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Development of Recombinant Immunoblot Assay Diagnostic Test Based on HIV-1 in Indonesia

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Background: High mutation rates in HIV-1 could affect the accuracy of diagnostic tests. Therefore, recombinant antigen that has an immunodominant and conserved region from HIV-1 need to be developed to detect HIV-1 infection in Indonesia.
Materials and methods: The recombinant antigens comprise of Gag (p24), Pol and Env (gp41). Each antigens was expressed in the *Escherichia coli* expression system and purified using Ni-NTA chromatography. The reactivity of purified antigen against HIV antibodies was tested against a group of 50 HIV-positive plasma samples and 45 HIV-negative plasma samples in a recombinant immunoblot assay (RIBA) platform test. Moreover, 21 of 50 HIV-positive samples and 3 of 45 HIV-negative samples were also tested using HIV blot 2.2 to compare RIBA with a commercial western blot kit. Ten HBV-positive and 10 HCV-positive plasma samples were used to check cross-reactivity with HIV recombinant proteins in RIBA.
Results: All HIV-positive samples (100%) tested with RIBA were reactive towards Gag (p24), Pol, Env (gp41). Otherwise, 3 of 21 HIV-positive samples assayed with HIV blot 2.2 were not reactive to Pol protein. All HIV-negative samples tested with RIBA and 3 HIV-negative samples tested with HIV blot 2.2 did not produce any bands of HIV antigens. Few HBV and HCV samples showed reactivity towards HIV recombinant proteins.
Conclusion: Each recombinant protein, Gag (p24), Pol, Env (gp41), could be expressed and purified, as well as had reactivity to HIV-positive samples in RIBA test. Therefore, RIBA can be used as a diagnostic test to detect HIV-1 infection in Indonesia.

Keywords: *diagnostic, HIV-1, immunodominant, recombinant immunoblot assay (RIBA)*

Introduction

Human immunodeficiency virus (HIV) is an agent that causes acquired immunodeficiency syndrome (AIDS). HIV-1 is responsible for the AIDS epidemic worldwide. This type has high variations because of its high genome mutation rates.[1] HIV-1 includes four groups, i.e. M (main), O (outlier), N (non-M, non-O), and P. HIV-1 group M has

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9 subtypes or clades, i.e. A-D, F-H, J and K. Subtypes of HIV-1 M differ at least 20% in amino acid composition of the envelope region and 15% in the area of Gag.[1,2] Subtype B is the most prevalent HIV-1 globally, especially in Europe and North America, while other subtypes are prevalent in Southeast Asia, including Indonesia. Differences in genome and protein sequences among subtypes of group M could affect the sensitivity in diagnostic tests, especially for non-B subtypes. Four subtypes of M group are found in Indonesia, namely subtype CRF01_AE, CRF02_AG, B and C. CRF01_AE dominates the HIV epidemic in Indonesia and has an infection percentage over 90%.[3,4] Genetic diversity in HIV-1 can decrease the success rate in diagnostic tests. Globally, misdiagnosis of HIV status using serologic tests happened in 6 out of 100 persons who were falsely identified as HIV-negative and 2 out of 100 persons would be falsely identified as HIV-positive.[5] Therefore, it is necessary to develop a diagnostic test that can detect HIV-1 subtypes in Indonesia more accurately to support the government policy to achieve AIDS-free Indonesia in 2030 and the UNAIDS 90-90-90 target.[6,7]

Previously, western blotting was one of the national testing strategies recommended by the World Health Organization (WHO). However, in order to scale-up HIV testing, prevention, and treatment, WHO recommends rapid diagnostic test or enzyme immunoassay other than western blotting which are simpler and lower in cost.[6] Notwithstanding, western blotting still can be used as a confirmatory test because of its high sensitivity and specificity for detecting anti-HIV antibodies. The western blot test is usually used to confirm the diagnosis results if an ELISA test is positive. In order to reduce indeterminate results, recombinant immunoblot assay (RIBA) can also be used as a supplemental confirmatory method for detecting HIV infections. Previous research showed that RIBA can reduce the window period to confirm early HIV infection.[8,9]

