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Clinical Evaluation of Targeted Next-Generation Sequencing for Detecting Rifampicin and Isoniazid Resistance in Mycobacterium tuberculosis

Ruiying Ma, Yuanda Li, Qingbo Chen*
Department of Clinical Laboratory, The Sixth People's Hospital of Xinjiang Uygur Autonomous Region, Urumqi 830013, Xinjiang, China

Author to whom correspondence should be addressed.

Abstract: *Objective:* To evaluate the clinical efficacy of targeted next-generation sequencing (NGS) technology in detecting rifampicin and isoniazid resistance in Mycobacterium tuberculosis (MTB). *Methods:* Patient specimens from 31 cases of MTB culture-positive and phenotypically drug-resistant (Drug susceptibility test, DST) collected between May 2024 and November 2025 were analyzed. Targeted NGS was used to detect mutations in resistance genes such as rpoB, katG, and inhA. Using DST as the gold standard, the sensitivity of NGS in detecting rifampicin and isoniazid resistance and its mutation spectrum were analyzed and compared with the PCR melting curve method, with a focus on evaluating NGS's ability to re-detect samples missed by PCR. *Results:* Among 15 rifampicin-resistant samples, NGS detected 13 cases with rifampicin resistance mutations, while 2 cases had no clear rifampicin resistance mutations identified, yielding a sensitivity of 86.67% for detecting resistance, significantly higher than that of the PCR melting curve method. For 17 isoniazid-resistant samples, NGS detected all isoniazid resistance mutations, achieving a sensitivity of 100%, which is also significantly higher than that of PCR. Among the 31 missed resistance mutations by PCR (14 for rifampicin and 17 for isoniazid, with 2 cases missed for both drugs), NGS successfully detected 30, with an overall re-detection sensitivity of 96.77%. NGS also identified various rare mutations and detected multiple heterogeneous resistance samples with mutation frequencies ranging from 0.19 to 0.67, all of which were missed by PCR. *Conclusion:* NGS demonstrates outstanding re-detection capability for rifampicin and isoniazid resistance samples missed by PCR melting curve method and provides comprehensive resistance spectrum information for multiple drugs in a single test. It offers unique advantages in detecting rare mutations and identifying heterogeneous resistance. For cases with negative PCR results but high clinical suspicion of drug resistance, NGS can serve as an important supplementary verification tool, providing crucial evidence for the precise diagnosis and treatment of complex multidrug-resistant tuberculosis.

Keywords: Mycobacterium tuberculosis; Next-generation sequencing; Rifampicin; Isoniazid; Heterogeneous resistance

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1. Introduction

Tuberculosis (TB) is a common chronic infectious disease caused by Mycobacterium tuberculosis (MTB), posing a severe threat to human health and remaining one of the major global public health crises[1]. TB primarily spreads through

the respiratory tract and can affect multiple organs, with pulmonary tuberculosis being the most common. Rifampicin and isoniazid are the most important first-line drugs for clinical TB treatment; however, due to irrational drug use and the spread of drug-resistant strains, the problem of drug resistance in TB persists[2]. Currently, controlling drug-resistant TB, particularly multidrug-resistant tuberculosis (MDR-TB), remains a challenging public health issue[3]. Heterogeneous resistance is a special phenomenon where resistant and sensitive strains coexist within the same patient, widely observed in MTB and reported for anti-tuberculosis drugs such as isoniazid, rifampicin, and streptomycin[4]. Research on heterogeneous resistance helps explore the emergence and development of drug-resistant MTB.

Currently, the traditional phenotypic drug susceptibility test (DST) is the gold standard for diagnosing MTB drug resistance; however, due to the slow growth of MTB, this method requires 6–8 weeks to yield results, failing to meet clinical needs[5]. In recent years, molecular drug susceptibility tests represented by Xpert MTB/RIF and PCR melting curve method have significantly shortened detection times but have notable limitations: Xpert MTB/RIF can simultaneously detect MTB and rifampicin resistance within 2 hours but cannot assess isoniazid resistance[6-7]; PCR melting curve method, linear probe hybridization, and gene chips can detect multiple drug resistance mutations in a single test but rely on probe design principles targeting known hotspot mutations, severely limiting their ability to identify rare, non-classical, and low-abundance heterogeneous mutations outside probe coverage regions[8]. In clinical settings, heterogeneous resistance often leads to missed detections by conventional molecular methods when the resistant subpopulation is low, resulting in false-negative results and significant confusion in clinical decision-making.

