

Clinical Relevance of Multiplex PCR for Respiratory Pathogens in Hospitalized Adults with Influenza-Like Illness

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ABSTRACT

Objective: Influenza-like illness (ILI) is a major cause of hospitalization during respiratory virus seasons. This study aimed to investigate the pathogens detected in patients presenting with ILI symptoms and to analyze the results in conjunction with clinical, laboratory, and radiological findings.

Materials and Methods: This retrospective, single-center study included 446 hospitalized adults with ILI between epidemiological week 40 of 2024 and week 20 of 2025. Respiratory pathogens were identified using a multiplex real-time polymerase chain reaction (PCR) assay detecting 18 viral and 5 bacterial pathogens. Demographic, clinical, laboratory, and radiological data were retrieved from electronic medical records. Disease severity was assessed using the CURB-65 score. Patients with *Streptococcus pneumoniae* detected by PCR were further evaluated according to radiological evidence of pneumonia.

Results: At least one respiratory pathogen was detected in 184/446 patients (41.2%), whereas no pathogen was identified in 262 patients (58.8%). The most frequently detected pathogens were *S. pneumoniae*, influenza viruses, and seasonal coronaviruses. The most common coinfection was influenza A plus *S. pneumoniae*. Among patients with pneumococcal PCR positivity, those with radiologically confirmed pneumonia were significantly older, had higher CURB-65 scores, and more frequently had concomitant influenza infection than patients without radiological evidence of pneumonia.

Conclusion: Multiplex molecular assays provide valuable information for respiratory pathogen detection, but molecular results, especially for bacterial pathogens such as *S. pneumoniae*, should be interpreted together with clinical and radiological findings.

Keywords: Adult, co-infection, influenza-like illness, molecular test, *Streptococcus pneumoniae*.



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INTRODUCTION

Influenza-like illness (ILI) is a general term used for respiratory infections caused by influenza and other respiratory viruses. These agents cannot be distinguished based solely on clinical findings.

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ILI imposes a significant economic burden by increasing healthcare costs and hospitalization rates.^{1,2}

According to the European Centre for Disease Prevention and Control (ECDC), ILI is defined by the acute onset of symptoms, with at least one systemic symptom (fever, headache, myalgia, or fatigue) and at least one respiratory symptom (cough, shortness of breath, or sore throat).³ However, these symptoms are not specific to any single pathogen and can occur in both viral and bacterial infections. Consequently, the frequent administration of empirical antibiotic therapy contributes to increasing antibiotic resistance and healthcare expenditures. This issue is particularly critical in countries where per capita daily antibiotic use is high.^{4,5}

The viruses most commonly responsible for acute respiratory infections include respiratory syncytial virus (RSV), influenza A and B viruses (IV), parainfluenza virus types 1, 2, and 3 (PIV), adenovirus (AV), coronavirus (CoV), rhinovirus (RV), enterovirus (EV), bocavirus (BoV), and metapneumovirus (MPV).⁶ Due to the numerous subtypes of these viruses and the lack of long-lasting immunity, diagnostic methods that directly detect viral nucleic acids are preferred.⁷ Although viral pathogens are the predominant cause of respiratory tract infections, several bacterial agents may present with clinical manifestations that overlap with those of ILI. *Streptococcus pneumoniae*, *Mycoplasma pneumoniae*, *Legionella pneumophila*, and *Bordetella pertussis* are therefore included in multiplex respiratory panels because of their clinical relevance and the value of early etiological identification in guiding patient management.⁸ Recently developed rapid molecular diagnostic methods allow pathogen detection within hours of specimen collection. These tests facilitate the timely initiation of appropriate antibacterial or antiviral therapy and enable optimal cohorting of hospitalized patients. Compared with conventional diagnostic methods, rapid molecular tests can identify pathogens 2–3 days earlier, thereby reducing inappropriate antibiotic use.⁹

The aim of this study was to retrospectively investigate the pathogens detected in patients presenting with ILI during the 2024–2025 winter season and to analyze the results in conjunction with clinical, laboratory, and radiological findings.

MATERIALS AND METHODS

Study Design and Setting

This retrospective, single-center observational study was conducted at Balıkesir Atatürk City Hospital between October 2024 and May 2025, corresponding to epidemiological week 40 of 2024 through week 20 of 2025. The study population consisted of hospitalized patients admitted to hospital wards or intensive care units with symptoms consistent with ILI.

