

kDNA and Molecular Typing of Leishmania spp. of Cutaneous Leishmaniasis Patients in Sistan and Baluchestan Province with Low Amount of Parasite

**Hadi Mirahmadi, Ph.D. ¹, Ahmad Mehravaran, Ph.D. ², Nasrin Rezaee, M.Sc.³, Saber Gholizadeh, Ph.D. ⁴,
Saber Raeghi, Ph.D.⁵, Elham-Sadat Roointan, Ph.D.⁶**

1- Assistant Professor, Infectious Diseases and Tropical Medicine Research Center, Tuberculosis Institute & Department of Parasitology and Mycology, Faculty of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran

2- Assistant Professor, Department of Parasitology and Mycology, Faculty of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran

3- Instructor, Infectious Diseases and Tropical Medicine Research Center, Tuberculosis Institute & Department of Parasitology and Mycology, Faculty of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran

4- Associate Professor, Department of Medical Entomology, School of Public Health, Urmia University of Medical Sciences, Urmia, Iran

5- Assistant Professor, Department of Laboratory Sciences, Maragheh University of Medical Sciences, Maragheh, Iran (Corresponding author; E-mail: Saber Raeghi saberraeghi@gmail.com)

6- Assistant Professor, Department of Parasitology, Shahid Beheshti University of Medical Sciences, Tehran, Iran

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Abstract

Background: Cutaneous Leishmaniasis, is endemically observed in different parts of Iran in two forms of anthroponotic and zoonotic. The identification of both species and the type of disease are beneficial for treatment and prevention. Microscopic identification of *Leishmania* species has not provided promising efficacy. The aim of this study was to determine the *Leishmania* species that are responsible for cutaneous leishmaniasis in Zahedan/ Iran by using PCR and PCR-RFLP techniques.

Method: Direct smears were obtained from cutaneous leishmaniasis suspected individuals with low parasitemia in cutaneous lesions referred to Zahedan health centers. Eventually, the DNA was extracted from smears using DNA extraction kit. PCR was used to amplify both *Leishmania* kinetoplastic DNA (kDNA) and ITS1 locus of ribosomal DNA. Additionally, PCR-RFLP on ITS1 products was conducted to determine parasite species.

Results: PCR-RFLP test (detecting ITS1 locus) on all positive samples in microscopic analysis led to the identification of *Leishmania major* in 52 samples (54.7%), and 43 cases were detected to have *Leishmania tropica* (45.3%). On the other hand, kDNA-PCR results indicated a frequency of 68 (55.7%) for *L. major* and 54 (44.3%) for *L. tropica*.

Conclusion: Due to the high frequency of kDNA in parasitic genome, PCR-kDNA compared to PCR-RFLP shows a higher efficiency and accuracy not only in identifying infection, but also in determining parasite species, especially among the patients with fewer lesions. This study also indicates that both *L. tropica* and *L. major* could be found in Zahedan, with a greater tropical leishmaniasis endemicity.

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Introduction

Leishmaniasis, a zoonotic infectious disease, is established by a protozoon, *Leishmania*, and transmitted through sandflies (*Phlebotomus* species) blood feeding into the human. Iran is one of the top ten places with a high prevalence of cutaneous leishmaniasis (1, 2), where *L. tropica* and *L. major* have been reported as etiologic agents capable of developing anthroponotic cutaneous leishmaniasis (ACL) and zoonotic cutaneous leishmaniasis (ZCL), respectively (3, 4).

The most important hyperendemic foci of cutaneous leishmaniasis in Iran are reported to be areas like Torkamansahra and Lotf-abad in northeast, Abardezh, Esfahan, and Yazd in center, Fars and Sistan and Baluchestan in south and southeast, and Ilam and Khuzestan in southwest Iran (5-7). With a high frequency in Sistan and Baluchestan, cutaneous leishmaniasis is one of the most prevalent endemic diseases reported in the majority of provinces of Iran (8-10). There are different *Leishmania* species that vary in terms of clinical symptoms, geographic features, vector, and animal reservoirs. Over the last few years, a series of studies have been performed in Iran on the epidemiology, vectors, reservoirs, and aimed to determine the type of *Leishmania* and its etiological components. The majority of these studies have used routine microscopic techniques (11, 12). Different regions of *Leishmania* -DNA are targeted in molecular methods. For instance, some genes like those of histones, and ribosomal-DNA (rDNA), included in organelles like mitochondria (13),

kinetoplast, and nucleus are utilized for molecular examinations (14-16). Due to the high stability, some regions of rDNA gene have been widely used in genetic diversity evaluations. This rDNA has shown a degree of variability among the closely related species.

