**Human Leukocyte Antigen-E Alleles are Associated with Hepatitis C Virus, Torque Teno Virus, and Toxoplasma Co-infections but are not Associated with Hepatitis B Virus, Hepatitis D Virus, and GB Virus C Co-infections in Human Immunodeficiency Virus Patients**

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**ABSTRACT**

**Context:** Data regarding the distribution of Human Leukocyte Antigen (HLA)-E alleles and their association with blood-borne pathogen infections/co-infections are limited for many populations, including Indonesia. **Aims:** The aim of this study was to analyze the association between HLA-E allelic variants and infection with blood-borne pathogens such as hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), torque teno virus (TTV), GB virus C (GBV-C), and Toxoplasma gondii (T. gondii) in Indonesian Javanese human immunodeficiency virus (HIV) patients. **Settings and Design:** A total of 320 anti-HIV-positive blood samples were analyzed for HBV, HCV, HDV, TTV, GBV-C, and T. gondii infection status and its association with HLA-E allelic variants. **Materials and Methods:** Nucleic acid was extracted from plasma samples and used for the molecular detection of HBV DNA, HCV RNA, HDV RNA, TTV DNA, and GBV-C RNA, whereas hepatitis B surface antigen, anti-HCV, immunoglobulin M and G (IgM and IgG) anti-T. gondii were detected through serological testing. The blood samples were genotyped for HLA-E loci using a sequence-specific primer-polymerase chain reaction. **Statistical Analysis Used:** Either the Chi-square or Fisher’s exact test was performed to analyze the frequency of HLA-E alleles and blood-borne pathogen infections in the population. Odds ratios (ORs) were calculated to measure the association between the antibodies found and the participants’ possible risk behaviors. A logistic regression analysis was used to assess the associations. **Results:** HLA-E*0101/0101 was associated with HCV/TTV co-infection (adjusted OR [aOR]: 3.5; 95% confidence interval [CI]: 1.156-10.734; \( P = 0.027 \)) and IgM/IgG anti-Toxo positivity (aOR: 27.0; 95% CI: 3.626-200.472; \( P = 0.001 \)). HLA-E*0103/0103 was associated with TTV co-infection (aOR: 2.7; 95% CI: 1.509-4.796; \( P = 0.001 \)). **Conclusions:** HLA-E alleles in Indonesian Javanese HIV patients were found to be associated with HCV, TTV, and toxoplasma co-infections.

**Key words:** Hepatitis C virus, Human immunodeficiency virus, Human leukocyte antigen-E, Torque teno virus, Toxoplasma

**INTRODUCTION**

Human leukocyte antigen (HLA)-E is a member of the nonclassical (Class Ib) major histocompatibility complex, which is defined by limited polymorphism and a restricted pattern of cellular expression.¹⁻³ The HLA-E gene consists of 7 exons and is expressed in most tissues.¹⁻³ HLA-E molecules are ligands for CD94/NKG2 receptors on natural killer (NK) cells and NK cytotoxic gene for immune recognition of self and non-self peptides.⁴⁻⁶ This process is known as KIR2DS1 and KIR2DL1, which are involved in the regulation of T cell responses.⁷⁻¹⁰

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T-lymphocytes (NK-CTL); binding results in the inhibition (NKG2A) or activation (NKG2C) of NK cell-mediated cytotoxicity. HLA-E also serves as a ligand for the αβ-T-cell receptor on CD8+ T lymphocytes (CTLs). Currently, the HLA-E*0101 and HLA-E*0103 alleles are frequently found in worldwide populations (approximately 50% each). HLA-E*0101 and HLA-E*0103 are differentiated by a G to A single-nucleotide (NT) substitution in the HLA-E a2 domain of exon 3 at codon 107, which encodes an amino acid substitution of arginine (HLA-E*0101) to glycine (HLA-E*0103). These differences might be the underlying causes of the different functions and mechanisms of these alleles.

