DEVELOPMENT OF MOLECULAR DIAGNOSIS METHOD FOR IDENTIFICATION OF HCV USING LAMP AND RT-PCR TECHNIQUES

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ABSTRACT: Hepatitis C is blood-borne viral disease worldwide. Chronic hepatitis C infection leads to cirrhosis of the liver, liver failure and liver cancer. Antiviral therapy exists and approximately 50% of infected individuals can be cured. The importance of low cost molecular diagnostic assays are especially important for the developing nations as they are already burdened with increasing number of hepatitis C patients who are generally economically backward. The aim of this thesis was to analyze blood samples using molecular technique like q-PCR and LAMP. Out of twenty samples analyzed q-RTPCR, twenty samples were positive and other twenty samples showed negative. In summary we have shown that LAMP is a rapid sensitive and specific method for detecting HCV in settings where real time PCR machine may not be available. The only limitation of the study is that we have used only ten clinically infected samples to compare with ten negative controls.
Key words: HCV, LAMP, RT-PCR and Molecular Diagnosis

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INTRODUCTION

Hepatitis C virus (HCV) infection is a global health problem which has affected around 170 million people worldwide and is one of the major causes of deaths related to liver cirrhosis and hepatocellular carcinoma [1]. HCV can be classified to seven major genotypes and 80 subtypes [2-4]. HCV genotypes vary in patterns of geographical distribution and therapeutic response. However, the geographical and genetic diversity of this RNA virus is constantly evolving because of rapid globalization. In India, HCV infection has been reported in 0%-21% population and responsible for 14%-26% cases of chronic liver disease. HCV infection is mostly transmitted through transfusion of blood or blood products. A high prevalence of HCV is found in many high-risk groups (HRG) exposed to blood or blood products like intra venous drug users (IDUs), patients with pediatric hematologic malignancies and those with thalassemia and hemophilia. India reported a higher percentage of blood donors (1%-1.5%) than in any developed country [5]. The importance of low cost molecular diagnostic assays are especially important for the developing nations as they are already burdened with increasing number of hepatitis C patients who are generally economically backward. The advent of molecular diagnostic approaches has allowed for the development of nucleic acid assays that are more sensitive and specific than antibody based technologies. The linking of these assays with appropriate detection systems, therefore, makes them highly desirable for detecting HCV RNA in patient samples. Molecular techniques not only help to detect HCV RNA but confirm active state of infection, i.e., the virus is in replicating state in the patient's body. In individuals falling in high risk diagnosis of HCV can give false negative results as these patients are already immuno-suppressed, in this scenario, molecular testing remains the best choice for detection. Molecular diagnostic assays are an integral part in the management of HCV patients.

Both qualitative and quantitative HCV molecular assays are used in the diagnosis of acute and chronic infection. The principle of qualitative HCV assays includes viral RNA isolation, complementary DNA (cDNA) synthesis, PCR amplification and detection of PCR amplicons. Qualitative HCV RNA test detects the presence of HCV circulating in the blood and is among the most sensitive tests available. Since HCV is a RNA virus, reverse transcription PCR is used to detect viral RNA. The viral genome is 9.6kb long, contains a single open reading frame that is translated to produce a single protein product, which is then further processed to produce functional proteins for viral replication and propagation. At the 5' and 3' ends of the viral RNA are the untranslated region (UTR) that are not translated into proteins but are important to translation and replication of the viral RNA. Most of the commercial and in-house PCR amplification strategies are targeted against the 5' UTR region as there is more than 90% sequence identity among different HCV genotypes, with some segments nearly identical among different strains [6]. The secondary and tertiary structures of this region are also largely conserved and this is one of the first regions which is transcribed of the first regions which is transcribed. Other than the 5' UTR region, the core and the 3' UTR region are also targeted for PCR based detection of HCV. A recent study showed that detection based on the sequence of the core region could reliably identify subtypes as well as major genotypes since the sequence divergence was greater than the divergence of the 5'UTR sequence. Though there are other regions like the E1, E2, NS2, which can be used as detection targets for PCR amplification but they are not in much use as there is a lack of conservation in the primer binding sites [7-9].