ITS1, a well-known member of ribosomal-DNA genes located between 5.8S and 18S coding regions, is successfully utilized for differentiation of species. Despite the other coding regions, this gene does not undergo translation and is capable of identifying intra-species diversity (17-19). kDNA consists of thousands of circular DNA transcripts (minicircle), each of which includes both conserved and variable regions. These regions are targeted in molecular and differential diagnosis (20). According to the high incidence rate of cutaneous leishmaniasis and lack of appropriate identification of this parasite in Zahedan, this study attempted to identify *Leishmania* genera by PCR technique (target genes: ITS-rDNA and kDNA) and to genotype positive samples by RFLP-PCR.

Materials and methods

Study area

Located in the southeast Iran, Zahedan is one of the largest cities of Iran and is the capital of Sistan and Baluchestan province, with a weather varying from mostly moderate to warm. It is also located in a longitude of 60° east and latitude of 29° north (figure 1). The time difference between Zahedan and Greenwich is +246 minutes.

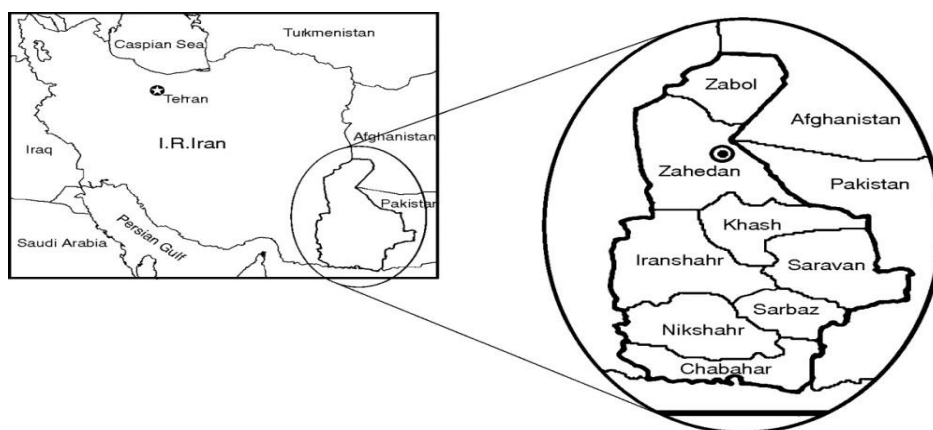


Figure 1. Map of Iran, Zahedan

Sample collection

This descriptive cross-sectional study was conducted on cutaneous leishmaniasis suspected patients between 2012 and 2017. Samples were collected in a sterile manner from individuals with suspected lesions who had been referred to the laboratory by physicians. They filled a form consisted of information like gender, number of lesions and their location, and probable history of going to endemic areas. The samples were obtained using a bistoury and from lower parts of lesions which included macrophage-containing tissues. These samples were then stained by Giemsa staining method and checked under the microscope with 1000x magnification. Positive samples in microscopic analyses were graded in a score range from +1 to +6, based on the average number of parasites in each microscopic field. Finally, 145 slides with low-parasitemia were selected for molecular assays.

Extraction of total genomic DNA

DNA extraction kit (Takapouzist, Iran DynaBio Blood/Tissue Genomic DNA Extraction Kit) was used to extract DNA from the stained smears. These extracted DNAs were then stored at -20°C .

kDNA detection by Nested-PCR

In order to determine different *Leishmania* species, Nested-PCR was used as a sensitive technique. The PCR process was conducted by using four pairs of primers (CSB2XF, CSB2XR) for the first step, and LIR 13ZF for the second step. Primers used in this study were designed based on conserved circular regions of kDNA (Minicircle). These sequences create a 750bp length band for *L. tropica* and a 560bp band for *L. major*. At the first step, the PCR mix consisted of 8ml MasterMix, 3ml DNA, 1ml primer, and 3ml distilled water. This PCR mix was then placed in the PCR instrument (Eppendorf Mastercycler Gradient). The first step consisted of an initial denaturation cycle, 95°C for 5 minutes, followed by 30 cycles of following program: 94°C for 30'' (denaturation), 55°C for 60'' (annealing), 72°C for 90' , and 72°C for 5' .

