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ORIGINAL RESEARCH

Direct sequencing for rapid detection of multidrug resistant *Mycobacterium tuberculosis* strains in Morocco

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Fathiah Zakham[1,4]
Imane Chaoui[1]
Amina Hadbae Echchaoui[2]
Fouad Chetioui[3]
My Driss Elmessaoudi[3]
My Mustapha Ennaji[4]
Mohammed Abid[2]
Mohammed El Mzibri[1]

[1]Unité de Biologie et Recherché Médicale, Centre National de l'Energie, des Sciences et des Techniques Nucléaires (CNESTEN), Rabat, [2]Laboratoire de Génétique Mycobacterienne, Institut Pasteur, Tangier, [3]Laboratoire de Tuberculose Institut Pasteur, Casablanca, [4]Laboratoire de Microbiologie, Hygiène et Virologie, Faculté des Sciences et Techniques, Mohammedia, Morocco

Background: Tuberculosis (TB) is a major public health problem with high mortality and morbidity rates, especially in low-income countries. Disturbingly, the emergence of multidrug resistant (MDR) and extensively drug resistant (XDR) TB cases has worsened the situation, raising concerns of a future epidemic of virtually untreatable TB. Indeed, the rapid diagnosis of MDR TB is a critical issue for TB management. This study is an attempt to establish a rapid diagnosis of MDR TB by sequencing the target fragments of the *rpoB* gene which linked to resistance against rifampicin and the *katG* gene and *inhA* promoter region, which are associated with resistance to isoniazid.

Methods: For this purpose, 133 sputum samples of TB patients from Morocco were enrolled in this study. One hundred samples were collected from new cases, and the remaining 33 were from previously treated patients (drug relapse or failure, chronic cases) and did not respond to anti-TB drugs after a sufficient duration of treatment. All samples were subjected to *rpoB, katG* and *pinhA* mutation analysis by polymerase chain reaction and DNA sequencing.

Results: Molecular analysis showed that seven strains were isoniazid-monoresistant and 17 were rifampicin-monoresistant. MDR TB strains were identified in nine cases (6.8%). Among them, eight were traditionally diagnosed as critical cases, comprising four chronic and four drug-relapse cases. The last strain was isolated from a new case. The most recorded mutation in the *rpoB* gene was the substitution TCG > TTG at codon 531 (Ser531 Leu), accounting for 46.15%. Significantly, the only mutation found in the *katG* gene was at codon 315 (AGC to ACC) with a Ser315Thr amino acid change. Only one sample harbored mutation in the *inhA* promoter region and was a point mutation at the –15p position (C > T).

Conclusion: The polymerase chain reaction sequencing approach is an accurate and rapid method for detection of drug-resistant TB in clinical specimens, and could be of great interest in the management of TB in critical cases to adjust the treatment regimen and limit the emergence of MDR and XDR strains.

Keywords: Morocco, *Mycobacterium tuberculosis*, multidrug resistance, *rpoB*, *katG, inhA* promoter

Introduction

Tuberculosis (TB) remains one of the most prevalent infectious diseases worldwide, especially in developing countries, where the highest burden of TB is found.[1] This is further exemplified by the high mortality and morbidity due to this disease. In this context, the emergence of multidrug resistant (MDR) TB (resistant to at least isoniazid and rifampicin) and extremely drug resistant TB (MDR plus additional resistance to a fluoroquinolone and any second-line injectable drug) cases has worsened the situation.[2]

Correspondence: Mohammed El Mzibri
Unité de Biologie et Recherches Médicales, Centre National de l'Energie, des Sciences et Techniques Nucléaires, BP 1382 RP Rabat, Morocco
Tel +21 25 3771 2751
Fax +21 25 3771 1846
Email mzibri@yahoo.com

According to reports from the World Health Organization (WHO), 250,000 TB patients were diagnosed in 2009,[3] and of these, 30,000 (12%) were notified as MDR TB. The WHO estimated that approximately 650,000 of the 12 million prevalent TB cases in 2010 represented MDR TB.[2] As for extensively drug resistant (XDR) TB, the first case was notified in South Africa in 2005, and by January 2010, 58 countries had reported at least one case of XDR TB.[4]

In Morocco, the incidence of TB has stagnated during recent years and was 81 per 100,000 in 2008 overall, but was significantly higher in several urban centers, or "hot spots". Of the roughly 28,000 new TB cases nationally each year, 12% are retreatment cases (failure treatment, relapse or chronic cases).[5] Among retreatment cases in Morocco, 12.2% are infected with MDR *Mycobacterium tuberculosis* (MTB) strains, whereas 0.4% of MDR TB are among new cases.

