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Original Article

Clinical Utility of Targeted Third-Generation Sequencing for Pathogen Detection in Community-Acquired Pneumonia: A Real-World Comparative Study of China

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Abstract

Objective: To evaluate the diagnostic performance and clinical utility of targeted third-generation sequencing (tTGS) for pathogen detection in community-acquired pneumonia (CAP), compared with targeted next-generation sequencing (tNGS) and conventional culture.

Methodology: We conducted a real-world, retrospective study including 356 CAP patients who were tested in parallel with culture, tNGS, and tTGS. Diagnostic sensitivity, pathogen spectrum, mixed-infection detection, and antimicrobial resistance (AMR) gene profiling were compared across methods. Clinical relevance was assessed by analyzing treatment adjustments informed by sequencing results.

Results: tTGS demonstrated the highest overall sensitivity (99.16%), surpassing tNGS (96.35%, $p < 0.05$) and culture (50.27%, $p < 0.01$). Across all samples, tTGS identified 135 microbial species — substantially more than tNGS (84 species) and culture (27 species) — including fastidious, rare, and slow-growing pathogens. tTGS also showed an improved ability to detect mixed infections (86.24% vs. 87.64% with tNGS; culture detected none). AMR gene detection was significantly higher with tTGS than with tNGS (58.99% vs. 48.88%, $p = 0.008$), and long-read sequencing enabled the identification of composite resistance patterns that tNGS missed. Among patients without underlying diseases, sequencing-guided therapeutic adjustment occurred in 67.87% of cases, with nearly 80% of AMR-positive patients demonstrating clinical improvement following regimen optimization.

Conclusions: tTGS provides markedly enhanced pathogen detection, greater sensitivity, and more comprehensive AMR profiling than both tNGS and culture. Its strong performance in identifying mixed infections and actionable determinants of resistance supports its incorporation into clinical diagnostic workflows for CAP. Further large-scale, prospective studies are warranted to validate its clinical impact and optimize its integration into routine practice.

Key words: Community-Acquired Pneumonia; Third-Generation Sequencing; Targeted Metagenomics; Antimicrobial Resistance; Mixed Infections.

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Introduction

Community-acquired pneumonia (CAP) remains a major global health challenge and a leading cause of morbidity and mortality across all age groups [1]. Despite advances in antimicrobial therapy, early and accurate pathogen identification remains a key barrier to optimal clinical management. The clinical manifestations of CAP are inherently nonspecific and often overlap with those of other respiratory illnesses, while mixed infections—reported in approximately 20–30% of cases—further complicate diagnosis and empiric treatment. As a result, inappropriate or delayed antimicrobial therapy remains common, contributing to poor outcomes and the growing threat of antimicrobial resistance [2].

Conventional diagnostic methods, including culture and targeted polymerase chain reaction (PCR), remain the cornerstones of pathogen identification in community-acquired pneumonia [3,4]. However, culture-based methods are limited by low sensitivity, prolonged turnaround time, and reduced detection of fastidious or slow-growing microorganisms [5-8]. PCR-based assays improve detection sensitivity and shorten diagnostic time, but their reliance on predefined targets restricts pathogen coverage and limits the identification of unexpected or emerging pathogens [9]. Moreover, both methods provide limited information for characterizing mixed infections and antimicrobial resistance (AMR) mechanisms [10-12]. Metagenomic sequencing has emerged as a powerful alternative for etiological diagnosis of infectious diseases. Next-generation sequencing (NGS) enables broad, unbiased pathogen detection, but its short-read nature may hinder resolution of complex genomic regions, mixed populations, and AMR determinants [13,14]. Although early-generation third-generation sequencing (TGS) platforms were limited by relatively high raw error rates and the absence of standardized analytical pipelines, which hindered their widespread clinical implementation [8,15,16], continuous technological

advancements and algorithmic improvements have substantially mitigated these constraints. Leveraging long-read sequencing, real-time data acquisition, and superior capability in resolving repetitive and complex genomic regions, TGS now enables more accurate genome assembly, comprehensive pathogen identification, and in-depth characterization of antimicrobial resistance (AMR) determinants, thereby offering distinct advantages over short-read sequencing approaches [7,14,17,18].

However, despite these promising findings, real-world evidence comparing tTGS with clinically established methods remains limited. Few studies have systematically evaluated its diagnostic sensitivity, pathogen coverage, detection of mixed infections, and clinical impact relative to targeted NGS (tNGS) and culture in a large CAP cohort [19,20].To address this gap, we conducted a comparative study assessing the diagnostic performance and clinical relevance of tTGS in 356 patients with CAP. Specifically, we compared tTGS with tNGS and conventional culture for pathogen detection, mixed-infection identification, AMR gene profiling, and downstream influence on therapeutic decisions. The findings aim to provide evidence for the potential integration of tTGS into routine diagnostic workflows for CAP.

Methodology

Study Design and Ethical Approval

This retrospective real-world study enrolled consecutive patients diagnosed with community-acquired pneumonia (CAP) at the First Hospital of Jilin University between May and September 2024. All patients underwent parallel pathogen detection using conventional culture, targeted next-generation sequencing (tNGS), and targeted third-generation sequencing (tTGS). Clinical, laboratory, and imaging data were extracted from electronic medical records for clinical correlation and outcome evaluation.

