

Optimization of Lyophilized LAMP and RT-PCR Reaction Mixes for Detection of Tuberculosis

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Abstract

Undoubtedly, one of the most infectious diseases in the world is tuberculosis. Key factor for tuberculosis control is to prevent possible contagion with rapid diagnosis and effective treatment. The culture method, which it takes several weeks to obtain results, is the gold standard method for laboratory diagnosis of tuberculosis. In order to prevent possible contagion of tuberculosis, diagnosis must be made in short time and treatment should be started as soon as possible. Normally, clinical samples are studied in advanced laboratories designed for this purpose. However, especially after the screening in rural areas, the transmission of the samples to the centers has many negative effects on the clinical material. Therefore, the latest trend molecular techniques in microbiological diagnosis are developing into point of care systems that can be applied in the field without laboratory infrastructure. The major challenge for molecular-based point-of-care tests is the need to store polymerase enzymes and some of the ingredients used in the cold chain. The aim of this study is to increase the resistance of the amplification reaction mixtures by lyophilizing the tuberculosis diagnosis. Lyophilization was performed on Loop-mediated isothermal amplification (LAMP) and Real-time PCR mixtures. For the lyophilization of LAMP and RT-PCR mixtures, two different experimental setups were tried from the literature except for the developed content. Chemicals such as stachyose, trehalose, glycerol and PEG 8000 are widely using as cryoprotectants. As a result, the developed content (0.5% PEG 8000, 2.0 % Stachyose) was determined the best cryoprotectant mixture. Accordingly, amplification mixtures can be produced with the developed lyophilization method and point of care kits can be developed.

Keywords: Lyophilized master mix, Loop-mediated isothermal amplification, LAMP, Real-time PCR, *M. tuberculosis*

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Introduction

Tuberculosis is a re-emerging infectious disease that causes death by outbreak (1). *Mycobacterium tuberculosis* which is the causative agent of tuberculosis infection is spread through droplet nuclei up to 5 microns in diameter (2). Depending on the environmental conditions, these small particles may remain suspended in the air for several hours. Besides, the infectious dose (<10 bacilli) is very low and therefore has the potential to be used in bioterrorism attacks (3). According to the World Health Organization (WHO), tuberculosis is one of the most important causes of death worldwide. Only in 2018, ten million fell ill with tuberculosis and 1.5 million died (4). Rapid diagnosis of tuberculosis has critical importance. However, gold standard method which is culture method takes a few weeks (5). But to prevent the spread of the disease, there is a critical need for quick, sensitive, reliable, point-of-care and economical methods for the laboratory diagnosis of tuberculosis (6).

DNA based amplification reactions and antigen-antibody based methods are available for the diagnosis of tuberculosis except for the culture method which is the gold standard (7). However, since the diagnosis of the disease takes a long time with the culture method, treatment is delayed. Also, because of the low sensitivity of the antigen-antibody

based methods, it is needed to the rapid and sensitive method for diagnosis of tuberculosis. Although nucleic acid based diagnostic systems are advantageous in terms of time and sensitivity, many of them have complicated protocols and depend on complex devices (8).

Molecular methods, especially PCR, have reached an important point for the diagnosis of tuberculosis (9). In addition to the commercially tuberculosis PCR detection kits, many laboratories have their own in-house methods (10).

However, PCR methods used in routine require specialists due to their complex processes and cold-chain transportation needs. Generally, PCR studies are performed with small volumes such as 1-10 μ l and the smallest error can have critical results (11). Therefore, the method to be developed should be as low as possible to minimize the person's error.

In recent years, scientists have been working on the use of DNA amplification reactions in the point of care tests (12). The loop-mediated isothermal amplification (LAMP) method developed by Notomi et al. (2000) is more sensitive than other PCR methods, because of using 4 or 6 different primers that recognize 6 or 8 different regions on the target gene (13,14). Due to its primers design and Bst DNA polymerase, the method does not require temperature cycling (15). Whereby its ability to perform at a constant temperature, it has become frequently used in point of care tests and microfluidic chips (16,17). Particularly, the fact that the detection of the products formed at the end of the reaction can be made with the naked eye or the lateral flow test strips makes it widely used in field studies (18).