In Morocco, as in other resource-limited countries, WHO guidelines recommend the diagnosis of TB by smear microscopy in all new TB cases, and by smear microscopy, culture, and drug susceptibility testing in retreatment cases.[6]

Currently, drug susceptibility testing is mainly based on the proportion method, which is mainly performed on Lowenstein–Jensen medium.[7] It can also be done using MGIT or BACTEC.[8] Culture-based drug susceptibility testing is widely recognized as a reference method but requires several weeks and multiple methodologies to complete.[7] Moreover, it is widely accepted that rapid drug susceptibility testing is critical in preventing emergence and expansion of drug resistance. Hence, there is an urgent need for new, rapid, and effective diagnostics to prevent the emergence and spread of MDR TB.

Drug resistance in MTB develops through spontaneous mutations in target genes, followed by selection of these resistant bacteria upon exposure to anti-TB drugs, which could lead to a sequential accumulation of mutations in specific genes for MTB and consequently MDR TB cases.[9]

Mutations in the rifampicin resistance determining region (RRDR) of the *rpoB* gene, encoding the β-subunit of DNA-dependent RNA polymerase, leads to resistance to rifampicin, which is the key first-line anti-TB drug.[10] Similarly, mutations in the *katG* gene and *inhA* promoter are largely responsible for resistance to isoniazid.[11]

Isoniazid resistance mutations are primarily found in the *katG* gene, encoding the catalase-peroxidase enzyme responsible for activating isoniazid,[12] the *inhA* gene, which encodes the molecular target *inhA* of the activated drug, and the promoter region of the mabA-inhA operon, resulting in overexpression of *inhA*.[13] Mutations in the *katG* gene and *inhA* promoter account for 50%–95% and 15%–34%, respectively, of isoniazid-resistant clinical isolates.[13]

During the past few years, molecular methods have been developed to identify drug resistance causing gene mutations.[14,15] One of latest techniques is the GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA, USA), which can detect mutations in the *rpoB* gene only; due to close association of rifampicin resistance and MDR TB, this technique has been used to detect MDR TB cases.[4] The technique has been thoroughly evaluated[16] and is used in many countries.[17] It has a sensitivity and specificity of 90.4% and 98.4%, respectively.[18]

Moreover, the WHO, as part of the "Stop TB 2006–2015 Strategy",[19] strongly recommends the integration of molecular approaches, especially GeneXpert MTB/RIF as the initial diagnostic test in individuals suspected of having MDR TB or TB associated with human immunodeficiency virus for countries most affected by TB.[4,19]

The polymerase chain reaction (PCR) sequencing-based strategy,[19] designed to detect mutations associated with drug resistance rapidly, is able to provide a same-day diagnosis from culture and even clinical samples with high sensitivity and specificity. This gold standard method can also detect new mutations that could be associated with drug resistance. Automated sequencing has been used by several groups in the clinical setting for detection of the most frequent mutations and has been found to confer excellent benefit for patient care.[20–22]

This study assessed the usefulness of the PCR sequencing approach for *rpoB* and *katG* genes and the *inhA* promoter region for rapid detection of MDR TB strains directly from sputum to guide treatment regimens for MDR TB. Further, this study would be of great interest when evaluating the efficiency with which the GeneXpert can identify MDR TB in Morocco.

Materials and methods

Sampling

The study was performed after gaining approval from the ethics committee of the Pasteur Institute in Casablanca, Morocco, and confidentiality of clinical and laboratory information on patients was preserved. Samples were collected at the Pasteur Institutes in Casablanca and Tangier. Casablanca is the biggest city and the economic capital of Morocco, which accounts for almost one fifth of the total cases of TB recorded in the country, according to the national anti-TB program.[19] The Pasteur Institute in Casablanca receives samples from different Moroccan cities. Tangier is a big city

in the north of Morocco and ranks fourth in the number of TB cases. The Pasteur Institute in Tangier receives samples from different cities in the northern region of the country.