The study protocol was approved by the Institutional Review Board of the First Hospital of Jilin University (Approval No. 2025-557). For this retrospective analysis using anonymized data, the requirement for written informed consent was waived.

Patient Selection

Inclusion Criteria

- Met the diagnostic criteria for CAP according to national guidelines;
- Underwent bronchoscopy or provided respiratory specimens (BALF, sputum, or throat swab) for pathogen detection.

Exclusion Criteria

Patients were excluded if they had:

- Known immunodeficiency disorders
- Rheumatic autoimmune diseases or malignant tumors
- Psychiatric disorders that impede cooperation
- Insufficient sample volume for sequencing
- Incomplete clinical information.

Sample Collection

Bronchoalveolar Lavage Fluid (BALF)

BALF samples were collected via fiberoptic bronchoscopy following standard procedures. After local anesthesia, 100–300 mL of sterile saline (37°C) was instilled in aliquots of 25–50 mL and immediately aspirated. Recovered fluid (usually 40–60% of instilled volume) was filtered through sterile gauze, documented, and transferred to sterile containers.

Throat Swabs

A sterile flocked swab was used to sample the posterior pharyngeal wall while avoiding the tongue and oral mucosa. Swabs were placed into viral transport media and sealed for downstream analysis.

Sputum Samples

Morning sputum was collected after oral rinsing. Patients were instructed to expectorate deep-airway secretions into sterile, leak-proof containers. Samples with < 1 mL volume or excessive saliva were rejected.

All specimens were transported under cold-chain conditions to ensure sample integrity.

Pathogen Detection Methods

Conventional Culture

Routine bacterial and fungal cultures were performed in the hospital microbiology laboratory using standard microbiological techniques. Identification and antimicrobial susceptibility testing were performed according to CLSI guidelines. Although all patients were scheduled for routine culture, only 185 specimens met the quality and quantity criteria required for reliable microbial cultivation.

Targeted Next-Generation Sequencing (tNGS)

Targeted next-generation sequencing was performed using a validated enrichment-based platform covering 258 respiratory pathogens and major antimicrobial resistance (AMR) genes. Briefly, nucleic acids were extracted, amplified using multiplex PCR

panels, and sequenced on a short-read sequencing platform. Bioinformatic analysis was conducted using curated microbial and resistance gene databases to generate pathogen and AMR profiles.

Targeted Third-Generation Sequencing (tTGS)

Targeted third-generation sequencing was performed at Dian Diagnostics Group using a nanopore long-read sequencing platform. Nucleic acids were extracted using automated systems, quantified by Qubit fluorometry, and amplified using targeted panels covering 354 respiratory pathogens and key AMR determinants. Long-read sequencing enabled direct detection of full-length resistance genes and composite resistance structures. Data analysis was conducted using validated pathogen and resistance annotation pipelines.

