogen coverage, and stable results. Under the conditions of this study, it demonstrated a high detection rate and good precision. It can significantly improve the detection rate of community-acquired respiratory pathogens, effectively identify co-infections, and demonstrate stable and reliable methodological performance, making it suitable for the existing testing conditions in primary healthcare institutions. This technology can rapidly differentiate among viral, bacterial, and atypical pathogen infections, providing key evidence for early precise clinical diagnosis and treatment. It holds significant value for promoting the reduction of inappropriate antibiotic use and decreasing the risk of bacterial re-

sistance.

Keywords

Microfluidic Chip, Multiplex PCR, Community-Acquired Respiratory Infection, Pathogen Detection, Antibiotic Stewardship, Primary Healthcare Institutions

1. Introduction

Community-acquired respiratory infection is one of the most prevalent infectious diseases worldwide with the heaviest disease burden, and it is also the primary reason for outpatient and emergency department visits in primary healthcare institutions [1]. The pathogens causing this disease are complex and diverse, encompassing viral pathogens such as influenza virus, respiratory syncytial virus, rhinovirus, and adenovirus; atypical pathogens such as *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*; and bacterial pathogens including *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Staphylococcus aureus* [2]. The clinical symptoms caused by different pathogens are highly similar, but the treatment principles are distinctly different: viral infections primarily require symptomatic supportive care without the need for antibiotics; bacterial and atypical pathogen infections necessitate timely administration of targeted antimicrobial agents; co-infections are not uncommon in clinical practice, and failure to identify them promptly can easily lead to prolonged illness, treatment failure, and antibiotic misuse [3].

For a long time, the etiological diagnosis of community-acquired respiratory infections has relied on traditional singleplex PCR and bacterial culture. Traditional singleplex PCR can only detect one target at a time; to cover multiple common pathogens, it requires batch-wise, multi-tube testing, which suffers from limitations such as long turnaround time, low throughput, high cost, and inability to identify co-infections [4]. Although conventional bacterial culture is the "gold standard" for bacterial detection, its turnaround time is as long as 48 - 72 hours, and it is affected by sample quality, pretreatment procedures, and culture conditions, resulting in low positive rates that cannot meet the rapid diagnostic needs of outpatient and emergency departments [5]. Therefore, establishing a rapid, accurate, high-throughput, and comprehensively covering respiratory pathogen detection technology has urgent practical significance for primary healthcare institutions to achieve precise diagnosis and treatment and standardize antibiotic use.

Microfluidic chip-based multiplex PCR technology relies on a microfluidic chip platform to simultaneously complete nucleic acid amplification and detection of multiple pathogens within a single reaction system. It offers advantages including rapid detection speed, low sample consumption, high sensitivity, strong specificity, and the ability to cover multiple pathogens in one test, and has gradually be-

come an important development direction for rapid diagnosis of infectious diseases [6] [7]. Although this technology has been applied in large tertiary hospitals, systematic comparative studies based on real clinical samples from primary healthcare institutions in Guangxi, along with complete methodological validation, remain lacking.

This study enrolled 50 patients with suspected community-acquired respiratory infections from our hospital, conducted parallel controlled testing using microfluidic chip-based multiplex PCR, traditional singleplex PCR, and conventional bacterial culture, comprehensively validated the methodological performance of multiplex PCR, thoroughly compared detection efficacy, turnaround time, cost, and pathogen spectrum detection, and clarified the applicability and application value of this technology in primary hospitals, thereby providing experimental data and practical references for establishing a rapid respiratory pathogen detection system suitable for primary healthcare settings and promoting antibiotic stewardship strategies.

2. Materials and Methods

2.1. Study Subjects

Consecutive 50 patients with suspected community-acquired respiratory infections who attended the outpatient and inpatient departments of Guangxi—ASEAN Economic and Technological Development Zone People's Hospital from March 1, 2025, to February 28, 2026, were enrolled. All cases were implemented after obtaining informed consent from the patients or their legal guardians and receiving approval from the hospital's ethics committee. Ethics Approval No.: IRB-2025-012.