Determination of positive, negative, or indeterminate results in RIBA follow the western blot test which requires three major proteins of HIV, i.e. Env, Gag, and Pol proteins. According to the WHO guidelines, the HIV test results are positive when there is serum reactivity towards 2 Env antigen, and 1 Pol antigen or 1 Gag antigen, whereas according to Center for Disease Control (CDC) guidelines, the HIV test results are positive when there is serum reactivity towards 1 Env antigen and 1 Gag antigen (p24). The test is negative if no bands are formed, whereas the result is indeterminate if it does not show results with positive or negative criteria.[2]

In this research, we developed RIBA that had recombinant proteins involving immunodominant and conserved epitopes from four dominant HIV-1 subtypes in Indonesia. There were three recombinant antigens from HIV-1 that had been developed. In the previous research, Pol and Env (gp41) were constructed based on four subtypes of HIV-1 and Gag (p24) were constructed from HIV-1 subtype CRF01_AE that were conserved for the other three HIV-1 subtypes. Hopefully, this method provides diagnostic confirmation test for HIV-1, especially for subtypes that are widely spread in Indonesia.

HIV-positive patients are at high risk of hepatitis B and/or hepatitis C co-infection because of common routes of transmission. The hepatitis B virus (HBV) and hepatitis C virus (HCV) infection could also be detected in blood samples.[10,11] Hence, we used HBV-positive and HCV-positive samples to observe cross-reactivity of HBV and HCV antibodies with the recombinant protein developed in this RIBA test. Antibody cross-reactivity can lead to incorrect diagnoses and false-positives should be considered when testing for HIV antibodies.[10]

Materials and methods

Expression and Purification of Recombinant Proteins

The gene sequences of recombinant proteins Pol, Env (gp41) and Gag (p24) used in this study had been codon-optimized to be expressed in *Escherichia coli* cells.[10,11] Pol and Env (gp41) were expressed in *E. coli* BL21, while Gag (p24) protein was expressed in *E. coli* BL21CP. Protein expression was induced using 1mM IPTG at 37°C for 4 hours. The recombinant proteins were purified using affinity column chromatography (Ni-NTA) in a native and denaturing conditions according to the QIA Expressionist™ protocol.[12] The protein concentration was measured using the Lowry method.

Samples Collection

The plasma or serum specimen with HIV infection were obtained from Subdit HIV Ditjen PPPL Kemenkes RI, RSUPN Dr. Cipto Mangunkusumo and Indonesian Red Cross and stored in Virology and Cancer Pathobiology Research Center (VCPRC). This study was approved by The Ethics Committee of the Faculty of Medicine, University of Indonesia. Informed consent was obtained from each study participant. The patients' samples were first used in HIV drug resistance research. There were a total of

50 HIV-1 positive samples that were used in this study and there was no recent HIV infection. Thirty-nine out of 50 HIV-1 positive samples had been genotyped and confirmed with viral load testing. The other 11 HIV-positive samples had not been genotyped, but had been tested and showed reactivity using Advia Centaur EHIV and CHIV (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA), Architect HIV (Abbott, Wiesbaden, Germany), Vironostika HIV Ag/Ab (bioMérieux, Marcy l'Etoile, France), Murex HIV Ag/Ab Combination (DiaSorin, Saluggia, Italy), and Genscreen Ultra Ag/Ab (Bio-Rad, Marnes-la-Coquette, France) immunoassays (Figure 1). The virus in HIV-positive plasma was inactivated by heating it in heat block 56°C for 30 minutes before being used for reactivity test against all three recombinant proteins that have been purified. We used 45 HIV negative plasma samples. Besides that, we used 10 HBV-positive plasma and 10 HCV-positive plasma samples to determine if there are cross-reactivity with HIV recombinant proteins (Pol, Env gp41 and Gag p24).