Clinical and Laboratory Data Collection

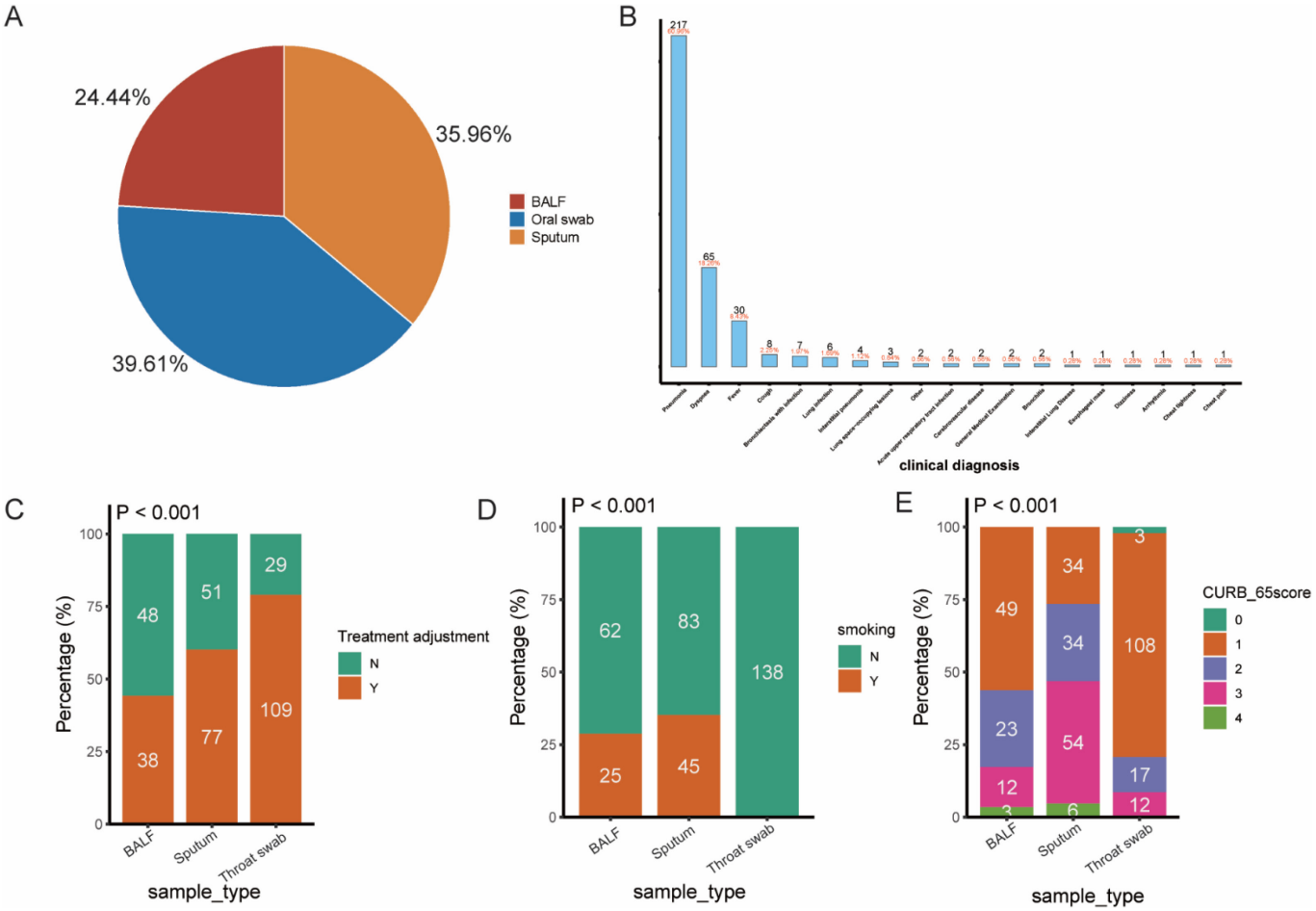
Clinical parameters, including symptoms, chest imaging, inflammatory biomarkers (CRP, PCT, WBC), CURB-65 score, antibiotic regimens, and clinical outcomes, were extracted for analysis.

To distinguish true infection from colonization—particularly for microorganisms commonly present in the upper respiratory tract—sequencing results were interpreted using integrated clinical adjudication. A detected organism was considered clinically significant if at least two of the following criteria were met:

- Compatible clinical symptoms and radiological findings;
- Elevated inflammatory biomarkers;
- High relative abundance (≥ 5% of microbial reads) or top-three absolute read counts;
- Clinical improvement following targeted antimicrobial therapy.

Mixed infection was defined as the detection of ≥ 2 clinically significant pathogens within the same specimen. AMR gene detection was based on curated

Figure 1. A: Pie chart of sample types; **B:** Bar chart of clinical diagnosis distribution. **C:** Stacked bar chart of sample types versus whether the treatment regimen was adjusted. **D:** Stacked bar chart of sample types versus smoking status. E) Stacked bar chart of sample types versus CURB-65 score.



resistance databases integrated into both sequencing pipelines, and the comparative analysis focused on overlapping gene panels.

Statistical Analysis

Statistical analyses were conducted using R software (version 4.4.1). Categorical variables were expressed as frequencies and percentages, while continuous variables were reported as mean ± SD or median (interquartile range, IQR). Detection sensitivity among culture, tNGS, and tTGS was compared using Fisher's test. A $p < 0.05$ was considered statistically significant.

Results

Patient Characteristics and Sample Distribution

A total of 356 patients with community-acquired pneumonia (CAP) were included in this study, comprising 192 males (53.93%) and 164 females (46.07%), with a median age of 39.5 years. Respiratory specimens consisted of throat swabs (39.61%), sputum samples (35.96%), and bronchoalveolar lavage fluid (BALF) (24.44%) (Figure 1A), reflecting routine clinical sampling practices in real-world CAP management.

The most common clinical diagnoses on admission were pneumonia (60.96%), dyspnea (18.26%), fever (8.43%), cough (2.25%), and bronchiectasis with infection (1.97%) (Figure 1B). Treatment regimens were adjusted based on microbiological findings in 224 patients (62.92%), indicating a substantial clinical reliance on pathogen detection results. A smoking history was documented in 19.66% of patients, and the median length of hospital stay was 8 days. Most patients presented with mild to moderate disease severity, with CURB-65 scores of 1–3 accounting for over 96% of cases (Table 1). Significant differences across specimen types were observed for treatment adjustment rates, smoking status, and CURB-65 scores (Figure 1C–E), suggesting that the sampling strategy was influenced by both clinical severity and patient characteristics.

Overall Diagnostic Sensitivity of Different Methods

Across all specimen types, targeted third-generation sequencing (tTGS) demonstrated the highest diagnostic sensitivity, identifying pathogens in 353 of 356 clinically confirmed CAP cases (99.16%). This performance exceeded that of targeted next-generation sequencing (tNGS), which detected pathogens in 343 cases (96.35%), and was markedly superior to conventional culture, which yielded positive results in only 93 of 185 cases (50.27%).

Table 1. Summary of Clinical Characteristics of Patients.