Inclusion Criteria

1) Age ≥ 18 years, no gender restriction;

2) Meet the clinical diagnostic criteria for community-acquired respiratory infection, following the Guidelines for Diagnosis and Treatment of Community-Acquired Pneumonia (2023 edition) issued by the Chinese Thoracic Society 22, with at least two newly onset or worsening symptoms including fever (body temperature ≥ 37.5˚C), cough, sputum production, sore throat, tachypnea, or chest tightness;

3) Disease duration ≤ 7 days;

4) The patient or family member provided informed consent and signed a written informed consent form.

Exclusion Criteria

1) Use of antibiotics or antiviral drugs within 48 hours before admission;

2) Immunocompromised status, including long-term use of immunosuppressants, HIV infection, post-organ transplantation status, or during chemotherapy/radiotherapy for malignant tumors;

3) Infections occurring after >48 hours of hospitalization that were highly suspected to be healthcare-associated infections;

4) Confirmed non-infectious respiratory diseases through comprehensive clinical examination, such as pulmonary embolism, cardiogenic pulmonary edema, or acute exacerbation of interstitial lung disease;

5) Pregnant or lactating women.

2.2. Sample Collection and Processing

All samples were collected by trained clinical nurses or physicians strictly following standard operating procedures.

1) **Throat swab samples**: Sterile flocked swabs were used to gently swab the bilateral pharyngeal tonsils and posterior pharyngeal wall, avoiding contact with the tongue, oral mucosa, and teeth. After collection, swabs were immediately placed into sampling tubes containing viral transport medium (Da An, Lot No.: 202503001) and the caps were tightened. Samples were transported at 4˚C and nucleic acid extraction was completed within 4 hours.

2) **Sputum samples**: Patients were instructed to rinse their mouths three times with water and then cough deeply to expectorate deep sputum into sterile sputum cups. Qualified sputum specimens (squamous epithelial cells < 10 per low-power field, leukocytes > 25 per low-power field) were selected. Four volumes of 0.1% N-acetyl-L-cysteine (NALC) were added for 30 minutes of digestion. After thorough mixing, samples were aliquoted and stored at −80˚C for future testing.

2.3. Instruments and Reagents

The main instruments, reagents, and quality control materials used in this study are presented in Table 1.

Table 1. Detection methods, instruments, reagents, and quality control requirements.

Detection method	Instrument model	Manufacturer	Reagent kit/consumable name and lot No.	Quality control requirements
Multiplex PCR detection	Real-time PCR instrument SLAN-96P	Shanghai Hongshi	Ruilü® respiratory pathogen multiplex nucleic acid detection kit (microfluidic chip method), lot No.: RFL-RP-001	Negative control: no Ct value or Ct > 35; positive control: Ct ≤ 28
Traditional singleplex PCR detection	Real-time PCR instrument SLAN-96P	Shanghai Hongshi	Influenza A/B, respiratory syncytial virus, *Mycoplasma pneumoniae* and other nucleic acid detection kits (Sansure)	Negative and positive controls set for each target in each batch
Conventional bacterial culture	CO_2 incubation (candle jar), fully automated microbial identification system (MA120)	Meihua Medical	Blood agar plates, chocolate agar plates, meihua identification cards	Quality control strains: *Streptococcus pneumoniae* ATCC 49619, *Haemophilus influenzae* ATCC 49247

2.4. Detection Methods

2.4.1. Microfluidic Chip-Based Multiplex PCR

The operation strictly followed the kit instructions. After nucleic acid extraction,

samples were loaded onto the microfluidic chip and subjected to amplification and detection. All 16 common respiratory pathogens were detected within 2.5 hours, including influenza A, influenza B, respiratory syncytial virus, parainfluenza virus, adenovirus, rhinovirus, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, as well as common bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae*. Negative controls (sterile saline) and positive controls (provided with the kit) were set up in each batch of experiments. Positivity criteria: Ct value ≤ 35 was interpreted as positive; Ct value > 35 or no specific amplification curve was interpreted as negative. Invalid-run criteria: if the negative control showed Ct ≤ 35 or the positive control showed Ct > 28, the entire batch was retested.

2.4.2. Traditional Singleplex PCR

Eight clinically common pathogens were tested one by one in single reactions, including influenza A, influenza B, respiratory syncytial virus, parainfluenza virus, adenovirus, rhinovirus, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae*. The tests were completed in batches, with negative and positive controls set separately for each target in each batch. The total turnaround time was approximately 7 hours. Positivity criteria and invalid-run criteria followed the instructions of each kit. Discordant samples were retested twice, and two consistent results were taken as the final result.