Western Blot

Overnight protocol was used according to HIV Blot 2.2 (MP Diagnostics, Singapore, Singapore) kit's manufacturer instruction.[13]

RIBA

Purified Pol, Env gp41 and Gag p24 proteins were run in SDS-PAGE. After that, the gel was prepared for the RIBA process using western blot method. After the transfer process was complete, the membrane was stained with Ponceau S to see the efficiency of protein transfer to the membrane. Then, the membrane strips were blocked with blocking buffer (5% skim milk in PBS-Tween 0.05%) and were given the primary antibodies from serum samples with final concentration of 1/10, and 1/200 for control serum from kit. The membrane strips were given the biotin-labeled secondary antibody with final concentration of 1/10.000, and strep-HRP with a final concentration of 1/10.000. The membrane strips were incubated for 1 hour at room temperature while rocking at 60 rpm or overnight at 40°C on each stage. In addition, the membrane strips were washed with PBS-Tween 0.05% for 15 minutes and repeated washing for 5 minutes on each stage. Thereafter, the strip was placed on top of the cellophane plastic and administered with Immun-Star™ HRP Substrate (Bio-Rad, Marnes-la-Coquette, France) (ratio 1: 1) and incubated for 1 min. Strips were visualized using ImageQuant Las 4000 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

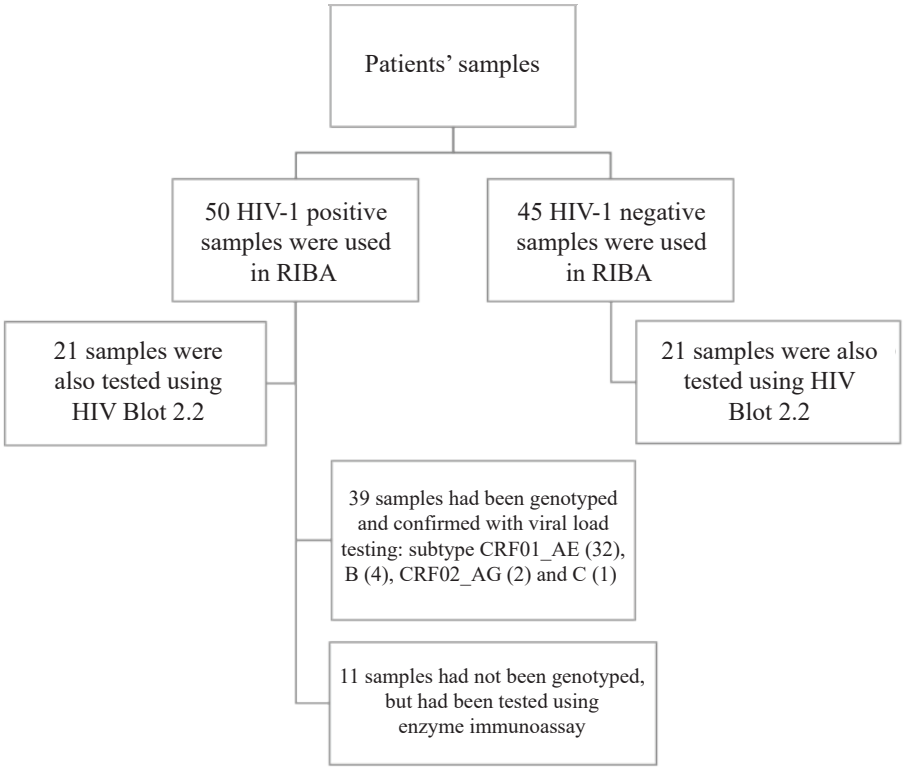

Figure 1. Flowchart of patients' samples used for RIBA and western blot.

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Results

Expression of Pol, Env (gp41) and Gag (p24) were analyzed on SDS-PAGE. The expression results of Pol, Env (gp41) and Gag (p24) after induced with 1 mM IPTG were optimal in 4 hours. All proteins then purified using affinity column chromatography (Ni-NTA) in a native and denaturing conditions. The purified proteins were analyzed on SDS-PAGE (Figure 2) and the concentrations were measured using the Lowry method. The Gag (p24) protein had a molecular weight about 27 kDa and concentration 1.36 mg/mL. The Pol protein had a molecular weight about 55 kDa with concentration 1.63 mg/mL, while the Env (gp41) protein had a molecular weight about 15 kDa with concentration 2.32 mg/mL.