Characteristics	Overall (n = 356)
Gender	
Female	164 (46.07%)
Male	192 (53.93%)
Age	
Mean (SD)	38.54 (29.81)
Median [IQR]	39.50 [7.00, 67.00]
Sample_type	
Throat swab	141 (39.61%)
Sputum	128 (35.96%)
BALF	87 (24.44%)
Smoking	
N	283 (79.49%)
Y	70 (19.66%)
Missing	3 (0.84%)
Treatment adjustment	
N	128 (35.96%)
Y	224 (62.92%)
Missing	4 (1.12%)
Hospital stay	
Mean (SD)	9.04 (5.56)
Median [IQR]	8.00 [7.00, 10.00]
Missing	7 (1.97%)
CURB_65 score	
0	3 (0.84%)
1	191 (53.65%)
2	74 (20.79%)
3	78 (21.91%)
4	9 (2.53%)
Missing	1 (0.28%)
TB-specific T cells	
positive	40 (11.24%)
negative	37 (10.39%)
Missing	279 (78.37%)
CRP	
Mean (SD)	37.98 (51.26)
Median [IQR]	16.50 [4.30, 48.50]
Missing	19 (5.34%)
PCT	
Mean (SD)	0.23 (0.62)
Median [IQR]	0.08 [0.05, 0.13]
Missing	18 (5.06%)
WBC	
Mean (SD)	8.8 (4.17)
Median [IQR]	8.11 [6.00, 10.44]
Missing	17 (4.78%)
Neutrophil ratio (%)	
Mean (SD)	66.65 (14.41)
Median [IQR]	67.00 [57.30, 76.90]
Missing	17 (4.78%)
Lymphocyte ratio (%)	
Mean (SD)	24.07 (12.65)
Median [IQR]	22.50 [14.60, 33.05]
Missing	17 (4.78%)
(1,3)-β-D-glucan (pg/mL)	
Mean (SD)	40.96 (13.89)
Median [IQR]	37.50 [37.50, 37.50]
Missing	291 (81.74%)
Galactomannan	
Mean (SD)	0.06 (0.03)
Median [IQR]	0.05 [0.04, 0.07]
Missing	327 (91.85%)
MP-IgM (COI)	
Mean (SD)	0.97 (2.27)
Median [IQR]	0.19 [0.05, 0.67]
Missing	75 (21.07%)
MP-IgG (AU/mL)	
Mean (SD)	87.7 (105.69)
Median [IQR]	35.80 [10.27, 121.00]
Missing	242 (67.98%)
CP-IgM (COI)	
Mean (SD)	0.13 (0.19)
Median [IQR]	0.06 [0.04, 0.12]
Missing	235 (66.01%)
CP-IgG (AU/mL)	
Mean (SD)	75.24 (72.75)
Median [IQR]	56.85 [24.60, 104.00]
Missing	276 (77.53%)

Table 2. Sensitivity Results Across All Sample Types.

Testing Method	Clinical confirmed cases		Sensitivity (%)
	Positive	Negative	
Conventional Culture (n = 185)			50.27
Positive	93	0	
Negative	92	0	
tNGS (n = 356)			96.35
Positive	343	0	
Negative	13	0	
tTGS (n = 356)			99.16
Positive	353	0	
Negative	3	0	

Statistical comparison showed that tTGS achieved significantly higher sensitivity than tNGS ($p < 0.05$) and culture ($p < 0.01$). These findings indicate that sequencing-based approaches, particularly long-read tTGS, are better able to capture the microbial complexity in CAP than culture-dependent diagnostics (Table 2).

Diagnostic Sensitivity Stratified by Specimen Type

When stratified by specimen type, diagnostic performance varied substantially among methods.

In sputum samples, detection rates were 100% for tTGS, 98.43% for tNGS, and 66.02% for culture. In throat swabs, tTGS achieved a detection rate of 100%, tNGS 97.16%. BALF samples exhibited lower overall sensitivity across all methods. Nevertheless, tTGS (96.60%) and tNGS (92.05%) markedly outperformed culture (30.49%).

These results suggest that, while BALF is often considered a high-quality lower respiratory specimen, sequencing-based detection remains strongly influenced by microbial load and specimen characteristics. Overall, sputum and throat swabs demonstrated higher detection sensitivity than BALF across sequencing platforms ($p < 0.05$) (Tables 3–5), suggesting that sample type significantly influences diagnostic performance.

Table 3. Sensitivity Results of Sputum Samples.

Testing Method	Clinical confirmed cases		Sensitivity (%)
	Positive	Negative	
Conventional Culture (n = 103)			66.02
Positive	68	0	
Negative	35	0	
tNGS (n = 127)			98.43
Positive	125	0	
Negative	2	0	
tTGS (n = 127)			100
Positive	127	0	
Negative	0	0	

Table 4. Sensitivity Results of Throat Swab Samples.

Testing Method	Clinical confirmed cases		Sensitivity (%)
	Positive	Negative	
tNGS (n = 141)			97.16
Positive	137	0	
Negative	4	0	
tTGS (n = 141)			100
Positive	141	0	
Negative	0	0	

Table 5. Sensitivity Results of BALF Samples.