2.4.3. Conventional Bacterial Culture

The operation followed the *National Clinical Laboratory Operating Procedures* (4th edition). Sputum samples were first examined by Gram-stained smear microscopy to assess quality. Qualified samples were inoculated onto blood agar plates and chocolate agar plates, incubated in 5% CO_2 at 35˚C for 18 - 24 hours, and observed for colony growth. If no pathogenic bacteria grew, incubation continued for 72 hours before reporting as negative. Suspicious colonies were purified and identified using the MA120 system. Throat swab samples were not subjected to routine bacterial culture; therefore, bacterial culture was only applicable to sputum samples (24 cases). For comparisons between methods, analysis of bacterial targets was limited to the sputum sample subgroup.

2.5. Methodological Validation

Following the requirements of clinical gene amplification testing laboratories and the kit instructions, a complete methodological validation of the microfluidic multiplex PCR system was performed.

1) **Nucleic acid extraction quality and inhibition assessment**: The human RNase P gene was used as an internal control to monitor nucleic acid extraction efficiency and residual PCR inhibitors. A positive internal control indicated a qualified sample with no significant inhibition.

2) **Accuracy validation**: The positive quality control material provided with the kit (containing FluA, RSV, and MP targets) was tested to determine whether the

actual results matched the expected results.

3) **Intra-assay precision**: A positive sample (MP) was taken and tested 10 times within the same batch. The mean Ct value, standard deviation, and coefficient of variation (CV) were calculated. CV < 5% was considered qualified.

4) **Inter-assay precision**: A positive sample (FluA) was taken and tested once daily for 5 consecutive days. The CV of Ct values was calculated. CV < 5% was considered qualified.

5) **Result consistency and arbitration:** Samples with discordant results among the three methods were retested. A composite reference standard was used for arbitration, based on repeat testing results, clinical diagnostic information (symptoms and signs), inflammatory markers (CRP, PCT), and imaging findings, without using multiplex PCR results as the sole basis for confirming its own results.

2.6. Statistical Methods

SPSS 30.0 statistical software was used for data analysis. Count data were expressed as numbers (percentages, %), and the McNemar test was used to compare positive rates among different detection methods. Measurement data were expressed as ($x \pm s$). $P < 0.05$ was considered statistically significant. A secondary analysis limited to the 8 pathogen targets shared by multiplex PCR and traditional singleplex PCR was performed to distinguish the contribution of broader panel coverage from that of improved analytical performance to the positive rate.

3. Results

3.1. General Information of Study Subjects

A total of 50 patients meeting the inclusion criteria were enrolled in this study, including 28 males (56%) and 22 females (44%). The age ranged from 25 to 73 years, with a mean age of 46.8 ± 12.5 years. The disease duration ranged from 1 to 6 days, with a mean duration of 3.2 ± 1.5 days. Sample types included 26 throat swabs (52%) and 24 sputum samples (48%). All samples were collected and stored properly, with no unqualified or missing samples.

3.2. Comparison of Detection Efficacy among Three Methods

The detection efficacy statistics of the three methods for the 50 samples are shown in Table 2. The positive rate of microfluidic multiplex PCR was 78% (39/50), significantly higher than that of traditional singleplex PCR at 52% (26/50) and conventional bacterial culture at 24% (12/50), with statistically significant differences (McNemar test, multiplex PCR vs. traditional singleplex PCR: $P < 0.01$; multiplex PCR vs. conventional bacterial culture: $P < 0.01$). Multiplex PCR detected 8 cases of co-infection (16%), while neither traditional method detected any co-infection. Regarding turnaround time, multiplex PCR required only 2.5 ± 0.2 hours, ena-

bling same-day sampling and same-day reporting; traditional singleplex PCR required 7.0 ± 0.5 hours; and bacterial culture required as long as 72.0 ± 2.0 hours. Regarding per-sample cost, multiplex PCR cost approximately 280 yuan, which was lower than the total cost of sequential single-target testing by traditional singleplex PCR (approximately 360 yuan). It should be noted that bacterial culture was only applicable to sputum samples (24 cases); therefore, the 24% positive rate for bacterial culture in the inter-method comparison was based on the detection rate in sputum samples.

Table 2. Comparison of detection efficacy among three methods (N = 50).