Women in Zimbabwe, who are homozygous for the HLA-E*0103 allele, have a 4.0-fold decreased risk for infection by human immunodeficiency virus 1 (HIV-1). This decreased risk might be achieved through mucosal immune response modulation by HLA molecular expression. Moreover, HLA-E*0103 is associated with better prognosis for HIV infection, although the mechanisms underlying this effect are unknown. In contrast, the HLA-E*0101/HLA-E*0101 genotype is associated with increased resistance to hepatitis C virus (HCV) infection. The protective role of HLA-E*0101 against HCV infection may be due to HCV-associated up-regulation of the inhibitory NKG2A receptor, which renders cells more vulnerable to inhibition through HLA-E/NKG2A interactions. Both HLA-E*0101 and HLA-E*0103 play protective roles in HIV and HCV infection. Discrepancies in the roles of different HLA-E alleles may be explained by the fact that, in contrast to HCV infection, HIV infection is associated with reduced NKG2A expression. Recently, the HLA-E variant HLA-E*0101 was also associated with an increased likelihood of HCV clearance. This association may help to predict sustained virological responses among HIV/HCV-co-infected patients undergoing therapy with PEGylated-interferon-alpha and ribavirin. HLA-E*0101 has a lower affinity for its natural ligands when compared to HLA-E*0103; thus, HLA-E*0101 is predicted to have better binding capabilities for HCV peptides that will later facilitate HLA-E-restricted CD8+ T-cell responses. This finding emphasized the positive role of HLA-E*0101 during HCV infection. In contrast, a study reported that HLA-E*0101 was associated with an increased risk of hepatitis B virus (HBV) infection, further indicating that HLA-E might have an influence on HBV infection. To date, we have a limited understanding of the role of HLA-E alleles in torque teno virus (TTV), GB virus C (GBV-C), and Toxoplasma gondii (T. gondii) infection. In HIV patients, co-infections involving blood-borne pathogens such as HCV, HBV, TTV, GBV-C, and T. gondii in Indonesian Javanese HIV patients. HLA-E alleles were found to be associated with HCV, TTV, and toxoplasma co-infections in Indonesian Javanese HIV patients.

MATERIALS AND METHODS

Study population

Since 2009, our group has been conducting a molecular epidemiology study of human blood-borne viruses and blood-borne pathogens (e.g., T. gondii) by collecting epidemiological-clinical data and blood samples from high-risk communities in Central Java, Indonesia. All participants were subjected to hematological and HIV assays, and then fractionated, aliquoted, and kept frozen until analysis. Written informed consent was obtained from all the individuals who participated in the study. Approval was obtained from the institutional ethics committee review boards of the Faculty of Medicine, Sebelas Maret University and the Dr. Moewardi General Hospital, Surakarta, Indonesia. All procedures were conducted according to the principles of the declaration of Helsinki. Through 2010, a total of 320 anti-HIV-positive blood samples were collected and used for the present study.

Serological markers for Toxoplasma gondii, hepatitis B virus, hepatitis C virus, and hepatitis D virus infections

The subjects’ plasma was separated from whole blood with ethylenediaminetetraacetic acid and screened for T. gondii, HBV, HCV, and hepatitis D virus (HDV) by serological assays as described previously. Briefly, the DRG T. gondii immunoglobulin M (IgM) Elisa Kit (DRG International, Inc., Springfield, NJ, USA) and the DRG T. gondii immunoglobulin G (IgG) Elisa Kit (DRG International, Inc., Springfield, NJ, USA) were used to detect IgM anti-Toxo and IgG anti-Toxo antibodies, respectively. An SERATEC Hepatitis B Quick Test (Gesellschaft für Biotechnologie GmbH, Göttingen, Germany) was used to detect hepatitis B surface antigen (HBsAg). An Ortho
HCV PA II (Ortho Diagnostics, Tokyo, Japan) and an HDV Ab ELISA (Diagnostic Automation, Calabasas, CA, USA) were used to detect anti-HCV and anti-HDV antibodies, respectively. All assays were performed according to the manufacturer's instructions. All samples were tested at least in duplicate.