Testing Method	Clinical confirmed cases		Sensitivity (%)
	Positive	Negative	
Conventional Culture (n = 82)			30.49
Positive	25	0	
Negative	57	0	
tNGS (n = 88)			92.05
Positive	81	0	
Negative	7	0	
tTGS (n = 88)			96.6
Positive	85	0	
Negative	3	0	

Pathogen Spectrum Identified by Different Diagnostic Modalities

The breadth of pathogens detected differed substantially among the three diagnostic modalities.

Conventional Culture

Among the 185 samples processed by conventional culture, 27 microbial species were identified, including 21 bacterial and 6 fungal species. The most frequently isolated organisms were *Klebsiella pneumoniae* (14.05%), *Candida albicans* (8.65%), *Pseudomonas aeruginosa* (5.95%), and *Acinetobacter baumannii* (4.32%). The limited diversity observed reflects the inherent constraints of culture-based diagnostics, particularly for fastidious or slow-growing organisms.

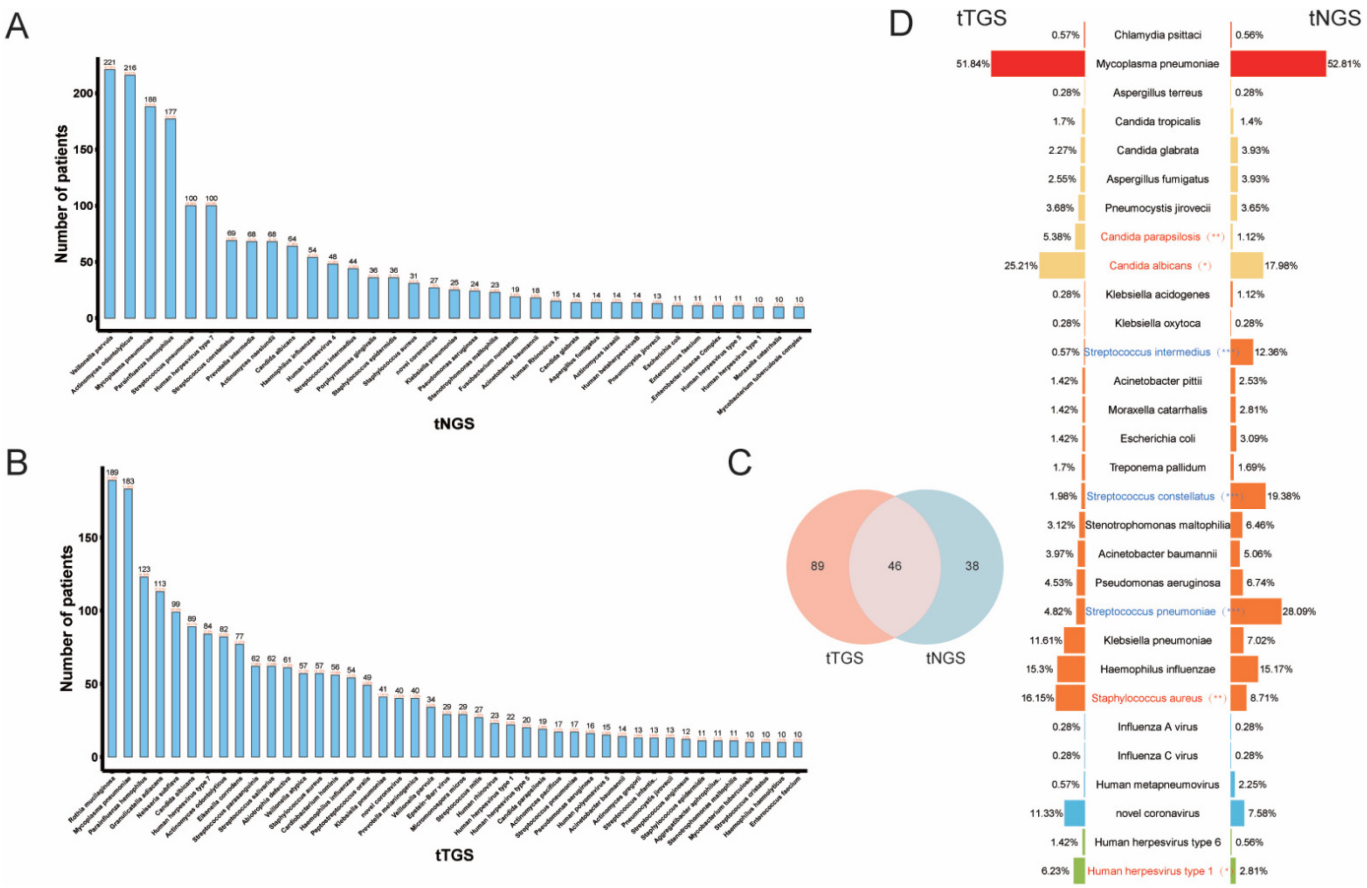
Targeted Next-Generation Sequencing (tNGS)

In contrast, tNGS identified a substantially broader pathogen spectrum across 356 samples, detecting a total of 84 microbial species, including 42 bacteria, 29 viruses, 9 fungi, 2 special pathogens, and multiple atypical pathogens such as *Mycoplasma pneumoniae* and *Chlamydia spp*. Notably, organisms traditionally regarded as oral or upper respiratory commensals—such as *Veillonella parvula* (62.08%), *Actinomyces viscosus* (60.67%), *Mycoplasma pneumoniae* (52.81%), *Haemophilus parainfluenzae* (49.72%), and *Streptococcus pneumoniae* (28.09%) — were frequently detected, suggesting complex microbial profiles in CAP patients (Figure 2A).