The first step product of PCR was diluted by distilled water (1:100 ratio) and utilized for the second step of PCR, with the same materials as the first PCR. The initiators of this step were ITS1F and ITS2R4, as shown in table 1. The total reaction volume was determined $30\mu\text{l}$, and the program was executed similar to the first step. Subsequently, products were

electrophoresed on the 2% ethidium bromide agarose gel and checked for the presence of 480bp bands.

RFLP for ITS-rDNA gene

ITS-1 positive samples were digested by *Hae*III (*Bsu*RI) enzyme which cuts the ITS1 sequence at the GG↓CC region.

Observation of bands was accomplished by electrophoresis of 5μl PCR-RFLP product in 1.5% ethidium bromide agarose gel. A 140bp and 340bp fragment was seen for *L. major*, with four particles (25bp, 38bp, 57bp, and 360bp) detected for *L. tropica* (figure 2).

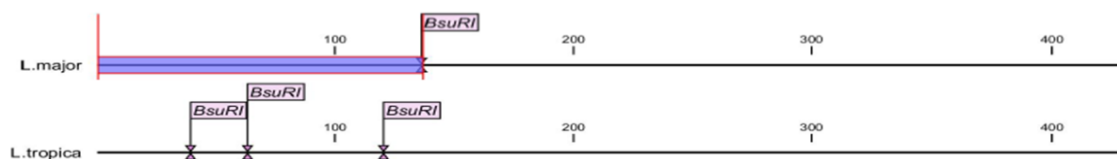


Figure 2. Regions developed following the action of *Bsu*RI restriction enzyme on *L. major* and *L. tropica* ITS-rDNA.

Table 1. The sequences of primers and the protocol used according to the studied genes

Method	Gene	Fragment (bp)	Primers	Enzymes	Annealing	NO. cycles	Cycle protocol
Nested-PCR (First stage)	kDNA		CSB2XF(5'-CGAGTAGCAGAACTCCCCTTA-3') CSB2XR (5'-ATTTTCGCGATTTTCGAGAACG-3')	---	55	30	Annealing :60s Extension: 90s
Nested-PCR (Second stage)	kDNA		LIR (5'-TCGCAGAAGCCCT-3') I3ZF(5'-ACTGGGTGGTAAAATAG-3')	---	60	30	Annealing :60s Extension: 35s
Nested-PCR (First stage)	ITS-rDNA	1100	IR 1 (5'-GCTGTAGGTGAA CCTGCAGCAGCTG GATCATT-3') IR2(5'-GCGGGTAGTCTGCCAAACACTCAGTCTG-3')	-----	58	37	Annealing :45s Extension: 90s
Nested-PCR (Second stage) & RFLP	ITS-rDNA	480	ITS1F (5'-GCAGCTGGATCATTTCC-3') ITS2R4 (5'-ATATGCAGAA GAGAGG AGG C-3')	<i>Bsu</i> RI (<i>Hae</i> III) with cut site GG↓CC; Blunt, 4h in 37 °C	58	37	Annealing :45s Extension: 90s

Results

Smears obtained from 145 *Leishmania* -suspected individuals referred to health centers were checked for the presence of Leishman bodies under the light microscope with 1000x magnification. Then, DNA was extracted from positive samples. kDNA amplification was performed through PCR. kDNA bands were detected in 122 positive (84.13%) and 23 negative (15.86%) samples. On the other hand, specific bands were detected for 95 positive samples (65.51%) and 50

negative samples (34.48%) in PCR-RFLP method used to amplify ITS-rDNA gene (following agarose gel electrophoresis), as shown in figure 3. From 95 positive samples treated with *Bsu*RI (also called *Hae*III) in which ITS-rDNA was successfully amplified, 52 cases were *L. major* and 43 cases were identified as *L. tropica*. Additionally, kDNA Nested-PCR analysis on 122 positive samples identified 68 samples as *L. major* and 54 samples as *L. tropica*.