KEY MESSAGES

- Multiplex PCR provides rapid and comprehensive pathogen detection and may support timely clinical decision-making. However, its diagnostic interpretation differs between viral and bacterial targets. In particular, *S. pneumoniae* detection from nasopharyngeal specimens may reflect colonization or, potentially, nonspecific detection, limiting its ability to establish pneumococcal pneumonia on its own.
- Multiplex PCR results should be interpreted together with clinical severity, radiological findings, and, when available, conventional microbiological data.
- This integrated approach may help distinguish probable pneumococcal disease from colonization and support more appropriate antimicrobial treatment decisions.

Ethical Approval

Ethical approval was obtained from Balıkesir Atatürk City Hospital Scientific Research Ethics Committee (Approval Number: 2025/07/77, Date: 24.07.2025). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Patients and Data Collection

A total of 446 patients with available respiratory polymerase chain reaction (PCR) panel results were included in the study. Patient demographic and clinical data, including age, sex, admission date, symptoms, comorbidities, laboratory findings, radiological findings, and clinical follow-up data, were retrieved from the hospital information management system and electronic medical records.

Computed tomography (CT) findings obtained concurrently with respiratory testing were evaluated through the Picture Archiving and Communication System (PACS). In addition, discharge summary reports were reviewed when available. The CURB-65 score was calculated for each patient (confusion, urea, respiratory rate, blood pressure, and age ≥ 65 years).

Patients were stratified into three age groups: 18–45 years, 46–64 years, and ≥ 65 years.

Diagnostic Criteria

ILI was defined as the presence of fever ($\geq 38^{\circ}\text{C}$) accompanied by respiratory symptoms such as cough and/or sore throat.

Definitions

Viral pathogens belonging to the same family were grouped for analysis (e.g., coronaviruses, including 229E, NL63, HKU1, and OC43).

Inclusion Criteria

Patients meeting the following criteria were included:

- Age ≥ 18 years
- Hospitalization due to ILI symptoms
- Availability of respiratory PCR panel results
- Complete clinical and laboratory data

Exclusion Criteria

Patients were excluded if they met any of the following criteria:

- Age < 18 years
- Fever lasting longer than 7 days within the previous 2 weeks
- Prior antibiotic use before hospital admission
- Outpatient status
- Incomplete or missing data

Clinical, Laboratory, and Radiological Investigations

Clinical findings, laboratory parameters, and radiological imaging results were evaluated retrospectively. Routine laboratory tests included hematological and biochemical parameters. Radiological evaluation primarily consisted of thoracic CT imaging when clinically indicated. Disease severity was assessed using the CURB-65 score. The score is based on five factors from which its name is derived:

- Confusion
- Urea
- Respiratory rate
- Blood pressure
- Age ≥ 65 years

Scoring was performed as follows: confusion (1 point), urea level > 7 mmol/L (1 point), respiratory rate ≥ 30 breaths/min (1 point), blood pressure (systolic < 90 mmHg or diastolic ≤ 60 mmHg) (1 point), and age ≥ 65 years (1 point). A total score of 0–1 indicates a low mortality risk, 2 points indicates a moderate mortality risk, and 3–5 points indicates a high mortality risk.

Sample Collection and Respiratory Panel Procedure

Respiratory PCR results obtained using the Bosphore Respiratory Pathogens Panel Kit v10 (Anatolia Geneworks) were analyzed.

Nasal, oropharyngeal, and nasopharyngeal swab samples were collected according to the manufacturer's recommendations. For nasopharyngeal swab collection, the patient's head was hyperextended, and a flocked swab was inserted through the

nostril into the posterior nasopharynx, rotated 2–3 times, and then withdrawn. The swab was immediately placed into a tube containing viral transport medium (VTM).

Samples were transported promptly to the laboratory. If storage was necessary, samples were maintained at room temperature (18–30°C) for up to 4 hours, at 2–8°C for up to 3 days, or at temperatures below –15°C for up to 30 days.