**Nucleic acid extraction and molecular detection**

Viral nucleic acid extraction and molecular detection were performed as previously described. Briefly, nucleic acids (DNA and RNA) were extracted from 200 μl of all plasma samples using the PureLink Viral RNA/DNA Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The nucleic acids were then aliquoted, and one aliquot was reverse-transcribed according to the SuperScript III First-Strand cDNA Synthesis Supermix Kit protocol (Invitrogen, Carlsbad, CA, USA). Molecular detection was performed by polymerase chain reaction (PCR) using the AmpliTaq Gold™ 360 DNA Polymerase Kit (Invitrogen, Carlsbad, CA, USA). A portion of the HBsAg gene was amplified by nested PCR. A portion of the NS5B region and a portion of the E1-E2 region of the HCV genome were amplified by nested PCR. HDV RNA was detected by amplification of a 400-bp-long region of the HDV genome proposed for the classification of HDV genotypes. A fragment of 344 bp from the 5' noncoding region of GBV-C was detected by nested PCR as described previously. TTV DNA was amplified by the nested PCR amplification of a conserved region of ORF2 as described previously. Internal amplification controls were included to exclude any false-negative results. Corresponding positive controls and one negative control (sterile water) were included for each group. To prevent PCR contamination, the reagent preparation, sample processing, and nested PCR assays were performed in rooms separate from those where the amplified products were analyzed. Aerosol-resistant pipette tips were used throughout the assays. The PCR products were subjected to electrophoresis in 2% agarose gels, which were stained with ethidium bromide and visualized under ultraviolet illumination. The specificity was confirmed by sequencing the amplicons. All samples were tested at least in duplicate.

**Human leukocyte antigen-E genotyping**

We extracted DNA using the High Pure PCR Template Preparation Kit (Roche Molecular Diagnostics, Manheim, Germany). The blood samples were genotyped for HLA-E loci using a sequence-specific primer (SSP)-PCR as described elsewhere, with internal amplification controls included to exclude any false-negative results. Briefly, to differentiate HLA-E alleles, the forward primers E*0101F (5-GGC TCG AGC TGG GGC CCG CCA-3) and E*0103F (5-GGC TCG AGC TGG GGC CCG CCG-3) paired with a common reverse primer (5-AGC CTG TGG ACC CTC TT-3) were used. We used the FastStart High Fidelity PCR System (Roche Molecular Diagnostics, Manheim, Germany) for the SSP-PCR. The PCR products were subjected to electrophoresis in 2% agarose gels, which were stained with ethidium bromide and visualized under ultraviolet illumination. All samples were tested at least in duplicate.

**Statistics**

The statistical analyses were performed using SPSS version 21 software (IBM Corp, Armonk, NY, USA). HLA-E allele frequencies were calculated with the direct counting method. The deviation from the Hardy-Weinberg equilibrium was quantified using the Chi-square test. Either the Chi-square or Fisher's exact test was performed to analyze the frequency of HLA-E alleles and blood-borne pathogen infections in the population. Odds ratios (ORs) were calculated to measure the association between the antibodies found and the participants’ possible risk behaviors. A logistic regression analysis was used to assess the associations between the HLA-E alleles, respondents’ genders, age, sexual orientation and activities (heterosexual with 1 partner, heterosexual with >1 partner, or homosexual), history of injection drug use (IDU), percentage of CD3+/CD4+ lymphocytes, CD3+/CD4+ lymphocyte absolute count, hemoglobin count, percentage of hematocrit, leukocyte count, platelet/thrombocyte count, erythrocyte count, and blood-borne pathogen infections found in respondents. There were 22 missing values for the variable sexual orientation and activities and two for all of the blood count variables. Logistic regression analysis was performed using the backward likelihood ratio method and selected by a stepwise algorithm (at each step, factors are considered for removal or entry: P value for an entry of 0.20 and exit of 0.15). All variables that had a P < 0.25 in the bivariate analyses were included in the logistic regression analysis. Adjusted ORs (aORs) were presented as the result of the logistic regression multivariate analysis, and 95% confidence intervals (CIs) were calculated for all data analyses. Results of the multivariate analyses were considered significant if the P value was below 0.05.

**RESULTS**

**Demographics**

A total of 320 HIV-positive blood samples were collected from 170 males (53.1%) and 150 females (46.9%) ranging...
from 19 to 72 years of age. Up to 85% (272/320) of the patients had received antiretroviral drugs. A total of 6.9% (22/320) of the subjects were IDUs. IgM anti-Toxo-, IgG anti-Toxo-, HBsAg, HBV DNA-, anti-HCV-, HCV RNA-, anti-HDV-, HDV RNA-, GBV-C RNA-, and TTV DNA-positive rates were 10.0% (32/320), 35.0% (112/320), 4.4% (14/320), 4.4% (14/320), 10.0% (32/320), 24.4% (78/320), 0% (0/320), 0% (0/320), 7.5% (24/320), and 30.6% (98/320), respectively [Table 1].