Despite the advantages of using DNA amplification methods for diagnostic purposes, there are still challenges that need to be overcome (19). Standardization of reaction mixture preparation is important for the accuracy and sensitivity of the results. It is difficult to ensure standardization, especially in field conditions, as the ingredients must be stored at -20 °C and work in the cold chain. Lyophilization is a protection method used in amplification reactions to make the mixtures resistant to environmental conditions (20). The lyophilization process, which is also used in commercial kits, extends the lifetime of PCR mixtures up to 1 year (21).

The aim of this study was to extend lifetime and resistant to environmental conditions of LAMP and RT-PCR mixtures via lyophilization. Accordingly, three different lyophilization methods were tried and results were compared.

Materials and methods

Bacterial strains and DNA isolation

Mycobacterium tuberculosis H37Rv reference strains were provided by Atatürk Chest Diseases and Chest Surgery Training and Research Hospitals. Reference strains grown on Lowenstein-Jensen medium was homogenized in physiological saline solution (0.9 % NaCl). DNA of the reference strains was extracted by QIAGEN QIAamp DNA mini kit, according to the manufacturer's instructions. The purity and quality of DNA were controlled using Implen NanoPhotometer (Implen GmbH, Germany).

LAMP and RT-PCR Assay

M. tuberculosis specific LAMP primers were designed using PrimerExplorer V5 according to IS6110 gene (GenBank accession no: X17348). LAMP reaction was performed in 25 μ l, containing 0.2 μ M each outer primers (F3 and B3), 0.8 μ M each inner primers (FIP and BIP), 20 mM Tris-HCl (pH: 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 5 mM MgSO₄, 0.4 mM dNTP, 2 U Bst polymerase and 5 μ l DNA sample. For isothermal amplification, samples were incubated at 71.4 °C for 60 minutes. After amplification, Bst polymerase inhibition was performed at 80 °C for 10 min. LAMP products were run 2% w/v agarose gel electrophoresis in 90V for 60 min. IS6 (5'-GGCTGTGGGTAG-CAGACC-3'), IS7(5'-CG GGTCCAGATGGCTTGC-3') primers and IS6110 Taqman probe (5'-(FAM)- TGTCGACCTGG-GCAGGGTTCG-(TAMRA)-3') were used in RT-PCR method described by Desjardin et al. (1998) (22). Real Time PCR was carried out in 25 μ l, containing 0.2 mM dNTP, 0.2 μ M IS6 and IS7, 0.1 μ M probe, 1 U Taq polymerase, 5 mM MgCl₂, and 5 μ l DNA sample. PCR reaction was performed with BioRad CFX96 with following conditions: 50 °C for 2 min, 95 °C 5 min, and 45 cycles of 94 °C 30 s and 68 °C 1 min.

The specificity, sensitivity, positive predictive value and negative predictive value were determined from the results based on a calculation previously reported (23).

Lyophilization of amplification mixtures

Two different cryoprotectant mixtures which described by Sharma et al. (2014) and Klatser et al. (1998) (20,24), except our mixture was used for LAMP and RT-PCR methods. The tested lyophilization contents in this study are given in table 1. Lyophilization was performed on the Christ Epsilon 2-6D LSCplus freeze dryer and the protocol is given in table 2. Am-

Table 1. Cryoprotectant mixtures and amount of ingredients

Mixtures	Cryoprotectants	Amount
1. mixture (Sharma et al. 2014)	PEG 8000	0.1 %
	Glycerol	2.0 %
	Trehalose	0.1 mM
2. mixture (Klatser et al. 1998)	Trehalose	5 %
3. mixture (This study)	PEG 8000	0-0.1-0.2-0.5 %
	Stachyose	0-1.0-2.0 %
	Glycerol	0-1.0-2.0 %
	Trehalose	0-1.0-2.0-5.0 %

Table 2. The protocol of freeze dryer

Stage	Steps	Pressure	Temperature State	Time
Pre-chill		760 Torr	Shelf at -55 °C	60 min.
Load tray		760 Torr	Hold at -55 °C	-
Vacuum		760 Torr-100 mTorr	Hold at -55 °C	-
Primary Drying	1	100 mTorr	Hold at -55 °C	420 min.
	2	100 mTorr	Ramp from -55 °C to -37 °C	120 min.
	3	100 mTorr	Hold at -37 °C	480 min
	4	100 mTorr	Ramp from -37 °C to +10 °C	360 min
	5	100 mTorr	Hold at +10°C	240 min
	6	100 mTorr	Ramp from +10°C to +25°C	120 min
	7	100 mTorr	Hold at +25°C	30 min
Secondary Drying	8	100 mTorr	Hold at +25°C	30 min

plification reactions were performed on a lyophilized mixture with 20 µl of molecular grade water and 5 µl of DNA sample (Tab. 1 and Tab. 2).