A total of 133 Moroccan patients with confirmed pulmonary TB were included in this study. Three consecutive sputum samples were collected from each patient as recommended by the WHO[23] and inoculated on Lowenstein–Jensen medium after decontamination by N-acetyl-l-cysteine.[24] Direct microscopic examination was performed by the Ziehl–Neelsen method. Part of each decontaminated sputum sample was used to extract MTB DNA for molecular analysis.

Drug susceptibility testing

Cultures obtained on Lowenstein–Jensen medium were collected and tested for drug susceptibility to rifampicin and isoniazid. Drug susceptibility testing was performed using the proportional method with Lowenstein–Jensen medium. The critical drug concentrations were 0.2 µg/mL for isoniazid and 40 µg/mL for rifampicin. The critical proportion of resistant bacillus necessary to define a resistant strain is 1% for the two tested drugs.[25]

Bacterial lysis

Specimens were first thawed and centrifuged at 6,000 *g* for one minute. For each specimen, the supernatant was discarded and the pellet was treated by heat shock treatment as described by Aldous et al.[26] The DNA thermolysate was used immediately for PCR amplification or stored at –20°C until use.

PCR for amplification of *rpoB and katG* genes and *inhA* promoter region

The *katG* and *rpoB* genes and the *inhA* promoter region were amplified by PCR using the corresponding primers (Table 1). Amplification reactions were performed in a total volume of 50 µL. The amplification mixture contained 0.5 mM of each primer, 2.5 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 25 mM $MgCl_2$, 1 unit of Hotstar Taq DNA polymerase (Invitrogen, Saint Aubin, France), and 5 µL of a DNA sample in 1 × Taq polymerase buffer. For the three target genes, the mixtures were denatured at 94°C for 7 minutes. Thirty-five cycles of PCR were then performed, with denaturation at 94°C for one minute, primer annealing for one minute at the corresponding melting temperature, and primer extension for one minute at 72°C. At the end of the final cycle, the mixture was incubated at 72°C for 7 minutes. For every reaction, a negative control in which DNA template was omitted from the amplification mixture and a positive control containing DNA from H37Rv strain was included. Amplicons were visualized after electrophoretic fractionation in 1.5% agarose gel in 1 × Tris/borate/ethylenediamine tetraacetic acid buffer and staining with ethidium bromide.

Sequencing reaction

Direct sequencing of amplicons was performed using a Big Dye Terminator kit version 3.1 (Applied Biosystems, Foster City, CA, USA) that includes dideoxynucleotides labeled with four fluorochromes of different colors. For each PCR product, both strands were sequenced in independent reactions using the above-mentioned primers. The resulting chromatograms were manually edited to ensure sequence accuracy and added to the alignment component of Molecular Evolutionary Genetics Analysis software (MEGA) version 5 (Tempe, AZ, USA).

Results

A total of 133 clinically symptomatic TB patients were enrolled in this study and sent for MTB and MDR TB investigation. Among them, 100 new cases had active and chemonaïve TB or had received treatment for less than a month. The rest (n = 33) were previously treated patients (drug relapse or failure, chronic cases) nonresponsive to anti-TB treatment after a sufficient duration of treatment. Male gender was more common than female gender, with 27.48% being women and 72.52% being men, giving a sex ratio of 2.64. The median patient age was 38 (range 15–80) years.

It was found that 13.5% of the patients were rifampicin-monoresistant (18/133), 7.5% were isoniazid-monoresistant

Table 1 Primers for polymerase chain reaction amplification

Gene	Size	Primer	Sequence	Temperature (°C)
rpoB[21]	123	TR8	TGCACGTCGCGGACCTCCA	58
		TR9	TCGCCGCGATCAAGGAGT	
katG[27]	419	RTB 59	TGGCCGCGGCGGTCGACATT	62
		RTB 38	GGTCAGTGGCCAGCATCGTC	
inhA promoter[27]	246	inhA P5	CGCAGCCAGGGCCTCGCTG	60
		inhA P3	CTCCGGTAACCAGGACTGA	

(10/133), and 8.3% were resistant to rifampicin and isoniazid (11/133). Moreover, nine MDR strains were isolated from previously treated patients, whereas only two MDR strains came from new TB cases.