A list of dual co-infections and several triple co-infections is provided in Table 1. Triple co-infections included in Table 1 have significant associations with specific HLA-E alleles.

The positive rates of TTV DNA/anti-HCV, TTV DNA/IgG anti-Toxo, TTV DNA/IgM anti-Toxo, anti-HCV/IgG anti-Toxo, and HCV RNA/IgM anti-Toxo were 4.4% (14/320), 11.9% (38/320), 3.1% (10/320), 3.1% (10/320), and 3.1% (10/320), respectively. The positive rates of TTV DNA/HBsAg, GBV-C RNA/IgM anti-Toxo, and TTV DNA/HBsAg/HBV DNA were all 1.9% (6/320), 1.3% (4/320), and 3.1% (10/320), respectively. In the logistic regression model, positive rates of TTV DNA were associated with the linear value of hemoglobin (g/dl) (aOR: 4.61; 95% CI: 2.185-9.719; \( P < 0.001 \)) and erythrocytes (million/μl) (aOR: 3.14; 95% CI: 1.643-6.002; \( P = 0.001 \)).

## Table 1: Demographics and human leukocyte antigen-E allele distribution among anti-human immunodeficiency virus-positive respondents

| Variables                              | Frequency (%) | HLA-E*0101/0101 (%) | HLA-E*0101/0103 (%) | HLA-E*0103/0103 (%) |
|----------------------------------------|---------------|---------------------|---------------------|---------------------|
| Gender                                 |               |                     |                     |                     |
| Male                                   | 53.1 (170/320)| 55.6 (20/36)        | 49.3 (68/138)       | 56.2 (82/146)       |
| Female                                 | 46.9 (150/320)| 44.4 (16/36)        | 50.7 (70/138)       | 43.8 (64/146)       |
| Sexual orientation and activities      |               |                     |                     |                     |
| Heterosexual with a partner            | 4.3 (1/298)   | 4.6 (1/36)          | 5.5 (1/36)          | 5.5 (1/36)          |
| Heterosexual with >1 partner           | 4.9 (1/298)   | 4.7 (1/36)          | 5.7 (1/36)          | 5.7 (1/36)          |
| Homosexual                             | 8.0 (2/298)   | 7.6 (1/36)          | 6.4 (1/36)          | 7.2 (1/36)          |
| History of IDU                         | 6.3 (2/298)   | 5.6 (2/36)          | 8.7 (1/36)          | 5.5 (1/36)          |
| Dual co-infections                     |               |                     |                     |                     |
| Anti-HCV (+)                           | 10.0 (32/320) | 5.6 (2/36)          | 8.7 (1/36)          | 12.3 (18/146)       |
| HBsAg (+)                              | 4.4 (1/320)   | 5.6 (2/36)          | 4.3 (1/36)          | 4.3 (1/36)          |
| IgM anti-Toxo (+)                      | 10.0 (32/320) | 11.1 (3/29)         | 10.1 (3/29)         | 9.6 (14/146)        |
| IgG anti-Toxo (+)                      | 35.0 (112/320)| 44.4 (16/36)        | 33.3 (43/138)       | 34.7 (50/146)       |
| HCV RNA (+)                            | 24.4 (78/320) | 27.8 (10/36)        | 30.4 (4/138)        | 27.8 (16/146)       |
| TTV DNA (+)                            | 30.6 (98/320) | 33.3 (12/36)        | 24.6 (13/36)        | 35.6 (32/146)       |
| HBV DNA (+)                            | 4.4 (1/320)   | 0 (0/36)            | 4.3 (1/36)          | 5.5 (1/36)          |
| GBV-C RNA (+)                          | 7.5 (2/320)   | 5.6 (1/36)          | 11.6 (2/36)         | 4.1 (1/36)          |
| HCV RNA/anti-HCV (+)                   | 3.8 (12/320)  | 5.6 (1/36)          | 5.8 (1/36)          | 4.1 (1/36)          |
| HBV DNA/HBsAg (+)                      | 3.8 (12/320)  | 0 (0/36)            | 4.3 (1/36)          | 4.1 (1/36)          |
| IgM/IgG anti-Toxo (+)                  | 1.9 (6/320)   | 11.1 (3/36)         | 0 (0/36)            | 1.4 (1/36)          |
| Triple co-infections*                  |               |                     |                     |                     |
| HCV RNA/TTV DNA (+)                    | 8.1 (26/320)  | 16.7 (5/36)         | 11.6 (16/36)        | 2.7 (4/146)         |
| HCV RNA/HBV DNA/anti-HCV (+)           | 9.4 (30/320)  | 11.1 (3/36)         | 13.0 (4/36)         | 5.5 (1/36)          |