MATERIALS AND METHODS
Sample and methodology used for the study
Blood samples from 10 HCV positive patients and blood samples from 10 HCV negative subjects
Extraction of plasma from blood
Whole blood was collected into commercially available anticoagulant-treated EDTA-treated (lavender tops). Cells are removed from plasma by centrifugation for 10 minutes at 1,000-2,000 x g using a refrigerated centrifuge. Centrifugation for 15 minutes at 2,000 x g depletes platelets in the plasma sample.

The resulting supernatant is designated plasma. Following centrifugation, it is important to immediately transfer the liquid component (plasma) into a clean polypropylene tube using a Pasteur pipette. The samples were maintained at 2-8°C while handling. The plasma was apportioned into 0.5 ml aliquots, and stored, at –20°C until ten samples were collected. It is important to avoid freeze-thaw cycles. Samples which are hemolyzed, icteric, or lipemic can invalidate certain tests.

Extraction of RNA from plasma
To nuclease-free 1.5 ml micro centrifuge tubes: 200 µl plasma from each of the twenty collected samples was added. 200 µl working solution, freshly prepared (carrier RNA-supplemented Binding Buffer) was added to it followed by 50 µl Proteinase K solution; and mixed immediately. It was incubated for 10 min at +72°C. 100 µl of Binding Buffer and mixed well. One High Pure Filter Tube was inserted into a Collection Tube and the entire sample was pipetted into the upper reservoir of the Filter Tube. The entire High Pure Filter Tube assembly was inserted into a standard table-top centrifuge and centrifuged for 1 min at 8,000 × g. After centrifugation the Filter Tube was removed from the Collection Tube; the flow through was discarded along with the Collection Tube. A new Collection Tube was kept underneath and 500 µl Inhibitor Removal Buffer was added to the upper reservoir of the Filter Tube and centrifuged for 1 min at 8,000 × g. After centrifugation the Filter Tube was removed from the Collection Tube; the flow through and the Collection

Tube discarded. A new Collection Tube was kept. 450 µl Wash Buffer was added to the upper reservoir of the Filter Tube and centrifuged for 1 min at 8,000 × g and the flow through was discarded. After the first wash and centrifugation: the flow through and the Collection Tube was discarded A new Collection Tube was kept and 450 µl Wash Buffer was added to the upper reservoir of the Filter Tube and centrifuged for 1 min at 8,000 × g and the flow through was discarded .The Filter Tube-Collection Tube assembly was spun it for 10 s at maximum speed (approx. 13,000 × g) to remove any residual Wash Buffer. The extra centrifugation time ensures removal of residual Wash Buffer. The Collection Tube was discarded and the Filter Tube was inserted into a nuclease free, sterile 1.5 ml micro centrifuge tube. To elute the viral nucleic acids: 50 µl Elution Buffer was added to the upper reservoir of the Filter Tube and centrifuged for 1 min at 8,000 × g. The micro centrifuge tube contains the eluted, purified viral nucleic acids. The eluted nucleic acids was directly used in PCR (10 – 20 µl DNA eluate) or RT-PCR (3.5 µl viral RNA).

Real time PCR method

Make sure that the tubes for standards & at least one negative control (Water, PCR grade) are included per PCR run. To generate a standard curve, all supplied Standards (HCV S 1-5) were used for each PCR run. Before each use, all reagents need to be thawed completely and mixed (by pipetting or by brief vortexing). Depending upon the number of samples a mix can be prepared as follows.

Pipette 10 µl of the Master Mix into each labeled PCR tube. Then add 15 µl of the earlier extracted RNA to each sample tube and mix well by pipetting up and down. Correspondingly, 15 µl of the Standards (HCV S1-5) must be used as a positive control and 15 µl of water (Water, PCR grade) as a negative control. Close the PCR tubes and transfer the same into real time PCR instrument.

Thermal profile

1.50ºC-15s

2.95ºC-10 min

3.95ºC-10 s

4.55ºC -20 s

5.72ºC-10 s

Steps 3-5 were repeated for 45 cycles.\

The run completed in about one and a half hours.

LAMP PCR

The HCV detection is based on the amplification of a single-copy 5' UTR sequence by means of the Reverse Transcription Polymerase Chain Reaction (RT-PCR) and for measuring of the amplification product concentration growth in the course of the PCR by means of the fluorescence marked probe (real-time PCR).