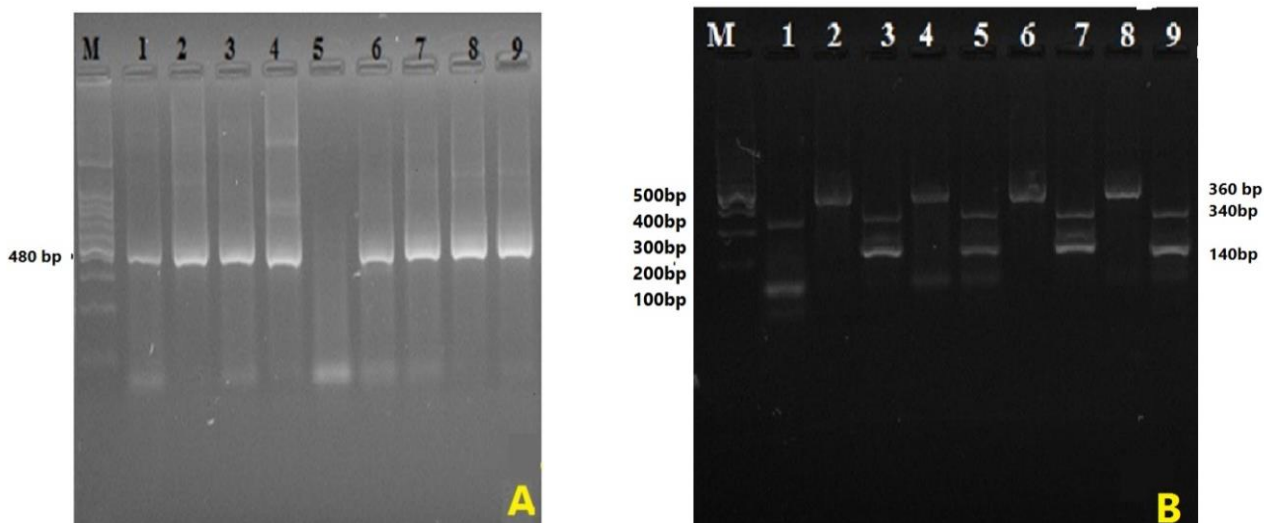


Figure 3. (A): Results of ITS-rDNA electrophoresis on 1% agarose gel, (B): Lines 2, 4, 6, and 8: enzyme-unexposed *Leishmania*, line 1: enzyme-exposed *L. tropica*, lines 3, 5, 7, and 9: enzyme-exposed *L. major*, M line: 100bp ladder

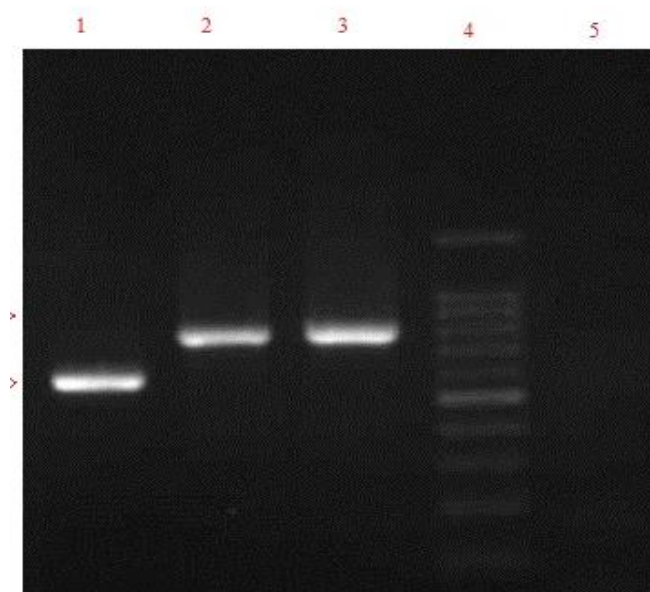


Figure 4. Result of kDNA electrophoresis on 1.5% agarose gel

Line 1: 560bp band of *L. major*, lines 2, 3: 750bp bands of *L. tropica* positive control and *L. tropica* patient sample, line 4: DNA marker (100bp), line 5: negative control