*Triple co-infections represent the triple co-infections significantly associated with specific HLA-E alleles, HLA-E: Human leukocyte antigen-E, IDU: Injection drug use, HCV: Hepatitis C virus, HBsAg: Hepatitis B surface antigen, TTV: Torque Teno virus, GBV-C: GB Virus C

Association of human leukocyte antigen-E alleles with blood-borne pathogen infections among anti-human immunodeficiency virus-positive subjects

HLA-E*0101 and HLA-E*0103 were found in 54.4% (174/320) and 88.8% (284/320) of the respondents, respectively, resulting in three possible genotypes:
HLA-E*0101/0101, HLA-E*0101/0103 (heterozygote),
and HLA-E*0103/0103.

The HLA-E*0101 allele was associated with positivity for HCV RNA (aOR: 1.9; 95% CI: 1.011-3.666; P = 0.046) and HCV RNA/TTV DNA (aOR: 4.0; 95% CI: 1.285-12.698; P = 0.017). In contrast, the HLA-E*0101 allele was inversely associated with positivity for TTV DNA (aOR: 0.4; 95% CI: 0.208-0.663; P = 0.001).

The HLA-E*0103 allele was inversely associated with positive rates of HCV RNA/TTV DNA (aOR: 0.3; 95% CI: 0.093-0.865; P = 0.027) and IgM/IgG anti-Toxo (aOR: 0.04; 95% CI: 0.005-0.276; P = 0.001). Respondents who tested positive for HBV DNA all possessed the HLA-E*0103 allele. Furthermore, all subjects who tested positive for HBsAg/HBV DNA possessed the HLA-E*0103 allele.

The frequency of HLA-E*0101/0101, HLA-E*0101/0103 (heterozygote), and HLA-E*0103/0103 were 11.3% (36/320), 43.1% (138/320), and 45.6% (146/320), respectively [Table 1]. The distribution of the HLA-E alleles was in concordance with the Hardy-Weinberg equilibrium (P > 0.05).

HLA-E*0101/0101 was associated with positivity for HCV RNA/TTV DNA (aOR: 3.5; 95% CI: 1.156-10.734; P = 0.027) and IgM/IgG anti-Toxo (aOR: 27.0; 95% CI: 3.626-200.472; P = 0.001). Respondents who possessed HLA-E*0101/0101 were all negative for HBV DNA.

HLA-E*0103/0103 was associated with positive rates of TTV DNA (aOR: 2.7; 95% CI: 1.509-4.796; P = 0.001). In contrast, HLA-E*0103/0103 was inversely associated with positive rates of HCV RNA (aOR: 0.5; 95% CI: 0.273-0.989; P = 0.046), HCV RNA/TTV DNA (aOR: 0.3; 95% CI: 0.079-0.778; P = 0.017), and HCV RNA/IgG anti-Toxo (aOR: 0.4; 95% CI: 0.150-0.948; P = 0.038). Unless otherwise stated, no statistical associations could be drawn.

DISCUSSION

This study is the first report on HLA-E allele distributions in Indonesia. We found that the HLA-E*0101/0101 frequency was very low compared to the frequencies of HLA-E*0103/0103 and HLA-E*0101/0103 (heterozygote).