All proteins were transferred to nitrocellulose membrane. Among 50 samples that used in this study, 32 HIV-positive samples identified as subtype CRF01_AE, 4 HIV-positive samples identified as subtype B, 2 HIV-positive samples identified as subtype CRF02_AG, 1 HIV-positive sample identified as subtype C and 11 HIV-positive samples were unknown. All HIV-positive plasma samples were 100% reactive to Pol, Env (gp41) and Gag (p24) proteins.

The bands of Pol (55kDa) were located between 39.8 kDa and 58.1 kDa. Gag (p24) (27 kDa) bands located between 20 kDa and 29 kDa. Env (gp41) protein (15 kDa) bands located between 14.3 kDa and 20 kDa (Figure 3A). Meanwhile, 45 HIV-negative plasma samples showed absent reactivity for Pol, Env (gp41) and Gag (p24) (Figure 3A).

Twenty-one samples from 50 HIV-positive plasma samples and 3 samples from 45 HIV-negative plasma samples were tested using HIV 2.2 Blot kit. Twenty-one HIV-positive plasma samples showed reactivity and the HIV-negative plasma showed absent reactivity. None of the samples tested in this study showed reactivity against HIV-2 protein antigens.

Most samples had reactivity towards all proteins when tested using RIBA and HIV Blot 2.2. However, sample HIV 3, HIV 4, and HIV 6 had reactivity towards proteins Gag and Env, but did not show reactivity towards Pol protein when tested with HIV Blot 2.2 (Table 1).

Ten HBV-positive plasma samples and 10 HCV-positive plasma samples were tested toward Pol, Env (gp41) and Gag (p24) in RIBA (Table 2). Two HBV-positive samples had reactivity towards Pol and Gag (p24) and three HBV-positive samples had reactivity towards Gag (p24) protein only. In HCV-positive samples, there were one sample that had reactivity toward Pol and two samples that had reactivity toward Gag (p24) protein.

Discussion

The use of the PQE80L vector having a 6xHis-Tag sequence can facilitate the binding of recombinant proteins to Ni-NTA.[14,15] Purification of Ni-NTA can be performed either on native or denaturing conditions apart from the tertiary structure of the protein, but depends on the nature and location of the target protein (located within the cytoplasm or in the inclusion bodies in cytoplasm). At first, the three recombinant proteins were prepared from cleared lysate with sonication to see the solubility of recombinant proteins. The results of SDS-PAGE analysis show that recombinant protein Gag (p24) was a dissolved protein, while Pol and Env (gp41) protein were not soluble. Recombinant proteins expressed in *E. coli* are usually present in soluble form, but proteins can form aggregates and inclusion bodies that are not soluble in the purification process at high expression levels. Proteins that form aggregates such as the Pol and

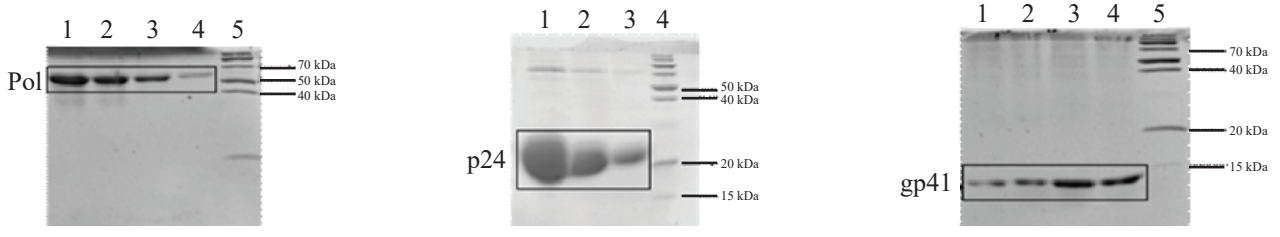

Figure 2. Purification of Pol, Gag (p24) and Env (gp41) proteins. Left: Purification of Pol protein with denature condition (Lane 1-4: elution 1-4; lane 5: protein marker). Middle: Purification of Gag (p24) protein with native condition (Lane 1-3: elution 1-3; lane 4: protein marker). Right: Purification of Env (gp41) protein with denature condition (Lane 1-4: elution 1-4; lane 5: protein marker).