Targeted Third-Generation Sequencing (tTGS)

tTGS revealed the most diverse microbial landscape, identifying 135 species across all samples. These included 90 bacterial species, 24 viruses, 15 fungi, and multiple atypical pathogens. The most frequently detected organisms were *Rothia mucilaginosa* (53.54%), *Mycoplasma pneumoniae* (51.84%), *Parainfluenza hemophilus* (34.84%), *Streptococcus mitis* (32.01%), *Neisseria subflava* (28.05%), and *Candida albicans* (25.21%) (Figure 2B). The expanded detection breadth of tTGS highlights its ability to capture a more comprehensive representation of the respiratory microbial environment in CAP.

Figure 2. A: Microbial distribution detected by tNGS. **B:** Microbial distribution detected by tTGS. **C:** Venn diagram showing microbial species detected by tTGS and tNGS. **D:** Detection rates and significance results of microorganisms identified by both methods; red text indicates significant detection in tTGS, blue text indicates significant detection in tNGS.



Comparative Analysis Between tNGS and tTGS

Both sequencing methods commonly detected thirty microbial species. However, significant differences were observed for seven organisms. tTGS showed higher detection rates for *Candida parapsilosis, Candida albicans,* and *Staphylococcus aureus,* as well as human herpesvirus 1 (HHV-1), whereas tNGS more frequently identified *Streptococcus intermedius, Streptococcus constellatus,* and *Streptococcus pneumoniae* ($p < 0.05$) (Figure 2C, D).

These platform-dependent discrepancies likely reflect fundamental technical differences between short-read and long-read sequencing strategies. The superior performance of tTGS in detecting *Candida species* and *HHV-1* may be attributed to its long-read capability, which enables improved resolution of repetitive genomic regions, complex genome architectures, and high GC-content sequences, thereby facilitating more accurate taxonomic assignment and read mapping for fungal and viral genomes. In addition, long-read sequencing is less affected by amplification bias and exhibits enhanced tolerance to structural variation, further improving the detection of organisms with large or repetitive genomes.

Conversely, the higher detection rates of *Streptococcus species* by tNGS may result from its optimized short-read library preparation, higher per-base accuracy, and targeted enrichment efficiency for bacterial genomic fragments, which collectively enhance sensitivity toward low-abundance bacterial pathogens. Moreover, the relatively small genome sizes and conserved genomic structures of *Streptococcus species* favor efficient capture and amplification during short-read sequencing workflows, potentially contributing to their preferential detection by tNGS.

Taken together, these findings indicate intrinsic platform-specific detection biases driven by differences in read length, sequencing chemistry, and library construction strategies. Such complementary detection profiles suggest that the combined application of short- and long-read sequencing technologies may provide a more comprehensive and balanced assessment of complex microbial communities in clinical metagenomic diagnostics.

Detection of Mixed Infection

Mixed infections were commonly identified by sequencing-based methods. tTGS detected polymicrobial infections in 303 cases (86.24%), while tNGS identified mixed infections in 312 cases (87.64%). In contrast, conventional culture failed to identify any mixed infections. In mixed-infection detection, tTGS significantly improves diagnostic yield compared with conventional culture, while demonstrating performance comparable to that of tNGS.

The most prevalent mixed-infection patterns involved combinations of bacteria, viruses, fungi, and *Mycoplasma pneumoniae*, underscoring the polymicrobial nature of CAP. These findings indicate that sequencing-based diagnostics provide a more comprehensive view of coexisting microorganisms that may contribute to disease pathogenesis (Table 6).

Consistency Analysis Between Diagnostic Methods

The highest concordance was observed between tNGS and tTGS, with an overall agreement rate of 96.07%. In comparison, concordance between culture and sequencing methods was markedly lower (51.89%).

Subgroup analysis by specimen type showed the strongest agreement between sequencing methods in sputum samples (98.43%), followed by throat swabs

Table 6. Detection of Mixed Infections by Different Methodologies.