Primer Design for LAMP Reaction: The oligonucleotide primers used for LAMP amplification of HCV were designed from 5` UTR region sequences. A set of six primers (two outer, two inner, and two loops) were designed using primer explorer software. However, we did not use loop primers in our experiment.

F3 5` -TCTTGGGAGAGCCATAGTG-3`

B3 5` -CATAAGCAAGCACCCTATCAG-3`

FIP-5` -GATCCAAGAAAGGACCCGGTTCGTTCCGGAACCGGTGAGTAC-3`

BIP 5` -CCTGGAGATTTGGGCGTGCTTTTAGTAGGGTTAGGGCCTTTCGC-3`
The LAMP reaction was carried out in a 25 ul reaction mixture composed of
1.6 M each of FIP and BIP,
0.2 M each of F3 and B3,
2.5 ulL Thermopol buffer (20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH4)2SO4, 9 mM MgSO4 and 0.1 % Triton X-100),
1.4 mMdeoxynucleoside triphosphate mix,
1 M Betaine (Sigma-Aldrich, St. Louis, MO, USA),
3 µl of target cDNA, and distilled water.

The mixtures were heated to 95ºC for 5 min, and then chilled on ice prior to addition of 8 U of Bst DNA polymerase large fragments (New England Biolabs). Immediately after addition of the polymerase, the mixture was incubated at 62 º C for 70 min in a heating block (SBH 130D; Stuart Scientific, Staffordshire, UK) and then heated at 80 º C for 10 min to terminate the reaction. Samples which the copy numbers of virus RNA had previously been quantified by real-time PCR were used as positive controls. LAMP products were subjected to 2 % agarose gel electrophoresis stained with EtBr and visualized under UV light.
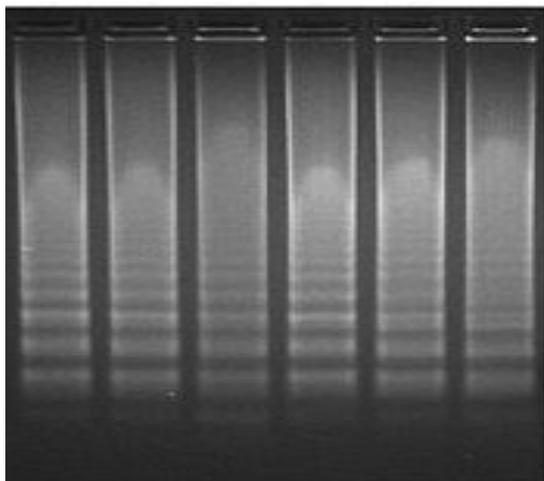
Figure 1: LAMP amplicons run on a 2% agarose gel

In addition, 1 ul of SYBR Green I (Invitrogen lot: 49743A) diluted 1:10 was added directly to the LAMP products. The solution turned green if LAMP reaction products were present, otherwise it remained orange; coloration was evaluated under natural light and UV light (302 nm; via handheld UV torch lamp).
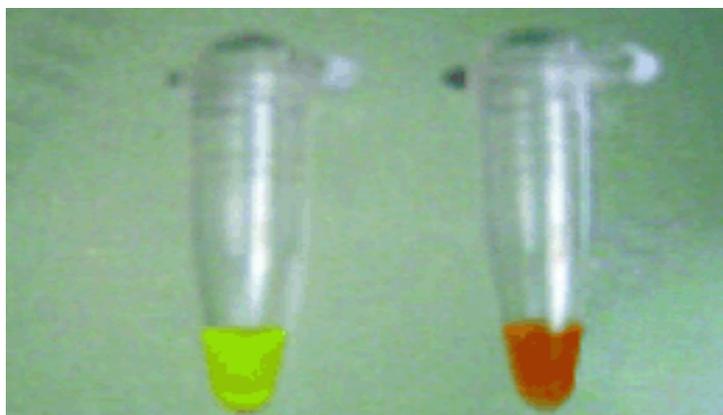