Real-time PCR analysis was performed in accordance with the manufacturer's instructions. Fluorescence detection was conducted using the FAM, HEX, Texas Red, and Cy5 channels. An endogenous internal control (Cy5) was used to evaluate sampling adequacy and procedural accuracy, while an exogenous internal control (Texas Red) was used to monitor nucleic acid extraction and PCR inhibition.

Threshold cycle (Ct) values were considered valid if ≤ 32 for internal controls and ≤ 28 for positive controls. Samples exceeding the threshold values in the FAM, HEX, Texas Red, or Cy5 channels were interpreted as positive. As results were reported qualitatively as positive or negative, analyses were based on qualitative data. Raw amplification data, Ct values, and amplification curves were not routinely archived and were therefore unavailable retrospectively.

The panel detected the following pathogens: parainfluenza virus (PIV) 1–4, enterovirus (EV), human metapneumovirus (hMPV), respiratory syncytial virus (RSV A/B), bocavirus (BoV), rhinovirus (RV), coronaviruses (229E, NL63, HKU1, OC43), adenovirus (AV), influenza A (H1N1, H3, H1), influenza B, parechovirus, *Haemophilus influenzae* type B, *Mycoplasma pneumoniae*, *Streptococcus pneumoniae*, *Legionella pneumophila*, and *Bordetella pertussis*.

Statistical Analysis

Statistical analyses were performed using SPSS version 22.0 (IBM Corp., Armonk, NY, USA). The normality of continuous variables (e.g., age and laboratory parameters) was assessed using the Kolmogorov–Smirnov and Shapiro–Wilk tests. As the variables were not normally distributed, continuous variables were expressed as median (interquartile range) and compared between groups (e.g., pathogen-positive vs. pathogen-negative patients) using the Mann–Whitney U test.

Categorical variables, including pathogen detection status (positive/negative), age groups, sex, and the presence of single- or dual-pathogen infections, were presented as frequencies and percentages. Comparisons between categorical variables were performed using the chi-square test (e.g., pathogen detection by age group and sex and dual-pathogen infection by sex).

A p value of < 0.05 was considered statistically significant.

Table 1. General characteristics of the patient population

Patient characteristics	Pathogen (–) (n=262) (%)	Pathogen (+) (n=184) (%)	p
Sex			0.900
Female	113 (43.1)	80 (43.5)	
Male	149 (56.9)	104 (56.5)	
Age group (years)			0.400
18–45	20 (7.6)	13 (7.1)	
46–64	59 (22.5)	33 (17.9)	
≥65	183 (69.8)	138 (75.0)	
Clinical setting			0.300
Ward	194 (74.0)	144 (78.3)	
Intensive care unit (ICU)	68 (26.0)	40 (21.7)	

Table 2. Distribution of mono- and co-infections by sex and age group

	Mono-infection (n=148) (%)	Co-infection (n=36) (%)	p
Sex			0.030
Female	70 (47.3)	10 (27.8)	
Male	78 (52.7)	26 (72.2)	
Age group (years)			0.090
18–45	12 (8.1)	1 (2.8)	
46–64	30 (20.3)	3 (8.3)	
≥65	106 (71.6)	32 (88.9)	

RESULTS

A total of 446 hospitalized adult patients with ILI were included in the study. Respiratory PCR testing identified at least one pathogen in 184 patients (41.2%), while no pathogen was detected in 262 patients (58.8%). The demographic characteristics of the study population are summarized in Table 1.

Pathogen detection was most frequent among patients aged ≥65 years, followed by those aged 46–64 years. The distribution of detected pathogens according to age group and sex is presented in Table 2. Both single- and dual-pathogen infections were most commonly observed in patients aged ≥65 years.

Among patients with dual-pathogen infections, males constituted the majority (72.2%), and this difference was statistically significant (p=0.030) (Table 2).