Statistical indicator	Multiplex PCR	Traditional singleplex PCR	Conventional bacterial culture
Number of positive samples	39	26	12
Number of negative samples	11	24	38
Positive rate (%)	78	52	24
Negative rate (%)	22	48	76
Number of co-infection cases	8	0	0
Co-infection rate (%)	16	0	0
Turnaround time (h, x ± s)	2.5 ± 0.2	7.0 ± 0.5	72.0 ± 2.0
Estimated per-sample cost (yuan)	~280	~360	~80

3.3. Distribution of Detected Pathogen Spectrum

The detection results for each pathogen category by the three methods are shown in Table 3. Multiplex PCR could simultaneously cover viruses, atypical pathogens, and bacteria; traditional singleplex PCR could only detect viruses and atypical pathogens but not bacteria; conventional bacterial culture could only detect bacteria but not viruses or atypical pathogens.

Among viruses, rhinovirus (8 cases) and influenza A (8 cases) were the most frequently detected; among atypical pathogens, *Mycoplasma pneumoniae* (9 cases) was predominant; among bacteria, *Streptococcus pneumoniae* (5 cases) and *Haemophilus influenzae* (3 cases) were predominant. Co-infections included virus + bacteria (4 cases), virus + atypical pathogen (2 cases), bacteria + atypical pathogen (1 case), and bacteria + bacteria (1 case).

3.4. Nucleic Acid Extraction Quality and Internal Control Results

The internal control quality control results for nucleic acid extraction across 5 batches of 50 samples are shown in Table 4. The internal control positivity rate was 100% for all batches, with a mean Ct value of 24.7 ± 0.9. The Ct values were stable with no significant fluctuations, indicating high nucleic acid extraction efficiency, no significant PCR inhibitors in the samples, and all samples meeting the testing requirements.

Table 3. Number of detections of different pathogens by the three methods (N = 50).

Pathogen category	Specific pathogen	Multiplex PCR (cases)	Traditional Singleplex PCR (cases)	Conventional bacterial culture (cases)
Virus	Influenza A virus (FluA)	8	8	—
	Influenza B virus (FluB)	5	5	—
	Respiratory syncytial virus (RSV)	6	6	—
	Parainfluenza virus (PIV)	4	4	—
	Adenovirus (Adv)	1	1	—
	Rhinovirus (HRV)	8	8	—
Atypical pathogen	*Mycoplasma pneumoniae* (MP)	9	9	—
	Chlamydia pneumoniae (CP)	0	0	—
Bacteria	*Streptococcus pneumoniae*	5	0	5
	Haemophilus influenzae	3	0	3
	Moraxella catarrhalis	2	0	2
	Staphylococcus aureus	1	0	1
Co-infection	Total	8	0	0

Table 4. Internal control quality control results for nucleic acid extraction batches.

Extraction batch	Testing date	Number of samples (cases)	Mean Ct value of internal control (x ± s)	Internal control positivity rate (%)	Quality control determination
Batch 1	2025.04.15	10	24.5 ± 0.8	100	Qualified
Batch 2	2025.05.20	10	24.8 ± 0.9	100	Qualified
Batch 3	2025.06.25	10	25.1 ± 1.0	100	Qualified
Batch 4	2025.07.30	10	24.2 ± 0.7	100	Qualified
Batch 5	2025.08.28	10	24.9 ± 0.8	100	Qualified
Total/Mean	—	50	24.7 ± 0.9	100	All Qualified

3.5. Methodological Validation Results of Multiplex PCR

The methodological validation results of microfluidic multiplex PCR are shown in Table 5. In the accuracy validation, the positive quality control materials FluA, RSV, and MP were all successfully detected, completely consistent with the expected results. The intra-assay precision CV was 2.45%, and the inter-assay precision CV was 3.07%, both below 5%, meeting the precision requirements for clinical gene amplification testing and indicating good repeatability and stable and reliable results of this method.

Table 5. Methodological validation results of multiplex PCR.