Table 2. Frequency distribution of kDNA ITS-rDNA based on the locations of lesions

Type of parasite	location of Lesion	face	Hand	Feet	Hand and face	Hand and trunk	Trunk	Hand and Feet	body	Head and face	Nose	Face and arms	Head and hand	Total
L. major	ITS-rDNA	10 (19.60)	15 (29.41)	13 (25.49)	2 (3.92)	1 (1.96)	3 (5.80)	0	5 (9.80)	2 (3.92)	0	0	1 (1.96)	52 (100)
	kDNA	14 (20.58)	18 (26.47)	14 (20.58)	2 (2.94)	3 (4.41)	5 (7.35)	1 (1.47)	5 (7.35)	3 (4.41)	1 (1.47)	1 (1.47)	1 (1.47)	68 (100)
L. tropica	ITS-rDNA	10 (22.72)	15 (36.36)	6 (13.67)	0	1 (2.27)	2 (4.54)	1 (2.27)	1 (2.27)	5 (11.36)	1 (2.27)	0	1 (2.27)	43 (100)
	kDNA	11 (20.35)	18 (33.3)	8 (14.8)	1 (1.85)	1 (1.85)	3 (5.55)	2 (3.7)	1 (1.85)	6 (11.1)	1 (1.85)	1 (1.85)	1 (1.85)	54 (100)
Negative	ITS-rDNA	3 (6)	11 (22)	8 (16)	4 (8)	3 (6)	6 (12)	3 (6)	3 (6)	2 (4)	2 (4)	4 (8)	1 (2)	50 (100)
	kDNA	1 (4.34)	8 (34.72)	5 (21.7)	1 (4.34)	0	3 (13.02)	0	1 (4.34)	0	0	4 (17.36)	0	23 (100)
Total	ITS-rDNA	23	41	27	6	5	11	4	9	9	3	4	3	145
	kDNA	23	41	27	6	5	11	4	9	9	3	4	3	145

Data have been presented as number (%)

Discussion

Leishmaniasis is one of the major health issues in Sistan and Baluchestan. It is an infectious disease transmitted to human through blood feeding of *Phlebotomus* (in the old world) and *Lutzomia* (in the new world) sandflies species. Iran is reported to be one of the top ten countries of the world with a high prevalence of cutaneous leishmaniasis (1). *L. major* is the major etiologic agent of zoonotic cutaneous leishmaniasis and a wide range of animals, particularly rodents, have been identified as reservoirs (4). On the other hand, *L. tropica* is well-known as the common etiologic agent of anthroponotic cutaneous leishmaniasis in Iran and the majority of other countries (4, 21). In respect to the positive results given by both the microscopic analysis and molecular techniques, molecular techniques were found to have higher sensitivity and specificity. Among the most applicable techniques, PCR showed a remarkable high

specificity and sensitivity (22), through which the process of determination of species can be achieved even in Giemsa-stained leishmaniasis samples. These direct smears from cutaneous leishmaniasis-suspected cases is easily obtained and represent a long-term accessibility for PCR method (23).

In this study, samples were collected from leishmaniasis-suspected patients in Zahedan who had very low level of parasites in samples. To identify *Leishmania* infection and isolation of parasite, direct smears from lesions and microscopic examination was used. Rapid diagnosis, facilitated sampling and transferring of samples, use of a single sample for both microscopic and molecular investigations, and the possibility of using smears provided a long time before the test are advantages of using these smears (24).

Studies on *Leishmania* infection has recently obtained particular importance (25). Two *Leishmania* genomic loci