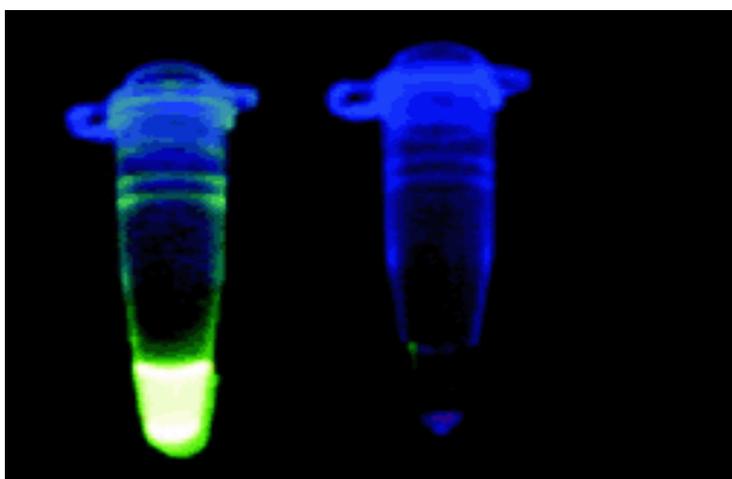**Figure 2: LAMP results when viewed under naked eye**

Figure 3: LAMP results when viewed under UV light

RESULTS AND DISCUSSION
Sample collection, RT-PCR and LAMP

Blood was collected from ten HCV infected subjects in EDTA vacutainers along with ten blood samples from healthy volunteers who were free of HCV infection. These ten individuals were outpatients to GYD clinic, Hyderabad.

The RNA was extracted from each sample as described in Methods. The RNA was subjected to RT-PCR using Genosens HCV quantification kit. The same samples were subjected to LAMP using primers designed for HCV. The samples were incubated at 62 º c for one hour in a water bath set to 62ºC.Thereafter the picogreen was added to each tube to visually observe the colour change as well observed under the uv light. All ten samples except one showed fluorescence.

Table 1: Patient blood samples showing positive to q--PCR amplification result.

Sample Number	RT-PCR result	LAMP result
1	+	+
2	+	+
3	+	+
4	+	-
5	+	+
6	+	+
7	+	+
8	+	+
9	+	+
10	+	+

Table 2: Patient blood samples showing Negative to q-PCR amplification results.

Sample Number	RT-PCR result	LAMP result
1	-	-
2	-	-
3	-	-
4	-	-
5	-	-
6	-	-
7	-	-
8	-	-
9	-	-
10	-	-

HCV quantitative assay is used to determine the number of international units of HCV RNA per millimeter of serum or plasma (IU/mL) in known HCV positive patients. Recently, real time PCR based detection systems have become widely available and are considered as the detection method of choice by many clinicians. The advantages of this technique are that they have a very low limit of detection, have a broad dynamic range. Several companies now market the real time PCR assays: the COBASs Ampliprep/Cobas Taq Man assay (CAP/CTM, Roche Molecular Diagnostics) and the real-time HCV assay (also named Accu Genes HCV, Abbott Molecular Inc., Des Plaines, IL, United States). These assays have the advantage of having a broad dynamic range of amplification, thus improving the limits of detection (LOD) to 10 IU/mL, and linear quantification up to 107 -108 IU/mL [10]. The quantitation of HCV viral RNA in Cobas Amplicor is performed using the HCV Quantitation .Standard. The HCV quantitation standard is a noninfectious armoured RNA construct of HCV sequences with identical primer binding sites as the HCV RNA target and a unique probe binding region that allows HCV Quantitation Standard amplicon to be distinguished from HCV target amplicon. The HCV Quantitation Standard is pipetted into each individual sample and control at a known copy number and is and is then amplified by PCR. The COBAS Taq Man HCV Test, v2.0 uses reverse transcription and PCR amplification primers against the highly conserved 5' untranslated region of the HCV genome [11].