Thermal stability and analytical sensitivity of lyophilized mixtures

The lyophilized LAMP and RT-PCR mixtures were stored at -20 °C, 4 °C and room temperature to reveal the thermal stability of lyophilized mixtures. Three sets of vials were used from each of the stored temperatures. Reaction mixtures were used for amplification on 1st, 2nd, 4th weeks and 2nd, 6th and 12th months.

Serial dilutions between 10⁵ CFU/ml and 10¹ CFU/ml of *M. tuberculosis* samples were used to determine the limit of detection of the lyophilized LAMP and RT-PCR mixtures. *M. tuberculosis* free sputum DNA was used as the negative control. The prepared dilutions were studied using both lyophilized mixtures and freshly prepared mixtures.

Results

Optimal cryoprotectant mixtures

For the lyophilization of LAMP and RT-PCR mixtures, three different cryoprotectant mixtures were tested. In the studies carried out as a result of the lyophilization process, described by Sharma et al., (2014) and Klatser et al., (1998) (20,24) contents were found to cause inhibition in both amplification reactions which can be shown in figure 2. The cryoprotectant mixture tested for the first time in this study is suitable for LAMP and RT-PCR studies. Accordingly, the cryoprotectant given in table 1 was tested at the indicated concentrations. As shown in figure 2, the cryoprotectant mixture proposed by Sharma et al., (2014) and Klatser et al., (1998) (20,24) has not been successful in our studies (Fig. 1 and Fig. 2).

Thermal stability of lyophilized mixtures

Lyophilized mixtures were stored at -20 °C, 4 °C and room temperature to protect from light. Lyophilized samples were tested in 3 replicates, both positive and negative, at 1, 2, 4 weeks, 2, 6 and 12 months. No inhibition was observed at -20 °C and 4 °C conditions for 12th month. However, the lyophilized samples

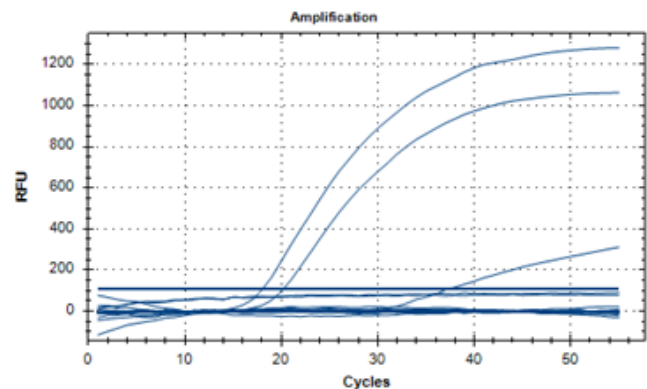


Figure 1. Results of different cryoprelyophilized mixture. 1) Freshly prepared mixture, 2) (0.5 % PEG 8000 + 2.0 % stachyose) Cryoprotectant mixture, 3) (0.5 % PEG 8000 + 1.0 % stachyose) Cryoprotectant mixture.

stored at room temperature did not work after 2 weeks (Fig. 3).

Analytical sensitivity of lyophilized mixtures

Serial dilutions between 10⁵ CFU/ml and 10¹ CFU/ml of the *M. tuberculosis* were used to measure the detection limit of lyophilized LAMP and RT-PCR assay. The limit of detection studies was performed in three replicates and parallel with lyophilized mixtures and the newly prepared mixtures. As a result, the detection limit of lyophilized and newly prepared mixtures was 10² CFU/ml in LAMP assay. The detection limit of lyophilized and newly prepared mixtures was 10¹ CFU/ml in RT-PCR assay (Fig. 4 and Fig. 5).