The distribution of pathogens detected by respiratory PCR testing showed that *S. pneumoniae*, influenza viruses, and

Table 3. Distribution of pathogens detected by PCR testing

Respiratory pathogen	Subtype	Number
<i>S. pneumoniae</i> (n=64)		64
Influenza (n=59)	Influenza A	26
	Influenza H3	26
	Influenza H1N1	3
	Influenza B	3
	Influenza H1	1
Coronavirus (n=32)	Coronavirus HKU1	19
	Coronavirus NL63	7
	Coronavirus OC43	5
	Coronavirus 229E	1
Rhinovirus/Enterovirus (n=28)		28
Respiratory syncytial virus (n=17)		17
Metapneumovirus (n=11)		11
Adenovirus (n=3)		3
Parainfluenza virus 2 (n=3)		3
Bocavirus (n=2)		2
Parechovirus (n=1)		1
Total		220

PCR : Polymerase chain reaction.

coronaviruses were the most frequently identified pathogens (Table 3). Among cases with co-detected pathogens, the distribution of respiratory pathogens is summarized in Figure 1, with the most common combinations being influenza A + *S. pneumoniae*, RV/EV + *S. pneumoniae*, and RSV + *S. pneumoniae*.

Patients with respiratory PCR positivity for *S. pneumoniae* were categorized based on radiological findings. Patients with imaging features consistent with typical pneumonia—such as lobar involvement, consolidation, or air bronchograms shortly after symptom onset—were classified as Group 2 (radiologically confirmed pneumonia). Patients without these radiological findings were classified as Group 1 (no radiological evidence of pneumonia).

Demographic data, laboratory findings such as CRP and WBC, CURB-65 scores, and clinical findings were analyzed for Groups 1 and 2 (Table 4). In the pneumonia group, median age, CURB-65 score, and the frequency of concomitant influenza infection were significantly higher.

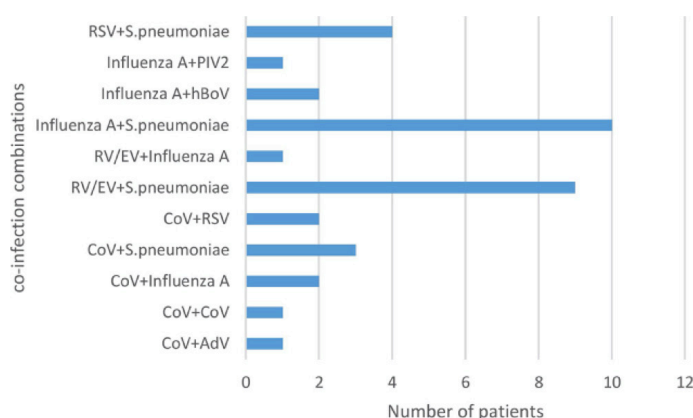
DISCUSSION

This study aimed to elucidate the demographic, epidemiological, clinical, and radiological characteristics of adults hospitalized with ILI during the 2024–2025 season.

Table 4. Evaluation of patients with pneumococcal PCR positivity according to radiological findings (n=64)

Variable	Group 1 (n=47)	Group 2 (n=17)	p
Age, median (IQR)	67 (59–75)	80 (69–90)	0.001
Sex (M/F)	30/17	11/6	0.900
CRP, median (IQR)	7.5 (2.02–13)	10.3 (5.94–18.85)	0.060
WBC, median (IQR)	11,870 (7,897–15,370)	10,880 (7,980–13,540)	0.800
CURB-65, median (IQR)	2 (0–2)	3 (2–3)	<0.0001
Co-infection with influenza	3 (6.4%)	7 (41.2%)	0.002
ICU admission	6 (12.8%)	6 (35.3%)	0.060

Group 1: Radiologically unconfirmed pneumonia (negative); Group 2: Radiologically confirmed pneumonia (positive). CRP: C-reactive protein; CURB-65: Confusion, urea, respiratory rate, blood pressure, and age ≥ 65 years; ICU: Intensive care unit; IQR: Interquartile range; M/F: Male/female; WBC: White blood cell.

**Figure 1.** Distribution of co-infection combinations.

During this season, 446 nasopharyngeal swab (NPS) samples were analyzed in our laboratory, with a positivity rate of 41.2%. In 2018, using a different version of the same device and kit, 3,074 NPS samples from both pediatric and adult patients across 13 hospitals in Türkiye were analyzed, yielding a positivity rate of 48%.¹⁰ In a study conducted in Taiwan using respiratory tract samples from hospitalized adult patients, similar to the patient population in our study, the positivity rate was 22%.¹¹ Mengelle et al.¹² reported a very high viral pathogen positivity rate of 88.7% in a pediatric population. Although PCR tests for respiratory infections are highly sensitive, positivity rates may vary depending on patient demographics, the duration and severity of symptoms, the analytical performance of the test, sample type, and seasonal factors.^{13,14} Consequently, positivity rates differ among studies.