Genotypic drug susceptibility testing of the 133 samples showed that seven were isoniazid-resistant (5.3%), 17 were rifampicin-resistant (12.8%), and nine had MDR TB (6.8%). Comparison of phenotypic and genotypic resistance showed that all strains harboring mutations in *rpoB* and *katG* genes and the *inhA* promoter were phenotypically resistant to rifampicin and/or isoniazid. All the mutations identified by partial sequencing of *rpoB* and *katG* genes and the *inhA* promoter region are listed in Table 2.

Our results show that the most recorded mutation in the RRDR of the *rpoB* gene was substitution of TCG > TTG at codon 531 (Ser531 Leu), accounting for 46.15% (Table 2). Other point mutations and deletions were found in a limited number of cases: Asp516Val, Asp516Tyr, 518AAC Δ was found in two cases each and GLn513Pro, Gln513Leu, Asp516His, His526Arg, His526Ser, Lys527Gln, Ser531Trp, and 520CCG Δ in one case each. Interestingly, no strain harbored more than one amino acid change.

Significantly, the only mutation found in the *katG* gene was at codon 315 (AGC to ACC) with a Ser315Thr amino acid change. Only one sample harbored mutation in the *inhA* promoter region and was a point mutation at the −15p position (C > T).

The distribution of genotypically resistant strains according to patient TB profiling is reported in Table 3. Strains monoresistant to isoniazid were found in both new cases (5%) and previously treated patients (6%). However, rifampicin-monoresistant strains prevailed in new cases (15%). As expected, the majority of MDR strains were isolated from previously treated patients (24.2%), especially chronic and drug relapse cases, whereas only one new case was infected with a genotypic MDR strain.

Discussion

Worldwide, the success of national programs against TB relies on rapid diagnosis and good management of drug-resistant TB cases. Indeed, emergence of drug-resistant isolates of MTB poses a serious threat to global TB control. In Morocco, and according to TB control program recommendations, drug susceptibility testing should be performed for previously treated cases (relapse, failure, and chronic) and patients coinfected with TB and human immunodeficiency virus, where MDR TB cases are highly likely and there is a strong likelihood of acquiring further resistance. Therefore, the decision regarding treatment is crucial.[19,28] Moreover, to improve the performance of the TB control program in Morocco, close evaluation of monitoring of treatment outcomes for relapse, failure, and default cases has been strongly recommended.[29]

MDR is a consequence of inappropriate chemotherapy, erratic drug supply, misuse of TB drugs, poor TB management, and lack of control. For these reasons, research efforts are directed towards finding ways to speed up the process of susceptibility testing, with limited cost and complexity.

According to WHO recommendations and in order to implement GeneXpert for genotypic MDR analysis, this study was planned to undertake direct detection of MDR strains by a PCR sequencing-based approach. This standard method for genotyping drug susceptibility was limited to three specific genes, ie, *rpoB, katG* and *inhA*, which are the

Table 2 Frequency of mutations identified by sequencing in the *kat*G and *rpoB* genes of multidrug-resistant *Mycobacterium tuberculosis* isolates

Gene	Position	Type of mutation	Amino acid change	Frequency	Total
rpoB	513	Substitution of CAA → CCA	Glutamine → Proline	1 (3.8%)	26
	513	Substitution of GAA → CTA	Glutamine → Leucine	1 (3.8%)	
	516	Substitution of GAC → GTC	Aspartate → Valine	2 (7.7%)	
	516	Substitution of GAC → TAC	Aspartate → Tyrosine	2 (7.7%)	
	516	Substitution of GAC → CAC	Aspartate → Histidine	1 (3.8%)	
	518	Deletion of AAC		2 (7.7%)	
	520	Deletion of CCG		1 (3.8%)	
	526	Substitution of CAC → CGC	Histidine → Arginine	1 (3.8%)	
	526	Substitution of CAC → AGC	Histidine → Serine	1 (3.8%)	
	527	Substitution of AAG → CAG	Lysine → Glutamine	1 (3.8%)	
	531	Substitution of TCG → TGG	Serine → Tryptophan	1 (3.8%)	
	531	Substitution of TCG → TTG	Serine → Leucine	12 (46%)	
katG	315	Substitution of AGC → ACC	Serine → Threonine	15 (93.75%)	16
inhA promoter	−15	Substitution of C → T		1 (6.25%)	