The Versant HCV quantitative test (Siemens Healthcare Diagnostics) which is HCV RNA assay based on signal amplification by branched DNA (bDNA). In this assay, single stranded DNA molecules are present; which acts as probe DNA molecules. Next an extender DNA molecule is added. Once the capture and extender molecules are in their proper place they are hybridized and the sample is added. The bDNA assay version 3.0 has been reported to have a lower detection limit of 615 IU/mL to 8 million IU/mL whatever the HCV genotype [12]. The advantage of RT-PCR is that it allows continuous monitoring of amplicon kinetics during the exponential phase before the amplification reaches its plateau. This allows for a good correlation between the initial numbers of template copies whereas in qualitative assays based on PCR, amplicon detection was at the end [13]. Thus the use of quantitation techniques have greatly enhanced the sensitivity and reliability in detection techniques.
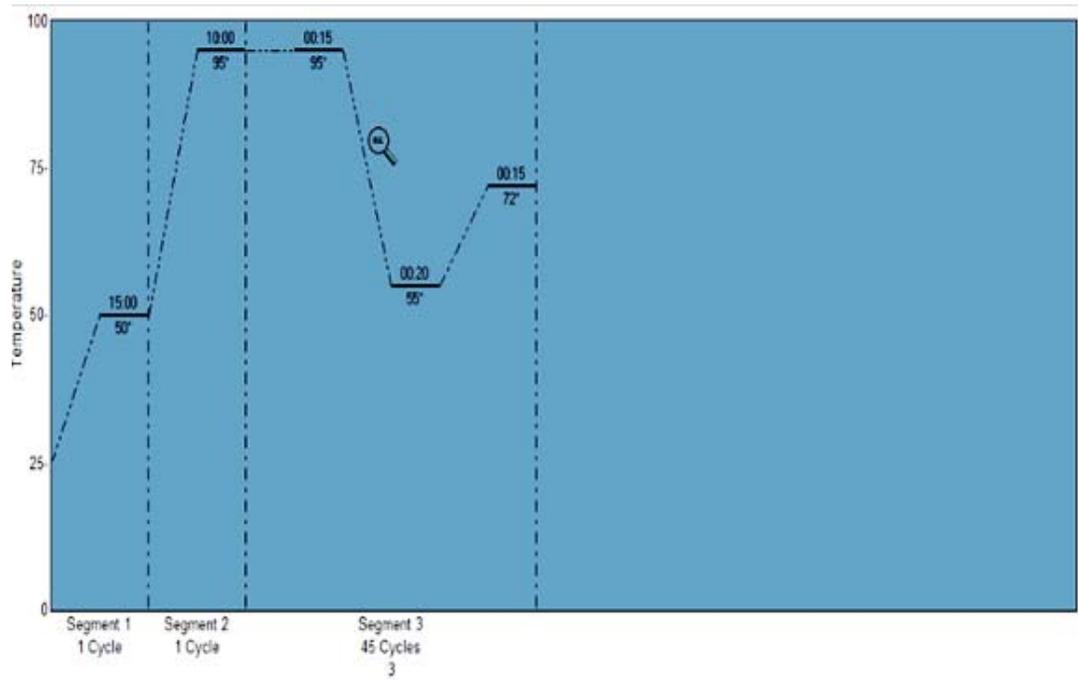

Figure 4: Thermal profile of HCV real time PCR amplification

Real-time PCR allows continuous monitoring of amplicon synthesis early during the reaction when amplification kinetics is exponential and before amplified product concentration reaches a plateau. This allows a good correlation between the initial number of template copies and the number of PCR cycles needed to detect amplified products, therefore leading to an accurate quantitation.

This constitutes a major difference with current RT-PCR for which amplicon detection and quantitation are performed at the end of the PCR, where determination of high RNA levels requires sample dilutions [14].

Another advantage is that the presence of polymerase inhibitors which induce underestimation of RNA concentrations, could be detected byreal-time PCR. Indeed, measurement of fluorescence after each elongation step during the exponential phase, allows calculation of the slope of the amplification curve. This slope is a direct function of the amplification efficiency and can therefore be used as an indicator of the presence of inhibitors.

The diagnosis of HCV infection depends mainly on detecting circulating antibodies to this virus, EIA 2.0 detects anti-HCV in approximately 90% of cases [15]. The third-generation EIA 3.0 is more sensitive than EIA2.0 and the predictive positive values are 0.52 versus 0.23[16]. EIA 3.0 detects antibody earlier in the course of infection [17], five to six weeks after the onset of hepatitis in 80% of patients.

Some limitations have been observed with EIA tests in that they could not differentiate among acute, chronic and past infection. In some acute cases there could be a long interval before seroconversion. In low risk groups such as blood donors, even the third-generation EIAS produce false positive results.