It is noteworthy that in our study, the most frequently detected agent in nasopharyngeal swab samples obtained from patients presenting with ILI findings was *S. pneumoniae*. In adults, nasopharyngeal carriage is theoretically important

for the development of invasive pneumococcal disease (IPD), although it is not considered a strong independent risk factor. A high nasopharyngeal microbial load may facilitate bacterial invasion and microaspiration into the lungs, leading to pneumonia. Evidence suggests that influenza virus can modify host cells to enhance pneumococcal adhesion, invasion, and disease potential.¹⁵ Among the 64 patients with pneumococcal detection, 17 (27%) had radiological findings compatible with pneumonia, while 47 (73%) did not, suggesting colonization. Influenza and *S. pneumoniae* coinfection was observed in 10 patients; 7 (41.2%) were in the radiologically confirmed pneumonia group, while 3 (6.4%) were in the colonization group. This difference was statistically significant ($p=0.002$). Similarly, Karabulut et al.¹⁶ reported a substantial rate of viral–bacterial co-detection (35.53%), highlighting the frequent etiological overlap encountered in routine clinical practice. Consistent with these findings, a meta-analysis of 27 studies showed that *S. pneumoniae* was the most frequently detected bacterial coinfecting pathogen (35%).¹⁷ Therefore, in patients with ILI, secondary pneumococcal infections, in addition to viral agents, should be considered, emphasizing the importance of pneumococcal vaccination during influenza seasons.

Multiplex PCR assays and syndrome-specific panels allow the simultaneous detection of multiple pathogens in a single sample. However, interpreting *S. pneumoniae* results on respiratory panels is challenging because of frequent nonpathogenic colonization of the upper respiratory tract mucosa or potential cross-reactivity with closely related streptococci such as *S. mitis* and *S. oralis*.¹⁸ In this study, the *lytA* gene was used for pneumococcal detection. Although useful, the *lytA* gene is not entirely specific, as homologous sequences exist in *S. mitis* and *S. oralis*, potentially causing false-positive results.^{19,20} Furthermore, the presence of pneumococcal target genes in nonpneumococcal streptococci isolated from oral specimens has raised concerns regarding the specificity of

qPCR-based approaches. Miellet et al.²¹ emphasized that the reliability of molecular pneumococcal detection depends on appropriate specimen collection, the selection of suitable target genes, and the interpretation of results within the clinical context. Despite the high sensitivity and rapid turnaround time of multiplex molecular assays, their role in routine clinical practice—particularly in distinguishing colonization from true infection and in comparison with conventional diagnostic methods—remains under debate. Current guidelines support the use of molecular pneumonia panels because of their superior sensitivity and faster pathogen detection; however, as highlighted by Walker et al.,²² their integration into clinical practice requires methodological standardization and careful interpretation of results in conjunction with clinical findings. Supporting these limitations, Simões et al.²³ reported that real-time PCR targeting *lytA* misidentified three isolates. Thus, PCR-based pneumococcal detection from colonized sites may yield inaccurate results.

In our study, the CURB-65 score was significantly higher in the radiologically confirmed pneumonia group, indicating that pneumococcal PCR positivity alone has limited diagnostic value but may support the likelihood of true pneumonia when combined with clinical scoring. The Turkish Thoracic Society recommends molecular diagnostics in hospitalized patients or those with severe pneumonia, emphasizing interpretation alongside the clinical presentation.²⁴

In their study of pediatric patients, Pernica et al.²⁵ showed that quantitative evaluation of *S. pneumoniae* in the nasopharynx would not provide practical benefit to clinicians attempting to distinguish viral from bacterial respiratory tract infections. Another pediatric case-control study suggested that a colonization density threshold of $>6.9 \log_{10}$ copies/mL yielded 64% sensitivity and 92% specificity for differentiating clinical pneumonia.²⁶ An important limitation of our study is that molecular test results were reported only qualitatively (positive/negative). Therefore, the relationship between Ct values and pathogen burden could not be assessed.