Table 3 Distribution of MDR according to the tuberculosis profile of patients

TB profile	n	Monoresistant strains		MDR strains
		Isoniazid	Rifampicin	
New cases				
Smear positive	72	5	12	1
Smear negative	28	0	3	0
Previously treated patients				
Chronic	7	0	0	4
Drug relapse	18	1	2	4
Drug failure	8	1	0	0
Total	133	7	17	9

Abbreviation: MDR, multidrug resistance.

main ones responsible for resistance to the anti-TB drugs rifampicin and isoniazid, and are widely associated with MDR TB strains.[9] Indeed, it is well documented that mutations in the *rpoB* gene, especially in RRDR, account for more than 95% of rifampicin resistance. Similarly, mutations in the *katG* gene at codon 315 exclusively confer high-level resistance to isoniazid in up to 75% of MDR TB strains.[9–11]

For both the *katG* and *rpoB* genes and the *inhA* promoter, the mutations or deletions found in this study have already been reported and are in concordance with previous published data.[20,21] The most recorded mutation located in the RRDR of the *rpoB* gene is the Ser531 Leu (TCG → TTG) substitution, which has been widely reported.[30,31] Interestingly, no double mutations were found in the samples analyzed. Our results clearly show that rifampicin monoresistance is relatively high. This resistance could be associated with other forms of isoniazid resistance, including other mutations in the *katG* gene or *inhA* promoter, or mutations in the *ahpC* gene, and other mechanisms of resistance. Moreover, this high frequency of rifampicin monoresistance could highlight that such resistance, as reported in previous studies, is not a rare event.[32] On the other hand, only the Ser315Thr point mutation was found in the *katG* gene and is the most frequent mutation in this gene reported worldwide.[11,31]

It is widely accepted that genotypic drug susceptibility testing has a high sensitivity and specificity as compared with conventional culture-based drug susceptibility testing, but is still unable to detect all drug-resistant strains.[11,33] Thus, the number of MDR strains detected by the PCR-based sequencing approach is underestimated. Of our 133 cases, nine were genotypically MDR, and the majority were isolated from previously treated patients. This is in agreement with reported data, and is of great interest for rapid detection of MDR strains, especially in critical cases, such as chronic and treatment failure or relapse patients, in which rapid diagnosis is mandatory for efficient chemotherapy.[34]

Moreover, eight MDR TB strains were isolated from 33 previously treated patients (21.1%). In 2004, 12.2% of previously treated patients were reported to be MDR.[3] Despite the limited number of cases, there is evidence that MDR cases have increased in Morocco, thereby threatening the success of the TB control program.