Discussion

The culture method is still the gold standard for the diagnosis of tuberculosis (25). However, in recent years, molecular tests have been studied for rapid diagnosis in the field. Lyophilization of the master mixes is an indispensable requirement for the developed molecular tests to be used in the field. There are

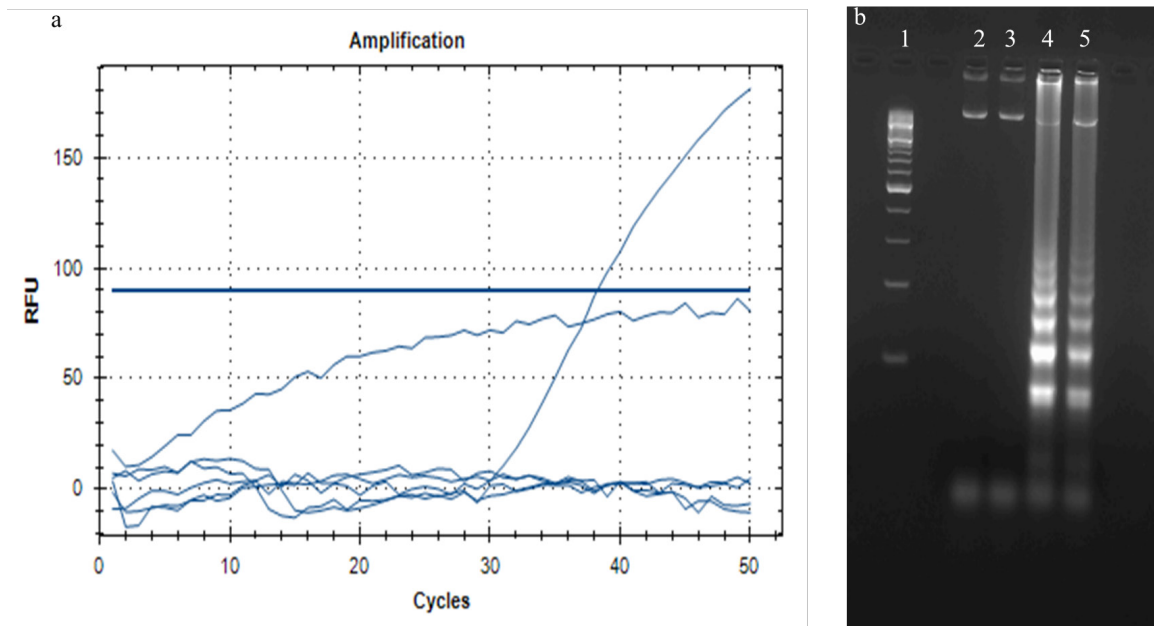


Figure 2. Results of lyophilized RT-PCR (a) and lyophilized LAMP (b) mixture. a: only 3. cryoprotectant mixture has positive result in RT-PCR assay. b: Results of LAMP assay 1) DNA ladder (100 bp, NEB), 2) 1. Cryoprotectant mixture, 3) 2. Cryoprotectant mixture, 4) 3. Cryoprotectant mixture 5) Freshly prepared mixture.

different stabilizers used for this purpose such as trehalose, PEG, Stachyose, glycerol (12,26). However, the stabilizers to be preferred must be compatible with the molecular method used. For the lyophilization of LAMP and RT-PCR mixtures, three different cryoprotectant mixtures were tested. In the study of Sharma et al., (2014) (24), the multiplex RT-PCR method was

developed for the diagnosis of foot and mouth disease virus (FMDV) and the optimization and validation of the lyophilization of the reaction content was performed. But, the cryoprotectant mixture proposed by Sharma et al., (2014) (24), has not been successful in our studies.