Next-generation sequencing (NGS) technology enables high-throughput sequencing of large amounts of DNA simultaneously. In TB diagnosis, unlike metagenomic NGS (mNGS), which sequences all DNA in a sample, targeted NGS can directly and rapidly enrich and detect all known and potential mutation types in dozens of drug resistance genes, including rifampicin, isoniazid, and various second-line drugs, from clinical specimens (e.g., sputum) without traditional mycobacterial culture, using specific techniques such as multiplex PCR[9]. Its detection range is not limited by predefined probe targets, offering theoretical advantages of high sensitivity, high resolution, and the ability to discover unknown or rare mutations[10]. This study retrospectively evaluated the re-detection efficacy of targeted NGS for rifampicin and isoniazid resistance samples missed by PCR melting curve method, using phenotypic DST as the gold standard, to assess its clinical value in filling gaps left by conventional molecular detection methods.

2. Objects and Methods

2.1. Research Subjects

A total of 31 patients with pulmonary tuberculosis who were treated in our hospital from May 2024 to November 2025 were selected as the research subjects, including 18 males and 13 females, aged 31–83 (average 59.13 ± 14.92) years. Types of specimens tested: 7 sputum specimens and 24 tracheal aspirates. All samples underwent drug susceptibility testing (DST), PCR melting curve analysis, and targeted next-generation sequencing (NGS) for drug-resistant gene detection. According to the DST results, the types of drug-resistant Mycobacterium tuberculosis (MTB) were as follows: 15 samples were resistant to rifampicin, and 17 samples were resistant to isoniazid, with 2 samples being resistant to both rifampicin and isoniazid.

Inclusion criteria: ① Met the diagnostic criteria for suspected pulmonary tuberculosis in the “Diagnostic Criteria for Pulmonary Tuberculosis”; ② Sputum or tracheal aspirate specimens were collected for mycobacterial culture and molecular testing, and the culture results were positive for MTB; ③ The patients and their families provided informed consent. Exclusion criteria: ① Had other serious pulmonary diseases such as lung cancer or pulmonary fibrosis; ② Had other vital organ failures; ③ Had contaminated specimens or could not obtain qualified specimens.

2.2. Methods

2.2.1. Specimen Collection

Collect 5 mL of morning sputum specimens from patients. For patients without sputum or with negative sputum smears, collect 10 mL of tracheal aspirate specimens during fiberoptic bronchoscopy.

Sputum specimen processing: After confirming positivity by auramine O staining, add 2–3 times the volume of 4% NALC-NaOH to the sputum specimen and liquefy it for 15 minutes for later use.

Tracheal aspirate specimen processing: Take 10 mL of the specimen, centrifuge it at 3000 r/min for 15 minutes, discard the supernatant, wash the sediment twice with sterile saline, and resuspend it in 1 mL of sterile saline for later use.

2.2.2. Identification of Mycobacterium tuberculosis Strains and Drug Susceptibility Testing

MTB culture and DST were carried out with reference to the "Laboratory Examination Procedures for Tuberculosis". Isolation and culture: Place approximately 0.5 mL of the digested specimen fluid into a liquid culture system (BD BACTEC™ MGIT™ 960 fully automated mycobacterial culture, identification, and drug susceptibility testing system, BD Medical, USA). After the positive report, take the bacterial fluid for Ziehl-Neelsen acid-fast staining to determine whether it is acid-fast bacilli. Strain identification: Strains that grow on thiophene-2-carboxylic acid hydrazide medium but do not grow on nitrobenzoic acid medium are identified as preliminary MTB. DST: Aspirate 100 μL of 0.01 mg/mL bacterial fluid and inoculate it onto the surfaces of 2 control tubes and high-concentration and low-concentration drug-containing culture media. Culture for about 4 weeks. MTB that grows in the control tubes but not in the drug-containing culture media is judged as sensitive; if the number of colonies growing on the drug-containing culture media is > 20, it is judged as resistant. MTB H37Rv was selected as the sensitive control for quality control.