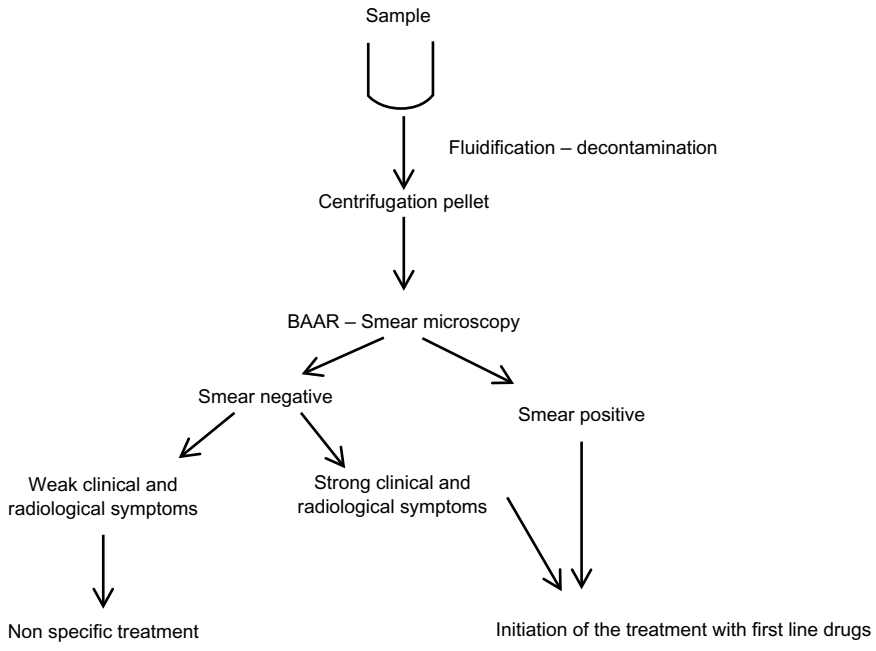

Figure 1 Management of suspected and new cases.
Abbreviation: BAAR, bacillus acido-alcohol resistant.

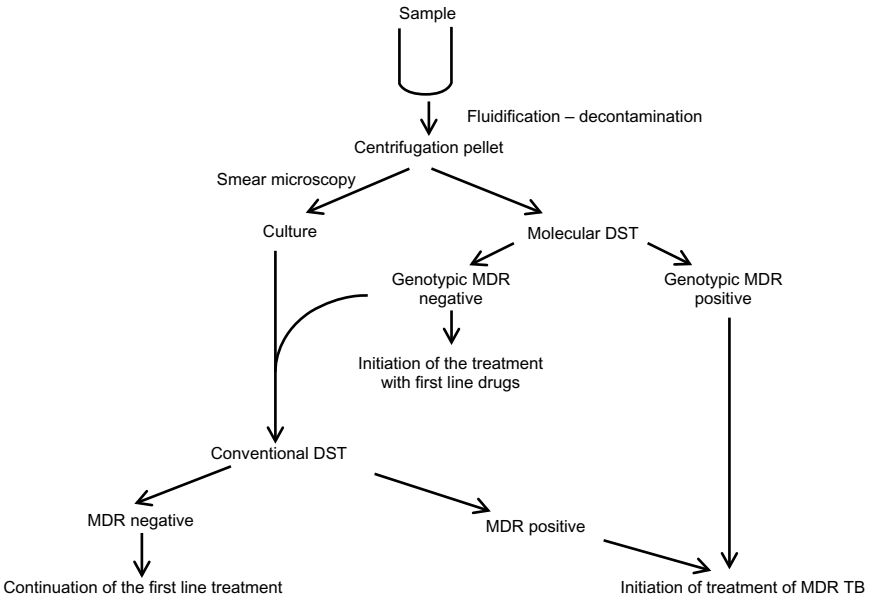

Figure 2 Place of genotypic DST in the global management of TB for specific cases: previously treated patients (chronic, relapse, failure, default), co-infection with HIV, patients with close contact to MDR TB cases.
Abbreviations: MDR, multidrug resistance; DST, drug susceptibility testing; TB, tuberculosis; HIV, Human immunodeficiency virus.

In spite of its high cost and cumbersomeness, automated sequencing is an excellent and valid method for accurate and rapid detection of drug-resistant TB in clinical specimens and a valuable method for the management of suspected MDR TB cases.[21] Significantly, one important advantage of sequence-based approaches is that the resulting data are virtually unambiguous, because the resistance-associated mutation is either present or absent.[14]

In Morocco, as in other countries with limited resources, introduction of the PCR-based sequencing approach to detect MDR cases should be of great benefit as a screening assay in the clinical setting for saving patients' lives and preventing dissemination of TB and MDR TB in the community. Based on our results and those from several international studies,[11,35–38] two algorithms associating conventional diagnosis, drug susceptibility testing, and genotypic drug susceptibility testing could be proposed (Figures 1 and 2) as an integrated model for reducing transmission of MDR strains in Morocco. Rapid detection of MDR strains can play a critical role in limiting the emergence of virtually untreatable XDR TB strains. In fact, the complications of drug resistance in MDR TB can lead to XDR TB.

In conclusion, implementation of molecular approaches for direct diagnosis of MDR TB, as a part of routine analysis in the laboratories of health care institutions, will be of great benefit in adapting treatment regimens, limiting dissemination of MDR TB strains, and limiting the emergence of XDR MTB strains for better management of TB in Morocco.

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Disclosure

The authors have no conflicts of interest in this work.

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