The HLA-E*0103 allele was found in most respondents, whereas the HLA-E*0101 allele was only found in approximately half of the respondents. These data are in concordance with the summary of HLA-E distributions worldwide reviewed in 2014. In Asian populations, the allele frequency of HLA-E*0103 was equal or higher than HLA-E*0101, whereas, in Western populations, the allele frequency of HLA-E*0101 was the highest. It was reported in 2003 that subjects of Thai origin, with a race and ethnicity arguably close to those of Indonesian subjects, had a higher frequency of HLA-E*0103 (either *01031 and *01032) than HLA-E*0101. This finding may contribute data regarding HLA-E allele distributions in Southeast Asia because the understanding of HLA-E distributions in various ethnic groups is still very limited.

In Indonesian HIV patients, HLA-E*0103/0103 was predicted as a risk factor for TTV infection, whereas the HLA-E*0101 allele may serve as a protective factor. However, the impact of HLA-E on TTV infection has not been reported. In the present study, HLA-E*0103/0103 was found to be inversely associated with HCV co-infection in HIV patients, implying that HLA-E*0103/0103 may have a protective effect against HCV infection in HIV-infected subjects. In the present study, respondents who were homozygous for HLA-E*0101/0101 had 2-fold decreased odds of HCV infection. Moreover, this allele exhibited a protective effect against co-infections associated with HCV among patients infected with HIV, as indicated by the screening results for HCV RNA/TTV DNA and HCV RNA/IgG anti-Toxo. Respondents who were homozygous for HLA-E*0103/0103 had 4-fold decreased odds of HCV/TTV co-infections and 2.5-fold decreased odds of HCV/latent toxoplasmosis co-infection. To the best of our knowledge, an association between the protective effects of HCV infection and related co-infections has not been previously reported. However, one study reported that HLA-E*0101/0101 provided resistance to HCV infection in German patients, which contradicts the findings of the present study. This discrepancy may be the result of the different races or ethnic groups included in the study populations. Different populations present varied immunogenetic profiles, partly caused by responses to continuous exposure to pathogens that have afflicted and continue to affect specific populations.

In contrast to the protective effect observed for the HLA-E*0103/0103 on HIV/HCV co-infections, possession of the HLA-E*0101 allele was predicted to be a risk factor for HCV co-infection in Indonesian HIV patients. Genotype HLA-E*0101/0101 was also predicted as a risk factor for HIV/HCV/TTV triple co-infection. No other statistical associations could be drawn between HLA-E*0101/0101 and blood-borne pathogen co-infections in the present study, possibly due to the low frequency of detection of HLA-E*0101/0101 (only 36 respondents were homozygous for HLA-E*0101).
All respondents genotyped with HLA-E*0101/0101 were found to be negative for HBV DNA, whereas all subjects suspected to be infected with HBV possessed the HLA-E*0103 allele. This finding might indicate that HLA-E*0101/0101 has a protective effect against HBV infection or that the HLA-E*0103 allele is a risk factor for such infection. In a previous study, HLA-E*0101 was reported as a risk factor for HBV infection.[21] Further research on the association between HBV DNA and HLA-E alleles is recommended to determine which allele affects HBV infection in specific populations.

The HLA-E*0103 allele was found to be inversely associated with the presence of IgM/IgG anti-Toxo in respondents’ sera. However, only 6 respondents tested positive for IgM/IgG anti-Toxo; all six respondents were anti-HIV-positive heterosexual women with only 1 sexual partner and no history of IDU. In contrast, women genotyped for HLA-E*0101/0101 had 27-fold increased odds of toxoplasmosis, as indicated by the detection of both IgM and IgG for *T. gondii*. An association between HLA-E alleles and *T. gondii* has not been previously reported.

It has been proposed that HLA-E might contribute to the regulation of antiviral immunity in blood-borne viral infections.[3] HLA-E*0101 and HLA*0103 were shown to differ in their surface levels, peptide affinity, and complex stability.[3] Thus, we propose that both types may exhibit different antiviral regulation mechanisms. In the present study, we found that the association between HLA-E alleles and HCV infections differed from those reported in previous studies. However, the mechanisms determining how specific HLA-E alleles regulate viral and/or parasite infections are still unclear. Identifying the alleles that are risk factors or protective factors for viral or parasite infections may contribute to future prevention measures and treatments.

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Conflicts of interest

There are no conflicts of interest.

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