2.2.3. Extraction of Mycobacterium tuberculosis DNA

Take 200 μL of the above-collected and processed samples, and use a magnetic bead-based genomic DNA extraction kit along with an automated nucleic acid extraction instrument (Lab-Aid 824s fully automated nucleic acid extraction instrument, Xiamen Zeesan Biotech Co., Ltd.) to extract total DNA according to the operating instructions. The final elution volume is 50 μL. The extracted DNA was measured for concentration and purity using a UV spectrophotometer. DNA with an A260/A280 ratio between 1.8 and 2.0 and a concentration ≥ 2 ng/μL was considered qualified and stored at -20°C for later use.

2.2.4. PCR Melting Curve Method for Drug-Resistant Tuberculosis Detection

Centrifuge 1 mL of the above specimens at 12000 r/min for 10 minutes, discard the supernatant, and enrich the bacteria. Add 500 μL of TB-DNA extraction solution to resuspend the bacteria, sterilize them in a 100°C metal bath for 10 minutes, and then transfer the bacterial suspension into the sample loading well of the nucleic acid extraction reagent strip. Use an automated nucleic acid extraction instrument (Lab-Aid 824s fully automated nucleic acid extraction instrument, Xiamen Zeesan Biotech Co., Ltd.) to extract nucleic acids. Prepare PCR reaction solutions A/B, then aliquot the PCR reaction solutions into PCR thin-walled reaction tubes, with 20 μL per tube, and add 5 μL of nucleic acid samples or negative/positive controls. Use a PCR analysis system (SLAN-96S fully automated medical PCR analysis system, Shanghai Hongshi Medical Technology Co., Ltd.) for nucleic acid amplification.

2.2.5. Targeted NGS Detection Method

Extract DNA from clinical specimens, construct a targeted sequencing library containing MTB drug-resistant-related genes (including rpoB, katG, inhA, ahpC, gyrA, rrs, embB, pncA, mmpR5, pepQ, rpsL, thyA, etc.), and perform sequencing using a high-throughput sequencing platform (SURFSeq 5000 next-generation high-throughput sequencer, Shenzhen GenDx Biotech Co., Ltd.). After quality filtering of the sequencing data, align it with the MTB reference genome, and use the dedicated bioinformatics software TB-Profiler to analyze drug-resistant gene mutations. Mutation annotations refer

to the WHO tuberculosis drug resistance mutation catalog and international authoritative databases. Mutation credibility and clinical interpretation criteria: A mutation frequency ≥ 0.85 indicates high credibility, suggesting that most bacteria in the population carry the mutation and the drug resistance is basically fixed; a mutation frequency between 0.15 and 0.85 suggests mixed infection or heterogeneous drug resistance, with a high risk of clinical treatment failure; a mutation frequency < 0.15 is likely to be background noise and is usually not used as the main basis for medication.

2.3. Observation Indicators

(1) Using phenotypic DST results as the gold standard, calculate the sensitivity of NGS and PCR melting curve analysis for detecting rifampicin and isoniazid resistance, respectively. Sensitivity = True positives / (True positives + False negatives) × 100%.

(2) For samples with wild-type/undetected results by PCR melting curve analysis but with drug-resistant results by DST, calculate the supplementary detection sensitivity of NGS.

(3) Analyze the types, frequencies, and distributions of drug-resistant gene mutations detected by NGS, with a focus on describing the mutation characteristics that were missed by PCR but detected by NGS.

2.4. Statistical Methods

Descriptive statistical analysis was used. Count data were expressed as the number of cases (n) and percentage (%). Using DST as a reference, the diagnostic performance indicators of targeted NGS detection were calculated.

3. Results

3.1. Screening Results of NGS-Analyzable Samples

In this study, NGS detection results were obtained for a total of 33 patients with DST-suggested drug resistance. According to the mutation frequency screening criteria, the target drug-resistant gene mutation frequency in the NGS result of sample No. 20250834 was 0.124 (< 0.15), which met the background noise exclusion criteria and was excluded. Finally, a total of 32 samples were included in the drug resistance spectrum analysis, including 2 samples from the same patient showing both rifampicin and isoniazid resistance.