Figure 5: Amplification plot of one of the sample showing HCVAmplification. The red curve is the internal standard.
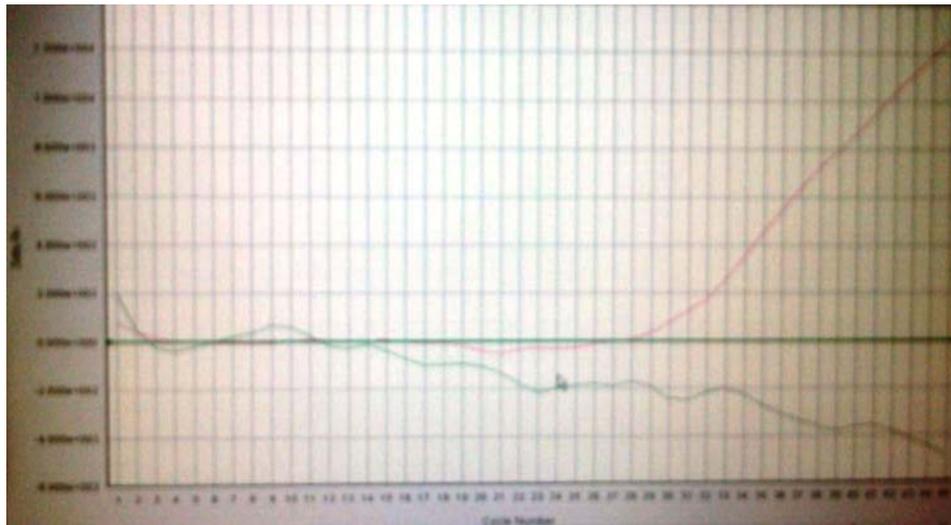

Figure 6: Amplification plot of a control sample from a healthy volunteer showing no amplification of the HCV virus. The red line shows amplification has taken place for the internal control.

Figure 7: Ten tubes showing positive amplification in all except one

HCVRNA could be detected many weeks before the appearance of anti-HCV and in some cases this may be the only evidence of HCV infection. Nested PCR is the most sensitive technique for the detection of HCVRNA. Primers specific for 5′ untranslated region (UTR) are the most sensitive because this region is highly conserved [18]. Detection of hepatitis C virus RNA by a two-stage polymerase chain reaction with two pairs of primers deduced from 5′-noncoding region [19].

In of HCV RNA positive cases, quantitation may provide prognostic information; lower HCV RNA levels appear to be associated with less symptomatic disease and with improved response to interferon therapy. Higher viral titres are associated with prolonged infection and are less responsive to treatment. Unlike most viral infections, antibodies against HCV do not appear within 5–10 days of the exposure to the infection. There is a seronegative window period (WP) of several months. Which means that most of the recently infected individuals will test HCV seronegative during the WP and thus will be missed.

The length of the WP depends, among other factors, on the general immune state of the patient and may last as long as 6 to 12 months in immune-compromised or immune suppressed patients. Immunosuppressive condition has been associated with HIV or HBV coinfection, organ transplants, and chronic hemodialysis. For example, there is a decrease of adaptive HCV-specific immune response in co-infection with HIV. Humoral immune responses to HCV during the acute phase were inhibited in the presence of active HBV replication, leading to poor antibody responses to HCV.

Antigen and nucleic acid amplification tests (NATs) allow for direct viral detection and have been shown to reduce the WP for detection of HCV infection by up to 60 days. Due to high cost of NAT methods pooled samples (10–96 per pool) [20] are used for donor screening in many of the developed countries. Due to the pooling, the samples are diluted, leading to cases where pre-seroconversion donations were negative by both Ab testing and pooled PCR D.

However, the failure to find HCV RNA in all clinical sera by these assays in this study may be due to the low level of RNA resulting from spontaneous viral clearance. Owing to its rapidity, sensitivity, specificity and simplicity, the HCV RT-LAMP is a suitable assay for diagnosing acute infection and screening blood donations for HCV infection, especially under conditions where sophisticated and expensive equipment are rarely available.

CONCLUSION

In conclusion we have shown that LAMP is a rapid sensitive and specific method for detecting HCV in settings where real time PCR machine may not be available. The only limitation of the study is that we have used only ten clinically infected samples to compare with ten negative controls.

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