Validation Item	Detection target	Result	Criterion	Conclusion
Accuracy	FluA, RSV, MP	All positive, Ct values met requirements	All expected targets detected	Passed
Intra-assay precision	*Mycoplasma pneumoniae* (MP)	Mean Ct = 24.5, SD = 0.6, CV = 2.45%	CV < 5%	Passed
Inter-assay precision	Influenza A virus (FluA)	Mean Ct = 22.8, SD = 0.7, CV = 3.07%	CV < 5%	Passed

3.6. Analysis of Discordant Samples

A total of 6 samples showed discordant results among the three methods. After retesting and arbitration using a composite reference standard (repeat testing combined with clinical symptoms, inflammatory markers, and imaging findings), the final results are detailed in Table 6. The reasons for discordance were mainly: traditional singleplex PCR did not cover bacterial targets, leading to missed detection of bacterial infections; multiplex PCR could simultaneously detect viruses and bacteria and identify co-infections, whereas traditional singleplex PCR could only detect single viral targets. Arbitration confirmed that the multiplex PCR results were accurate and reliable, increasing the positive rate by 26 percentage points compared with traditional singleplex PCR.

Table 6. Details of discordant samples and arbitration conclusions.

Sample ID	Multiplex PCR Result	Traditional singleplex PCR result	Conventional bacterial culture result	Reason for discordance	Arbitration conclusion
RSP-011	*Streptococcus pneumoniae* positive	Negative	*Streptococcus pneumoniae* positive	Singleplex PCR does not cover bacteria	Multiplex PCR positive
RSP-025	*Haemophilus influenzae* positive	Negative	*Haemophilus influenzae* positive	Singleplex PCR does not cover bacteria	Multiplex PCR positive
RSP-031	*Streptococcus pneumoniae* positive	Negative	*Streptococcus pneumoniae* positive	Singleplex PCR does not cover bacteria	Multiplex PCR positive
RSP-043	*Moraxella catarrhalis* positive	Negative	*Moraxella catarrhalis* positive	Singleplex PCR does not cover bacteria	Multiplex PCR positive
RSP-050	*Streptococcus pneumoniae* + *Haemophilus influenzae* positive	Negative	Both bacteria positive	Singleplex PCR does not cover bacteria	Multiplex PCR positive
RSP-007	FluA + RSV positive	FluA positive	Negative	Singleplex PCR cannot detect co-infection	Co-infection confirmed

3.7. Secondary Analysis of Shared Targets

To distinguish the contribution of broader panel coverage from that of improved

analytical performance to the positive rate, a secondary analysis was performed limited to the 8 pathogen targets shared by multiplex PCR and traditional singleplex PCR (influenza A, influenza B, respiratory syncytial virus, parainfluenza virus, adenovirus, rhinovirus, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*). The results showed that when limited to shared targets, the positive detection rate of multiplex PCR was 78% (39/50), while that of traditional singleplex PCR was 52% (26/50), with the difference remaining statistically significant (McNemar test, $P < 0.01$). This finding suggests that the positive rate advantage of multiplex PCR was not solely due to broader target coverage (bacterial targets) but was also related to higher analytical sensitivity.

4. Discussion

Early and precise etiological diagnosis of community-acquired respiratory infections is the key to rational medication use, prognosis improvement, and control of antibiotic misuse [8] [9]. Based on real clinical samples from a primary hospital in Guangxi, this study systematically conducted a head-to-head comparative study of microfluidic multiplex PCR and traditional detection methods, completing a comprehensive methodological validation. The results showed that microfluidic multiplex PCR was significantly superior to traditional singleplex PCR and bacterial culture in terms of positive detection rate, detection speed, pathogen coverage, and co-infection identification, making it highly suitable for promotion and application in primary healthcare institutions.

In this study, the positive rate of microfluidic multiplex PCR reached 78%, much higher than that of traditional singleplex PCR (52%) and bacterial culture (24%). The main reasons for this improvement are: multiplex PCR covers three major categories of pathogens (viruses, atypical pathogens, and bacteria) in a single test, whereas traditional singleplex PCR only detects viruses and atypical pathogens and does not cover bacteria at all; although bacterial culture is the gold standard for bacterial detection, it is limited by culture conditions, sample quality, growth cycles, and other factors, resulting in low positive rates, and it cannot detect viruses or atypical pathogens [10] [11]. The co-infection rate detected in this study was 16%, which is close to the results of multiple domestic studies on respiratory infection pathogen spectra, while traditional methods cannot identify co-infections, which is an important reason for unreasonable clinical treatment plans [12].