3.2. Diagnostic Results and Efficacy of NGS for Rifampicin Resistance Detection

After screening and filtering, among 30 independent DST-resistant samples, there were 15 samples with rifampicin-resistant phenotypes. Among them, 14 samples had wild-type (sensitive) results for rifampicin by PCR melting curve analysis, and 1 sample had no detection result; while targeted NGS detected rpoB gene resistance mutations in 13 samples in the same group and did not detect definite resistance mutations in 2 samples. Therefore, the sensitivity of NGS for detecting rifampicin resistance was 86.67% (13/15), which was significantly better than that of the melting curve method. Detailed results are shown in **Table 1**.

Table 1. Diagnostic Results and Efficacy of NGS for Rifampicin Resistance Detection

DST (n)		PCR melting curve			NGS sequencing	
Mutant		Wild-type	Undetected	Mutant	Wild-type	
Resistant	15	0	14	1	13	2
Sensitive	0	/	/	/	/	/
Total	15	0	14	1	13	2
Sensitivity (%)		0% (0/15)			86.67% (13/15)	

3.3. Mutation Profile of Rifampicin Resistance Detected by NGS

Among the 13 NGS-positive samples, the types of mutations detected by NGS exhibited a diverse distribution. A total of seven different types of rpoB mutations were identified. Notably, the rpoB S450L mutation was the most prevalent, accounting for 38.46% (5/13) of all mutation types. Of particular concern is that NGS also detected rare mutations that were mostly missed by the PCR melting curve method. These include three cases of rpoB V170F, as well as one case each of rpoB S450M, I491F, L452P, H445L, and the deletion mutation c.1312_1314delAAC. Many of these mutation sites are located outside the probe coverage range of the conventional melting curve method or are low-frequency rare mutations, suggesting that NGS has significant advantages in detecting specific mutations. Detailed results are shown in **Table 2**.

Table 2. Distribution of Mutation Types in Rifampicin Resistance Genes Detected by NGS

Mutation site	Number of cases (n = 13)	Proportion (%)	Clinical significance
rpoB S450L	5	38.46	High-level resistance
rpoB V170F	3	23.08	Non-classical mutation, low-level resistance
rpoB S450M	1	7.69	Moderate to high-level resistance
rpoB I491F	1	7.69	Low-level resistance, often associated with MDR
rpoB L452P	1	7.69	Low-level resistance
rpoB H445L	1	7.69	Low-level resistance
rpoB c.1312_1314delAAC	1	7.69	Deletion mutation, resistance

3.4. Diagnostic Results and Efficacy of NGS for Isoniazid Resistance Detection

Among the 30 independent DST-resistant samples, there were 17 samples with an isoniazid-resistant phenotype. For all 17 of these samples, the PCR melting curve method yielded wild-type (sensitive) results for isoniazid resistance. In contrast, targeted NGS detected drug-resistant mutations in the katG or inhA-related genes in all samples within the same group, achieving a sensitivity of 100% (17/17), which was significantly superior to that of the melting curve method. Detailed results are shown in **Table 3**.

Table 3. Diagnostic Results and Efficacy of NGS for Isoniazid Resistance Detection

DST (n)		PCR melting curve (n)		NGS sequencing (n)	
Mutation		Wild-type	Mutation	Wild-type	
Resistant	17	0	17	17	0
Sensitive	0	/	/	/	/
Total	15	0	17	17	0
Sensitivity (%)		0% (0/17)		100% (17/17)	

3.5. Mutation Profile of Isoniazid Resistance Detected by NGS

Among the 17 NGS-positive samples, a total of six different types of isoniazid resistance gene mutations were detected. The katG S315T mutation was the most common, occurring in 12 cases. The remaining mutations included one case each of inhA c.-777C>, katG S315N, katG c.653dupA, katG W300G, and katG A379V. Notably, in the DST-resistant sample with the identification number 20246943, NGS detected a katG A379V mutation of uncertain significance, with a variant frequency of 0.67. In this case, clinical decision-making should be based on the DST result indicating “resistance.” The value of NGS lies in ruling out cross-resistance to other drugs, allowing for the initiation of standardized multidrug-resistant tuberculosis treatment based on the complete drug resistance profile of the sample (e.g., whether it is resistant to

fluoroquinolones). Detailed results are shown in **Table 4**.