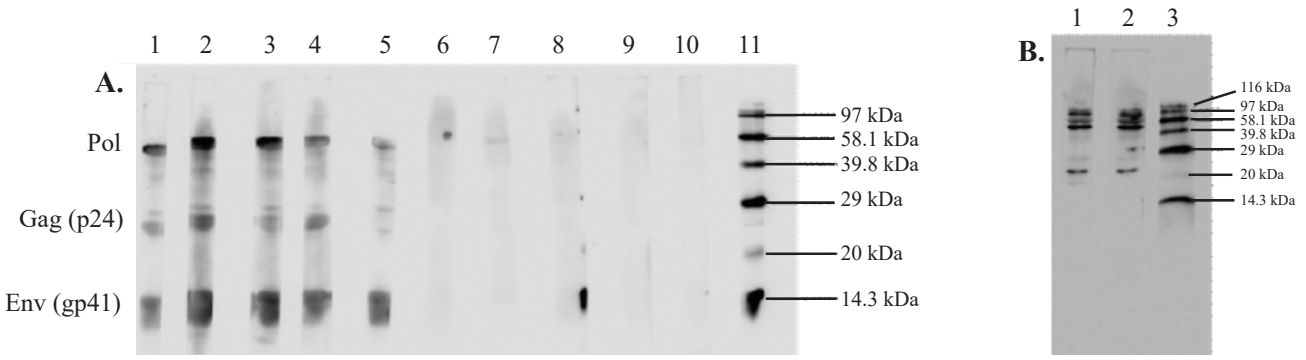

Figure 3. RIBA test result. A: Lane 1 -5: HIV-positive samples against three recombinant proteins; lane 6-10: HIV-negative samples against three recombinant proteins; lane 11: biotin marker B2787. B: Lane 1-2: rabbit serum with antibody to *E. coli* protein; lane 3: biotin marker B2787.

Table 1. Comparison of HIV Blot 2.2 kit and RIBA test.

No.	Samples	HIV Blot 2.2 Kit			RIBA		
		pol[(a)]	gag[(b)]	env[(c)]	Pol (IDR)	p24	Env (gp41 IDR)
1	HIV 1	+	+	+	+	+	+
2	HIV 2	+	+	+	+	+	+
3	HIV 3	−	+	+	+	+	+
4	HIV 4	−	+	+	+	+	+
5	HIV 5	+	+	+	+	+	+
6	HIV 6	−	+	+	+	+	+
7	HIV 7	+	+	+	+	+	+
8	HIV 8	+	+	+	+	+	+
9	HIV 9	+	+	+	+	+	+
10	HIV 10	+	+	+	+	+	+
11	HIV 11	+	+	+	+	+	+
12	HIV 12	+	+	+	+	+	+
13	HIV 13	+	+	+	+	+	+
14	HIV 14	+	+	+	+	+	+
15	HIV 15	+	+	+	+	+	+
16	HIV 16	+	+	+	+	+	+
17	HIV 17	+	+	+	+	+	+
18	HIV 18	+	+	+	+	+	+
19	HIV 19	+	+	+	+	+	+
20	HIV 20	+	+	+	+	+	+
21	HIV 21	+	+	+	+	+	+

(+): reactive; (-): not reactive; (a): Pol protein (p31, p51 or p66); (b): Gag protein (p55, p24 or p17); (c): Env protein (gp160, gp120 or gp41).

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Table 2. Reactivity of HBV and HCV positive samples towards Pol, Env (gp41) and Gag (p24).

No.	Samples	Recombinant Proteins		
		Pol	Env (gp41)	Gag (p24)
1	HBV 1	-	-	+
2	HBV 2	+	-	+
3	HBV 3	-	-	+
4	HBV 4	+	-	+
5	HBV 5	-	-	+
6	HBV 6	-	-	-
7	HBV 7	-	-	-
8	HBV 8	+	-	-
9	HBV 9	-	-	-
10	HBV 10	-	-	-
11	HCV 1	-	-	-
12	HCV 2	-	-	+
13	HCV 3	-	-	-
14	HCV 4	-	-	-
15	HCV 5	+	-	-
16	HCV 6	-	-	-
17	HCV 7	-	-	-
18	HCV 8	-	-	-
19	HCV 9	-	-	+
20	HCV 10	-	-	-

(+): reactive; (-): not reactive.