Types of Mixed Infections	tTGS (n = 356)	tNGS (n = 356)	Conventional Culture (n = 185)
Virus	2 (0.56%)	1 (0.28%)	0 (0.00%)
Bacteria	44 (12.36%)	43 (12.08%)	67 (36.22%)
Fungus	0 (0.00%)	0 (0.00%)	22 (11.89)
Mycoplasma	4 (1.12%)	0 (0.00%)	0 (0.00%)
Virus + Mycoplasma	1 (0.28%)	0 (0.00%)	0 (0.00%)
Bacteria + Fungus	17 (4.78%)	9 (2.53%)	0 (0.00%)
Bacteria + Chlamydia	1 (0.28%)	0 (0.00%)	0 (0.00%)
Bacteria + Virus	37 (10.39%)	55 (15.45%)	0 (0.00%)
Bacteria + Mycoplasma	62 (17.42%)	87 (24.44%)	0 (0.00%)
Bacteria + Virus + Mycoplasma	54 (15.17%)	78 (21.91%)	0 (0.00%)
Bacteria + Fungus+ Chlamydia	1 (0.28%)	1 (0.28%)	0 (0.00%)
Bacteria + Fungus+ Mycoplasma	33 (9.27%)	11 (3.09%)	0 (0.00%)
Bacteria + Fungus+ Virus	68 (19.10%)	58 (16.29%)	0 (0.00%)
Bacteria + Virus + Chlamydia	0 (0.00%)	1 (0.28%)	0 (0.00%)
Bacteria + Fungus+ Virus + Mycoplasma	28 (7.87%)	12 (3.37%)	0 (0.00%)
Bacteria + Virus + Fungus + Mycoplasma	1 (0.28%)	0 (0.00%)	0 (0.00%)
Negative	3 (0.84%)	0 (0.00%)	96 (51.89%)

(97.16%) and BALF (90.61%). These results indicate high consistency between short-read and long-read sequencing platforms, while also highlighting the limited overlap between sequencing-based and culture-based diagnostics.

Antimicrobial Resistance Gene Detection

Antimicrobial resistance (AMR) genes were detected more frequently by tTGS than by tNGS. Specifically, tNGS identified resistance determinants in 174 samples (48.88%), whereas tTGS detected AMR genes in 210 samples (58.99%), representing a statistically significant difference ($p = 0.008$) (Table 7).

Both methods most commonly detected macrolide resistance–associated mutations in *Mycoplasma pneumoniae*. Importantly, tTGS uniquely identified complex resistance profiles involving multiple genes in four patients, suggesting an advantage of tTGS in long-read sequencing for resolving linked or composite resistance structures (Table 8).

Clinical Impact of Sequencing-Guided Therapy

To rigorously control for potential confounding effects of underlying diseases and prior medication exposure, we restricted our analysis to patients without pre-existing conditions and without a recent history of medication use. Among 221 patients without underlying diseases or confounding treatments, sequencing results informed therapeutic adjustments in 150 cases (67.87%). Among AMR-positive patients with complete clinical follow-up and no underlying diseases (n = 106), enabling robust outcome analysis, antimicrobial regimens were frequently adjusted, predominantly toward doxycycline- or rifamycin-based therapies.

Following regimen optimization, nearly 80% of AMR-positive patients showed clinical improvement, supporting the practical clinical value of sequencing-guided pathogen and resistance profiling in CAP management.

Discussion

Community-acquired pneumonia (CAP) continues to impose a substantial global health burden, and timely identification of causative pathogens is essential for guiding appropriate antimicrobial therapy [1].

Table 7. Results of Antimicrobial Resistance Gene Types Detected by Different Testing Methods.

Testing methods	Number of samples
tNGS	
23S rRNA A2063G	159
OXA-23	5
NDM	2
KPC	3
CTX-M	3
OXA-48	1
mecA	1
tTGS	
MP_23SrRNA_V	183
CTX_M	12
mecA	10
NDM	4
OXA-23	4
VIM	1

However, conventional diagnostic methods—including culture and PCR [21]—remain limited by restricted detection breadth [22], prolonged turnaround time [23], and low sensitivity [24,25], especially for fastidious [26] or slow-growing organisms [27,28]. In recent years, sequencing-based diagnostic approaches have shown considerable promise in overcoming these limitations [29,30]. In this real-world study, we systematically compared targeted third-generation sequencing (tTGS) with targeted next-generation sequencing (tNGS) and conventional culture across a large cohort of CAP patients. We demonstrated clear advantages of tTGS in multiple diagnostic dimensions [14,31].

Diagnostic Sensitivity and Pathogen Detection Performance

Our findings show that tTGS achieved the highest overall sensitivity (99.16%), exceeding both tNGS (96.35%) and culture (50.27%). These results are consistent with previous studies reporting superior sensitivity of long-read sequencing technologies for respiratory pathogen detection. The ability of tTGS to detect a broader range of microorganisms—including rare, difficult-to-culture, or slow-growing species—likely contributes to its enhanced performance. One of the most prominent advantages of tTGS observed in this study was its broader pathogen spectrum. tTGS identified 135 microbial species, substantially more than tNGS (84 species) and culture (27 species), including fastidious bacteria, viruses, fungi, and atypical pathogens. This expanded detection breadth is

Table 8. Detection of Resistance Genes in 4 Patients Using Different Testing Methods.

patient_ID	tNGS Drug resistance results	tTGS Drug resistance results	Sample Type
P161	23S rRNA A2063G	MP_23SrRNA_V; mecA	**Throat Swab**
P241	23S rRNA A2063G	MP_23SrRNA_V; mecA	**Throat Swab**
P328	23S rRNA A2063G	MP_23SrRNA_V; mecA	**Sputum**
P341	23S rRNA A2063G	MP_23SrRNA_V; VIM	BALF

particularly relevant for CAP, which often involves complex polymicrobial interactions and atypical organisms that may evade conventional diagnostic techniques.