During the 2024–2025 season, according to the WHO global influenza report, influenza A(H1N1)pdm09 predominated in the Americas, Western Africa, Northern Europe, and Oceania, while influenza A(H3N2) predominated in East and South Africa and in West, East, and South Asia. Influenza A(H1N1)pdm09 and influenza A(H3N2) were identified as co-dominant in Southeast Asia.²⁷ In Türkiye, according to Sentinel ILI surveillance from September 30, 2024, to April 20, 2025, influenza A and its subtype A(H3N2) were the most frequently detected.²⁸ Unlike the surveillance data, our study identified influenza A using the MP2 gene region without subtyping, and influenza A and A(H3) were detected at similar frequencies.

In Türkiye, Sentinel Surveillance from September 30, 2024, to April 20, 2025, showed that the most frequent non-influenza respiratory viruses were RV and CoV. In our study, following influenza, the most frequently detected pathogens were CoV, RV, RSV, and hMPV. Kaaijk et al.²⁹ similarly reported seasonal coronaviruses and rhinoviruses as the most common pathogens after influenza in adults aged >60 years. Although hMPV incidence decreased during the COVID-19 pandemic, positivity rates returned to pre-pandemic levels over three consecutive seasons.³⁰

In our study, 47 patients (73%) were PCR positive for *S. pneumoniae*, yet radiological and culture findings did not provide corresponding evidence. While molecular tests are now the reference standard for viral detection,⁷ PCR interpretation for typical bacterial pathogens such as pneumococcus is challenging because of nasopharyngeal colonization. Strålin et al.³¹ showed that sputum samples were superior to nasopharyngeal aspirates for determining pneumococcal etiology in community-acquired pneumonia by PCR. Consistent with this observation, Pickens emphasized the limited value of multiplex PCR testing performed on upper respiratory tract specimens for the etiological diagnosis of bacterial pneumonia, as bacterial pneumonia primarily affects the lower respiratory tract.³² Nevertheless, when interpreted in the appropriate clinical context, rapid and sensitive multiplex molecular panels may still support the diagnosis and management of patients with community-acquired pneumonia.

One limitation of our study is that the rapid respiratory panel was validated only for nasopharyngeal specimens. Although lower respiratory tract specimens may be more appropriate for determining the bacterial etiology of pneumonia, their processing requires additional extraction steps, which may prolong turnaround time. Consequently, nasopharyngeal specimens are frequently preferred in routine clinical practice because they are easier to obtain and allow more rapid reporting. Furthermore, PCR positivity does not necessarily indicate active infection, and qualitative molecular results alone cannot reliably distinguish infection from colonization. In a study by Søgaaard et al.,³³ respiratory panels demonstrated high sensitivity and specificity but a low positive predictive value for the detection of bacterial pathogens, suggesting that molecular detection of bacterial targets may have limited ability to distinguish true infection from colonization. Quantitative or semiquantitative molecular assays may provide additional diagnostic value by estimating bacterial burden. Endicott-Yazdani et al.³⁴ demonstrated, using real-world data, that semiquantitative respiratory panel results may be useful for assessing the clinical significance of detected microorganisms. Nevertheless, molecular bacterial burden should not be

interpreted in isolation but rather in conjunction with clinical findings, radiological features, and culture results. In the present study, Ct values were not systematically recorded; therefore, the association between molecular signal intensity and the clinical relevance of detected bacterial pathogens could not be evaluated. Future prospective studies reporting Ct values together with clinical, radiological, and conventional microbiological findings may provide additional diagnostic value and contribute to distinguishing active infection from colonization. However, Ct values should be interpreted in the context of specimen type, assay platform, and the overall clinical presentation.

CONCLUSION

In conclusion, multiplex PCR assays are valuable for rapid respiratory pathogen detection; however, *S. pneumoniae* positivity in nasopharyngeal samples should be interpreted cautiously, as it may represent colonization rather than active infection. Clinical and radiological correlation remains essential for accurate interpretation.

Ethics Committee Approval: Ethics committee approval was obtained from Balıkesir Atatürk City Hospital Scientific Research Ethics Committee (Approval Number: 2025/07/77, Date: 24.07.2025).

Informed Consent: Informed consent was waived by the ethics committee due to the retrospective nature of the study.

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