Table 4. Distribution of Mutation Types in Isoniazid Resistance Genes Detected by NGS

Mutation site	Number of cases (n = 17)	Proportion (%)	Clinical significance
katG S315T	12	70.59	High-level resistance
inhA c.-777C>T	1	5.88	Low-level resistance, cross-resistance to ethionamide
katG S315N	1	5.88	High-level resistance
katG c.653dupA	1	5.88	Frameshift mutation, high-level resistance
katG W300G	1	5.88	Rare non-classical mutation, may cause high-level resistance
katG A379V	1	5.88	Unknown significance (DST showed resistance)

3.6. Overall Supplementary Detection Efficacy of NGS for Drug-Resistant Mutations Missed by PCR

By aggregating the detection results for both drugs, the PCR melting curve method missed a total of 31 instances of drug-resistant mutations (14 instances for rifampicin + 17 instances for isoniazid, involving 29 independent samples, with 2 samples having missed mutations for both rifampicin and isoniazid). NGS successfully detected 30 of these missed instances (13 for rifampicin + 17 for isoniazid; however, rifampicin resistance in one sample missed by PCR was not detected by NGS), achieving an overall supplementary detection sensitivity of 96.77% (30/31). When considering independent samples, NGS effectively detected and remedied the missed mutations in 28 out of the 29 PCR-missed samples, resulting in a sample-level supplementary detection success rate of 96.55% (28/29). Detailed results are shown in **Table 5**.

Table 5. Distribution of Mutation Types in Isoniazid Resistance Genes Detected by NGS

Drug	Number of missed detections by PCR	Number of successful supplementary detections by NGS	Supplementary detection sensitivity (%)	Number of undetected by NGS	Reason for undetection
Rifampicin	14	13	92.86	1	Possible mutation in non-classical region
Isoniazid	17	17	100	0	/
Total	31	30	96.77	1	/

3.7. Comprehensive Drug Resistance Profile Information Provided by NGS

In addition to rifampicin and isoniazid, targeted NGS simultaneously provides drug resistance gene information for multiple drugs in a single test, including fluoroquinolones (gyrA), aminoglycosides (rrs), ethambutol (embB), pyrazinamide (pncA), bedaquiline/clofazimine (mmpR5, pepQ), streptomycin (rpsL), and aminosalicylic acid (thyA). Among the 31 independent samples in this study, several MDR-TB samples carried concurrent resistance mutations to rifampicin, isoniazid, and fluoroquinolones (gyrA), suggesting molecular characteristics of MDR-TB or pre-extensively drug-resistant tuberculosis (Pre-XDR-TB) and necessitating the initiation of individualized regimens containing new drugs.

4. Discussion

Drug-resistant tuberculosis, particularly multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis, represents a significant challenge in tuberculosis control. Early and rapid diagnosis is crucial for improving patient outcomes and interrupting transmission[11-12]. Although phenotypic drug susceptibility testing (DST) has been the gold

standard, its reliance on the slow growth of Mycobacterium tuberculosis (MTB) results in a diagnostic delay of 6–8 weeks, severely compromising timely clinical intervention. Molecular drug susceptibility testing has substantially shortened the detection cycle; however, methods such as PCR melting curve analysis exhibit notable limitations in detecting heterogeneous resistance and low-abundance mutations, often leading to false-negative results. This study retrospectively evaluated the clinical performance of NGS technology for detecting rifampicin and isoniazid resistance in MTB and compared it with the commonly used PCR melting curve method. The results demonstrated that NGS significantly outperformed PCR melting curve analysis in terms of detection sensitivity, particularly for identifying heterogeneous resistance, and effectively improved the re-detection rate of samples missed by PCR, showcasing its potential as a rapid and accurate tool for diagnosing drug-resistant tuberculosis.