A notable observation was the difference in detection patterns between tNGS and tTGS for certain microorganisms. tTGS detected higher rates of *Candida spp.*, *Staphylococcus aureus*, and HHV-1, while tNGS more frequently identified members of the *Streptococcus anginosus* group and *Streptococcus pneumoniae*. These discrepancies may reflect inherent differences between long-read and short-read sequencing technologies, particularly in resolving repetitive genomic regions, GC-rich loci [32], or highly conserved genes [33]. Long-read sequencing may also reduce primer bias and improve genome coverage [34]. In contrast, short-read sequencing offers higher per-base accuracy [35], which may benefit the detection of organisms with conserved or repetitive structures [36]. These technical differences warrant further study to optimize pathogen identification pipelines across platforms.

Mixed Infection Detection

Mixed infections were common in our cohort, with sequencing methods identifying co-infections in more than 85% of cases. In contrast, conventional culture detected no mixed infections, highlighting a major limitation of culture-based diagnosis in polymicrobial respiratory diseases. The most frequent mixed-infection patterns involved combinations of bacteria, viruses, fungi, and Mycoplasma pneumoniae, underscoring the complex microbial ecology of CAP. Accurate detection of mixed infections is clinically important because co-pathogens can modify disease severity [37,38], alter host immune responses, and influence therapeutic strategies [39]. The substantially higher detection rate of mixed infections by tTGS suggests that long-read sequencing may be particularly valuable in settings where polymicrobial involvement is suspected.

Antimicrobial Resistance Gene Profiling

Rapid identification of antimicrobial resistance (AMR) determinants is essential for guiding precision therapy [40]. Consistent with recent epidemiological trends, macrolide resistance in *Mycoplasma pneumoniae* was frequently detected, reinforcing concerns about rising rates of resistance among respiratory pathogens [18]. The superior long-read capability of tTGS also translated into improved profiling of antimicrobial resistance. tTGS detected a significantly higher proportion of AMR genes than tNGS and uniquely identified composite resistance gene structures in multiple cases. These findings highlight the clinical relevance of long-read sequencing for resolving linked resistance determinants and complex genomic arrangements that may be fragmented or missed by short-read technologies.

Importantly, sequencing results influenced clinical management. Among patients without underlying diseases, nearly 70% underwent treatment modification based on sequencing findings, and 80% of AMR-positive patients improved following regimen optimization. These observations suggest that sequencing-guided therapy has tangible clinical benefits and may contribute to more rational antibiotic use.

Study Limitations

Several limitations warrant consideration. First, the retrospective single-center design may limit generalizability. Second, although integrated clinical adjudication was applied, distinguishing colonization from true infection—particularly in upper respiratory specimens—remains challenging. Third, variations in sample types and microbial load may influence detection sensitivity. Future prospective multicenter studies incorporating standardized sampling protocols and host-response biomarkers are needed to further validate the clinical utility of tTGS.

Future Directions

Prospective, multicenter studies with standardized sampling methods are needed to validate the diagnostic accuracy and clinical utility of tTGS. Integrating host-response biomarkers with sequencing data may further improve pathogen interpretation and help differentiate colonization from infection. Additionally, cost-effectiveness analyses will be crucial for determining how sequencing-based diagnostics can be incorporated into routine clinical workflows.

Conclusions

In summary, this real-world study demonstrates that targeted third-generation sequencing enhances pathogen detection compared with conventional culture and offers diagnostic performance comparable to that of targeted next-generation sequencing. Its ability to characterize complex microbial communities and antimicrobial resistance determinants supports its potential integration into clinical workflows for managing community-acquired pneumonia. Larger prospective multicenter studies are warranted to further validate its clinical and economic impact.

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Data Availability
The sequencing data mentioned in this paper are available in the Genome Sequence Archive (GSA) under project HRA015268. Other data are available from the corresponding author or the first author (Name: Ye Yuan/Nianhong Lu; Email: yuanye@jlu.edu.cn/lunianhong@jlu.edu.cn) upon reasonable request.

Ethics and Consent to Participate
This study was conducted in accordance with the ethical standards of the Declaration of Helsinki. It was approved by the Institutional Review Board (IRB) / Ethics Committee of The First Hospital of Jilin University (Approval No. 2025-557). For retrospective studies or those using anonymized data, the ethics committee waived the requirement for informed consent, as appropriate.

Authors Contributions
All authors contributed to the conception and design of the study. Nianhong Lu, Jin Guo, Caihong Liu, and Jiangyuan Wang performed the experiments and collected the data. Danhua Wang analyzed and interpreted the data. Xue Li and Ye Yuan drafted the manuscript. All authors critically revised the manuscript for important intellectual content, approved the final version to be published, and agree to be accountable for all aspects of the work.

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Conflict of interest
No conflict of interest is declared.

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