extensively used in molecular investigations are kDNA and ITS-rDNA. With thousands of circular-DNA transcripts, each of which consisted of both conserved and variable regions, kDNA is well-characterized as a useful tool for molecular diagnosis and species determination. Furthermore, ITS-kDNA with numerous copies in the genome of parasite and repeated sequences is highly applicable for taxonomic classification (23). In this study, molecular methods were used to amplify *Leishmania* gene (by PCR and nested-PCR) and this amplification was then approved by enzymatic digestion accomplished through RFLP. Mirzaie et al reported three haplotypes of *L. major*, a similar haplotype of *L. tropica* and two haplotypes of *L. major* in rodents of Esfahan and Fars using microsatellite genes, ITS-rDNA and methods such as sequencing and nested-PCR (17). In addition, in an effort to determine *Leishmania* species by RFLP-PCR targeting ITS with LITSR and S.L58 primers, Bensoussan et al. indicated that this method is capable of detecting 74% of positive samples (26). Parvizi et al in 2008, designed ITS-rDNA and kDNA with the ability to identify genetic strains of *L. major* in sandflies of Iran by amplification and sequencing (18). In an attempt to determine cutaneous leishmaniasis species from DNA of cutaneous leishmaniasis smears taken from 47 patients by RFLP-PCR, *L. tropica* and *L. major* were detected in 20 and 27 samples, respectively (27). Based on the comparison with sequences recorded in global gene bank, samples isolated from cutaneous leishmaniasis patients in Zahedan, were shown to be *L. major* and *L. tropica*. Sharbat-khori et al. reported resemble results in their attempt to identify *Leishmania* by using microscopic and molecular methods (ITS-rDNA sequencing, semi-nested PCR that amplified minicircle kDNAs) in patients suspected to Leishmaniasis in northern cities of Iran in 2014

(28). In a similar study by Ramazany, the results did not indicate any significant difference between men and women in terms of numbers of patients with *L. major*, *L. tropica*, and negative cases (16). As it is seen in figure 3, Chi-square test showed a higher frequency for both genes of *L. major* in men (49 cases). These results are similar to those of Sharifi-Rad et al. study (32). In addition, the number of patients with *L. major* was higher than number of patients with *L. tropica*. The differentiation between *L. major* and *L. tropica* achieved in this study was arisen from intra-gene variations and generation of gene particles with various sizes. The results of the present study confirmed *L. major* and *L. tropica* as etiologic agents of cutaneous leishmaniasis in Zahedan. Studies conducted in Iran have shown that zoonotic cutaneous leishmaniasis is a growing health issue in endemic loci like Zahedan (30). Furthermore, all species and strains found in samples were determined by molecular techniques (based on direct smears). However, methods used to differentiate different *Leishmania* species based on morphological and microscopic characteristics failed to provide promising results and led to the use of molecular methods. Over the last few years, a range of molecular methods, e.g. PCR, have been broadly developed to reinforce the differentiation and identification of various *Leishmania* species (9, 29). Some of genes present in organelles like mitochondria, nucleus (rDNA) and kinetoplast have been found as appropriate targets for identification of species (31). Studies on kDNA have shown a higher specificity and accuracy. Notably, ITS-rDNA sequence analysis for species determination of *Leishmania* have found these areas highly conserved and stable (10). It should also be noted that high evolutionary rate of ITS sequence (which even shows variability between species of a single genus) leads to the high

efficacy of PCR in targeting this sequence and detecting a variety of genus and species of *Leishmania*. Despite the greater sensitivity of kDNA vs. ITS-rDNA in diagnosing infection, the high reliability of RFLP-PCR on ITS-kDNA gene has introduced it as the most appropriate tool for highly effective determination of different *Leishmania* species (31, 32).

In the current study, hands were the most affected organs of the body followed by face, legs and the rest of organs. Cutaneous lesions of leishmaniasis are more frequently found in organs with high exposure to sandflies' biting. In anthroponotic form, face is the most involved organ whereas in zoonotic form the lesions are most commonly seen on hands and legs, similar to what Yazdanpanah et al. have reported (33). Our results also indicated that the number of lesions developed by *L. major* was higher than that by *L. tropica*.

Zahedan is reported as one of the most important loci of zoonotic cutaneous leishmaniasis. Molecular epidemiologic evidence in Zahedan have revealed that the most dominant parasite species is *L. major*. The importance of *Leishmania* species determination and its impact on preventive strategies

have been resulted in applying a variety of methods with the aim of differentiating these species.

Conclusion

The results of the current study indicated a high endemicity rate of cutaneous leishmaniasis in Zahedan. PCR methods were successfully used to determine different species that cause cutaneous leishmaniasis, even for samples provided over several years before the test. These results also confirmed the necessity of PCR method directly on clinical samples e.g. direct smears, in both negative cases and determination of species. Therefore, additional studies are required to provide better preventive and control policies.

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