Env (gp41) were further purified in denaturing condition by adding denaturing agents such as 6M GuHCl and 8M urea or detergent to dissolve the protein.[15,16]

Elution of recombinant proteins has been successfully carried out, but the proteins still contained contaminant proteins from *E. coli* cells. This phenomenon often occurs in histidine-tagged recombinant proteins because *E. coli* and chaperon proteins have histidine residues. This allows bond formation between *E. coli* and resin proteins during the purification process.[16,17] The contaminants in recombinant proteins indicate that the affinity purification process with Ni-NTA in both native and denaturing conditions was still not effective. Another purification method is required to purify the recombinant protein from contaminant protein.

Previous research showed that RIBA test was able to avoid false-positive test results in low-risk populations, reduce uncertainty test results in high-risk populations and had high sensitivity and specificity to detect HIV-1 infection.[3,8,9,18,19] In addition, RIBA was able to detect various HIV-1 subtypes. Subtypes of HIV-1 group M differ in amino acid composition ±20-30% in the envelope region and 15% in the Gag region. In RIBA, all HIV-1 positive samples had positive results and all HIV-1 negative samples had negative results. The sensitivity and specificity of RIBA was 100%, but it needs further tests with larger sample size (>100) to obtain higher confidence. This study also showed that the recombinant proteins used in RIBA test had better reactivity than proteins in HIV Blot 2.2 western blot kit. Twenty-one HIV-AIDS samples showed reactivity toward Pol, Env (gp41) and Gag (p24) protein in RIBA test (21/21). Meanwhile, the reactivity of the samples to Gag and Env proteins in HIV Blot 2.2 kit were 100% (21/21), but reactivity towards Pol protein only 85.71% (18/21).

The weakness of RIBA test is contamination of protein from *E. coli* cells that were used to produce recombinant proteins. The test results using rabbit anti-*E. coli* serum samples showed reactivity bands. Figure 3B showed some *E. coli* protein bands located between 39.8 kDa and 58.1 kDa, 20 kDa and 29 kDa, which are the same size as Pol and p24 proteins. *E. coli* proteins, such as GroES (10.39 kDa), Fur (16.79 kDa), SlyD (20.85 kDa), CA (25.10 kDa), RplB (29.86 kDa), DnaJ (41.10 kDa), GroEL (57.35 kDa), and DnaK (69.11 kDa) are usually carried away during purification.[16] They have histidine residue so they can adhere to resin in Ni-NTA/IMAC affinity chromatography. Other than that, patients with a history of *E. coli* co-infection may produce false-positive test results and reduced specificity.

All HIV-negative samples did not show reactivity toward the recombinant proteins in RIBA test nor to the HIV proteins in the kit. However, some strips showed dark colors. This may be caused by sample contamination.[20]

The cross-reactivity test using HBV-positive and HCV-positive samples showed reactivity to Pol and Gag (p24), while no reactivity to Env (gp41) was observed. The result was negative or indeterminate for HIV-1 RIBA (Table 2), but the reactive results showed that false-positive and cross-reactivity may occur in HIV diagnosis. In cases of acute HBV or HCV infection, a false-positive result with an HIV test result is highly probable. In addition, if HIV infection is at a late stage characterized by a decreased $CD4^+$ T cell count, the chances of co-infection with HBV and HCV are higher.[10,11]

This study had some limitations. We did not count the $CD4^+$ T cell in HIV positive samples. We also did not know the stage of infection in HBV and HCV samples. Further studies are needed to determine the cause of cross-reactivity in RIBA HIV-1 test as well as implement quality control and quality assurance during recombinant protein development.[10]

Conclusion

Gag (p24), Pol, Env (gp41) recombinant proteins could be expressed and purified. RIBA that had those recombinant proteins had better reactivity compared to HIV Blot 2.2 kit. RIBA can be used as a potential diagnostic test to detect HIV-1 infection in Indonesia. Further research is needed to purify the target protein from contaminant protein to prevent non-specific test results. Apart from that, Gag (p24), Pol, Env (gp41) recombinant proteins may also be used as a component in other antibody detection methods that detect HIV-1 in human serum, plasma or whole blood specimens, such as rapid test or enzyme immunoassays recommended by the WHO.

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