Undoubtedly, diagnostic mixes developed as lyophilized

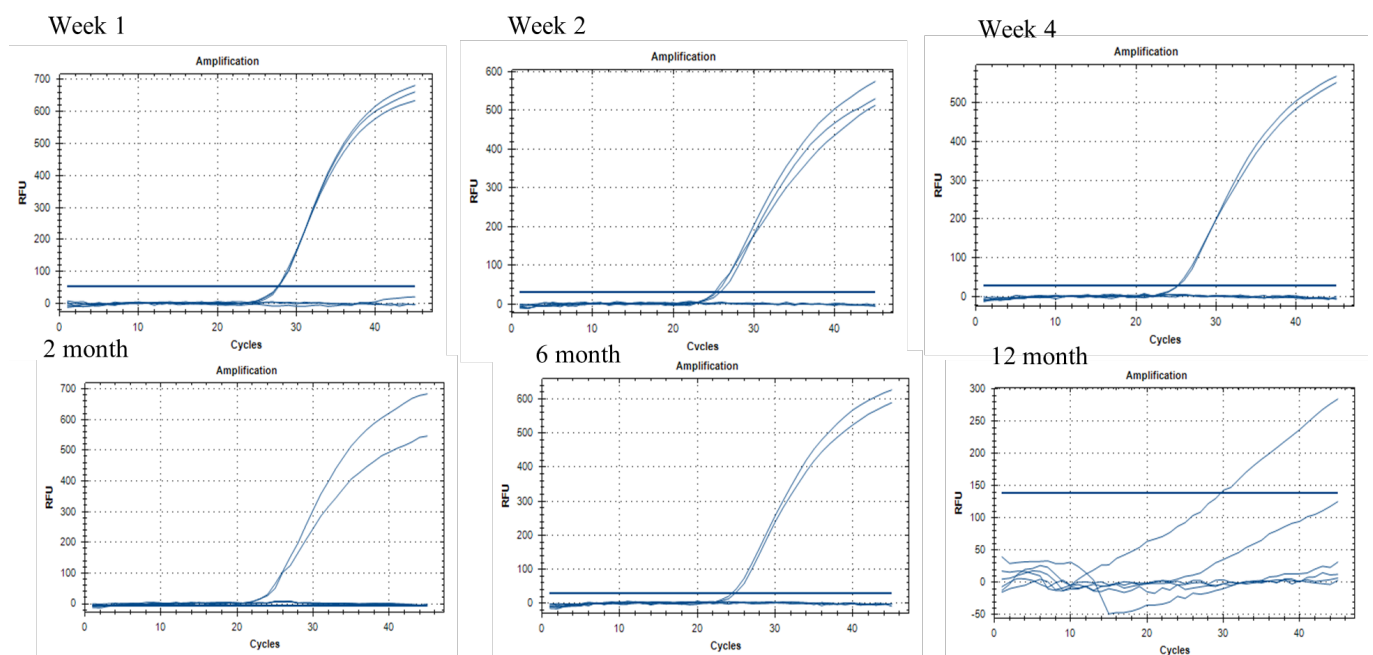


Figure 3. RT-PCR results of thermal stability of lyophilized mixtures

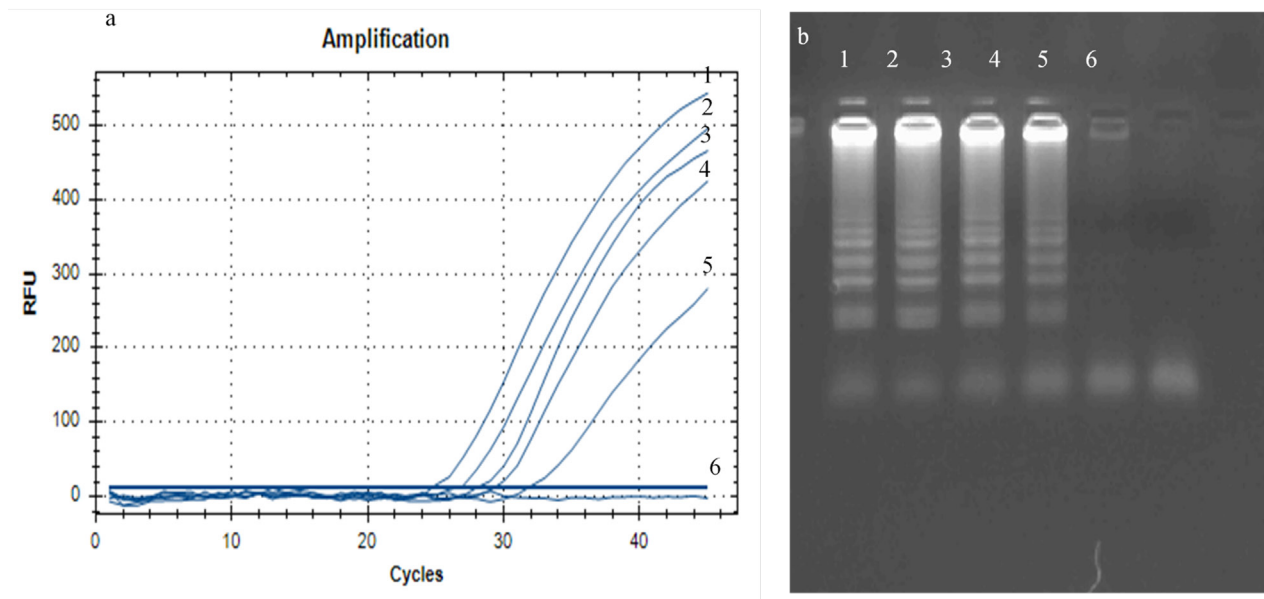


Figure 4. Detection limit results of lyophilized RT-PCR (a) and LAMP (b). a: 1) 10^5 CFU/ml, 2) 10^4 CFU/ml, 3) 10^3 CFU/ml, 4) 10^2 CFU/ml, 5) 10^1 CFU/ml, 6) negative control. b: a: 1) 10^5 CFU/ml, 2) 10^4 CFU/ml, 3) 10^3 CFU/ml, 4) 10^2 CFU/ml, 5) 10^1 CFU/ml, 6) negative control



Figure 5. An image of lyophilized mixture in PCR tubes.

have a longer life than non-lyophilized mixes (27). Nevertheless, even if they are lyophilized, these mixtures have certain periods in which they can maintain stability. The stability of the lyophilization developed by this study was evaluated up to 12 months at 3 different temperatures (-20°C , 4°C and room temperature). All lyophilized samples were assayed with 3 replicates and positive and negative samples. Studies were performed at the 1st, 2nd, 4th weeks and 2nd, 6th and 12th month after lyophilization. Any inhibition was not observed at -20°C and 4°C conditions for 12 months but the lyophilized samples stored at room temperature did not work after 2nd weeks. Chua et al. (2011) reported that the lyophilized PCR mixture remains stable for 7.4 months at 24°C . But unlike our study, the thermal stability of the lyophilized PCR assay was estimated using accelerated aging techniques with elevated temperatures also known as the Q10 method in Chua et al., (2011) study. Furthermore, the thermal stabilization of the lyophilized amplification mixture determined at room temperature for 2 weeks is enough for field studies. In a different study, the stability of the lyophilized mixture was studied at 3 different temperatures (4