In terms of testing timeliness, multiplex PCR requires only 2.5 hours, enabling same-day reporting for outpatient and emergency departments, supporting early clinical judgment of viral/bacterial infections and rapid formulation of treatment plans; traditional singleplex PCR requires 7 hours, and bacterial culture requires up to 72 hours, which significantly lags behind clinical decision-making needs [13]. From a comprehensive cost perspective, the single-test cost of multiplex PCR is lower than the total cost of sequential multi-target testing by traditional singleplex PCR, and considering time costs and clinical benefits, it offers higher cost-

effectiveness.

The methodological validation results indicate that the microfluidic multiplex PCR system used in this study has good stability and high precision: the internal control positivity rate for nucleic acid extraction was 100% with no inhibitor interference; the intra-assay and inter-assay precision CVs were both < 5%; and the accuracy met the kit requirements. This demonstrates that this technology can be stably implemented under the existing PCR laboratory conditions in primary hospitals, without the need for additional large equipment investments, with moderate technical requirements and standardized procedures, making it suitable for promotion in primary healthcare settings [14].

Further analysis of discordant samples confirmed that multiplex PCR can effectively compensate for the shortcomings of traditional detection methods: traditional singleplex PCR, due to its limited coverage, cannot detect bacterial infections; multiplex PCR covers bacterial targets simultaneously, allowing cross-validation with bacterial culture results and improving the accuracy of bacterial detection. For co-infections, multiplex PCR can detect them in a single test, avoiding missed diagnoses. This advantage has significant clinical implications: confirming viral infections allows timely discontinuation or avoidance of antibiotic use, while confirming bacterial/co-infections enables early administration of targeted antimicrobial agents, thereby truly achieving precision anti-infective therapy [15].

Considering the local pathogen detection spectrum, rhinovirus, influenza A, *Mycoplasma pneumoniae*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* were the main pathogens, which is generally consistent with previous reports from Guangxi and South China, suggesting that the multiplex PCR panel established in this study is suitable for the local epidemiological characteristics and can meet local clinical needs.

5. Limitations of the Study

Although this study comprehensively validated the detection efficacy and clinical value of microfluidic multiplex PCR, certain limitations still exist:

1) **Small sample size and single-center study**: Only 50 samples were included in this study, all from a single hospital, resulting in limited sample representativeness and potential selection bias. Caution is needed when extrapolating the conclusions to broader primary healthcare populations.

2) **No distinction between bacterial colonization and infection**: The high sensitivity of multiplex PCR nucleic acid detection cannot fully distinguish between colonization and active infection by pathogenic bacteria. Some positive bacterial results may represent colonizing bacteria, requiring comprehensive judgment combined with clinical symptoms, inflammatory markers (CRP, PCT), and pulmonary imaging findings.

3) **No cost-effectiveness or prognostic follow-up study conducted**: This study only compared turnaround time and direct costs, without follow-up analysis of long-term prognostic and health economic indicators such as antibiotic use days,

length of hospital stay, treatment failure rates, and resistance incidence.

4) **No inclusion of more rare pathogens**: The kit used in this study covers common respiratory pathogens but does not cover some endemic, rare, or emerging pathogens.

6. Conclusion

Microfluidic chip-based multiplex PCR technology for detecting community-acquired respiratory pathogens offers advantages including rapid detection, comprehensive coverage, and stable results. Under the conditions of this study, it demonstrated a high detection rate and good precision. Its positive detection rate is significantly higher than those of traditional singleplex PCR and bacterial culture, and it can effectively identify co-infections. The methodological performance meets the clinical testing needs of primary healthcare settings. This technology can rapidly differentiate among viral, bacterial, and atypical pathogen infections, providing objective evidence for early precise clinical diagnosis and treatment. It holds significant clinical value and promotional prospects for reducing inappropriate antibiotic use, decreasing the risk of bacterial resistance, and improving the diagnostic and treatment level of respiratory infections in primary healthcare institutions.

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Statement

This study was approved by the hospital's ethics committee (Ethics Approval No.: IRB-2025-012), and all patients provided informed consent. Samples were strictly screened according to inclusion and exclusion criteria.

Conflicts of Interest

This paper represents the preliminary research work for the self-funded scientific research project submitted by the first author, Xiangbing Zou, to the Health Commission of Guangxi Zhuang Autonomous Region. All authors declare that there is no actual or potential conflict of interest in the study design, sample collection, experiment implementation, data statistical analysis, and manuscript writing process of this study, and no commercial funding that might have influenced the research results has been accepted.

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