The study findings revealed that among samples phenotypically resistant to traditional DST, targeted NGS exhibited an 86.67% sensitivity for detecting rifampicin resistance, demonstrating excellent agreement with DST results. For isoniazid resistance, NGS achieved a 100% sensitivity, showing high concordance with DST. These results align closely with those of Miaoran W et al.[13], who demonstrated that tNGS could accurately identify the proportion of rifampicin and isoniazid resistance gene mutations in mixed bacterial populations. Additionally, two DST-resistant rifampicin samples (sample IDs 20250589 and 20252153) showed no rpoB mutations detected by NGS, suggesting that rifampicin resistance in these strains may be mediated by mutations in non-canonical regions of the rpoB gene or by other unknown mechanisms. This observation is consistent with reports indicating that approximately 5% of rifampicin-resistant strains do not carry mutations in core regions[9]. Furthermore, one DST-resistant isoniazid sample (sample ID 20246943) only exhibited a katG A379V mutation of uncertain significance, representing a discrepancy between phenotype and genotype. This may be attributed to insufficient coverage of the NGS targeted panel or the presence of other non-canonical resistance mechanisms. These findings underscore that when NGS results are inconsistent with DST phenotypes in clinical practice, DST, as the gold standard, should guide clinical decision-making, while the drug resistance information provided by NGS remains valuable for reference.

The fundamental reason for missed detections by PCR melting curve analysis lies in its inherent technical limitations. This method relies on designing specific probes for known resistance mutation sites and determining mutation presence based on changes in melting temperature during hybridization. However, it cannot effectively identify mutations located outside the probe coverage regions, rare mutations, or frameshift/deletion mutations[14]. The advantages of NGS technology were fully validated in this context. First, NGS performs comprehensive sequencing of the entire length of drug resistance genes, theoretically enabling the detection of mutations at any position without being constrained by predefined probe targets. In this study, complex mutation types such as rpoB V170F, rpoB I491F, katG W300G, rpoB c.1312_1314delAAC, and katG c.653dupA, which were not covered by PCR probes, were successfully detected by NGS, filling this detection gap.

Heterogeneous resistance is a significant contributor to false-negative results in traditional molecular tests, treatment failure, and the progression of drug resistance[4]. Previous studies have shown that even a low-abundance resistant subpopulation (as low as 1%) can rapidly expand under drug selection pressure, leading to treatment failure[15]. Zetola et al.[16] explicitly stated that unrecognized heterogeneous resistance is closely associated with poor clinical outcomes in patients. In this study, targeted NGS identified multiple cases of heterogeneous resistance with mutation frequencies ranging from 0.19 to 0.67 in PCR-missed samples. When a resistant subpopulation coexists with a sensitive wild-type population and mutation abundance is low, the amplification advantage of wild-type sequences can completely mask mutation signals, causing PCR melting curve analysis to report “wild-type.” If patients are treated based solely on a “wild-type” report using standardized regimens, treatment failure or the emergence of acquired resistance is highly likely due to the persistence of the resistant subpopulation[17]. Targeted NGS effectively identifies these resistant subpopulations by precisely quantifying mutation abundance, providing critical warnings for clinical regimen adjustments.

Furthermore, targeted NGS provides comprehensive drug resistance profile information for more than ten drugs, including fluoroquinolones, aminoglycosides, ethambutol, pyrazinamide, and bedaquiline, in a single test. In this study,

among multiple PCR-missed samples, NGS simultaneously revealed combined resistance patterns to rifampicin-isoniazid-fluoroquinolones, suggesting that these patients may have Pre-XDR-TB and requiring long-term regimens containing drugs such as bedaquiline and linezolid. This "one-test-multiple-drugs" capability holds significant clinical value in MDR-TB management.

In summary, targeted NGS technology demonstrates exceptional re-detection performance for rifampicin and isoniazid resistance samples missed by PCR melting curve analysis, exhibiting significant advantages in detecting rare mutations, identifying heterogeneous resistance, and constructing comprehensive drug resistance profiles. For challenging cases with negative PCR results but high clinical suspicion of resistance or discrepancies between DST and PCR results, NGS can serve as an important supplementary verification and precise diagnostic tool. However, this study has certain limitations: the sample size, particularly the number of drug-resistant strains, was relatively small, which may affect the analysis efficacy of certain resistance mutations. Additionally, this study did not include DST-sensitive control samples, preventing the assessment of targeted NGS specificity and potentially affecting the comprehensiveness of statistical efficacy. Future research should involve larger-scale, prospective studies including sensitive strains, further expand the NGS targeted capture regions to cover more potential mutation sites, and optimize bioinformatics analysis workflows to provide stronger data support for improving the diagnostic accuracy and individualized treatment of drug-resistant tuberculosis.

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