$^{\circ}\text{C}$, 25°C and 37°C), over 30 days (28). According to Khazani et al., (2017), the lyophilized PCR mixture was stable for 30 days. Here, it can be said that the type and amount of cryoprotectants used may be important to the stabilization of mixtures.

The infectious dose of many infectious microorganisms is quite low (29). It is very important to determine the infective dose of these pathogens with the methods to be developed for the patient's health. For this purpose, the LOD is determined during the kit development stages. In this study, determination of the limit of detection of the lyophilized master mix was performed. As a result, the detection limit of the lyophilized LAMP master mix was 10^2 CFU/ml and the RT-PCR master mix was 10^1 CFU/ml. Here, it is seen that the lyophilization process does not affect the detection limit of both two methods. In the lyophilization study of the PCR mixture used in the detection of *S. aureus* by Nagaraj et al. (2018) (26), the limit of detection was determined as 10^6 CFU/mL. Although the limits of detection are different in the studies, especially, parallel results between lyophilized and fresh mixtures demonstrate an important advantage of lyophilization processes.

In conclusion, this study provides a suitable lyophilization process for LAMP and RT-PCR mixtures used in the detection of *M. tuberculosis*. 0.5 % PEG 8000 and 2.0 % stachyose were found to be suitable as cryoprotectant mixtures. After the stabilization study, it was found that the stabilization of the lyophilized mixture was 2 weeks at room temperature and 12 months for the other two temperatures (-20°C and 4°C). Finally, the lyophilization process used in this study did not reveal any negative effects on the detection limits of LAMP and RT-PCR methods. In this study, the cold chain requirement of amplification mixtures, which is an important obstacle for the detection of tuberculosis in the field, was terminated.

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