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Association of IL-1β +3954 G>A and IL-6 -174 G/C polymorphisms in congenital toxoplasmosis

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Toxoplasmosis is caused by infection with the protozoan parasite *Toxoplasma gondii*, that has the capacity to infect all warm-blooded animals worldwide. The purpose of this investigation was to determine the distribution of genotypes and alleles in miscarriages woman as a result of *Toxoplasma gondii* infection associated with interleukin-1β and interleukin-6 polymorphisms. A total of 125 miscarriage women suspected of toxoplasma infection and 50 healthy pregnant without previous miscarriage as control were enrolled in this study. The cases were screened for anti-toxoplasma IgM and IgG by ELISA test. Among the 125 miscarriage women, only 50 were positive to anti-*Toxoplasma gondii* IgG and IgM antibodies. The present study focused on assay the genotypes at IL-6 -174 G/C and IL-1β +3954 G>A to establish the associations between genetic polymorphisms and infection with *Toxoplasma gondii*. Results showed that the altered IL-1β GA, AA genotypes were high significant elevated in miscarriage women with toxoplasmosis (P=0.03), OR = 10 and 95% confidence intervals (1.32-81.48); (P=0.0007), OR = 0.07 and 95% confidence interval (0.01-0.32). The genotype GC at IL-6 (G/C) appears to be highly correlated with infection (P=0.01); OR = 3.18 and 95% confidence interval, (1.22- 8.30). In terms of allelic heterogeneity, C alleles were significantly more common in infected than uninfected cases for IL-6, while A allele is common in IL-1β single nucleotide polymorphisms (P=0.050). Furthermore, this study demonstrates that there is a strong and highly significant association between two forms of single nucleotide polymorphisms and the increased risk for toxoplasmosis. Genotypes of these polymorphism should be considered when evaluating genetic effects on toxoplasmosis incidence. However, to improve the prediction of this disease predisposition, a further study based on a larger cohort of patients is warranted.

Palabras clave: *Toxoplasma gondii*; Single nucleotide polymorphisms; genotype.

Introduction

Toxoplasma gondii is an obligate intracellular parasite that can infect almost all cells with nucleus in warm-blooded animals.(1) It leads to congenital diseases of the fetus and newborns upon first exposure to *T. gondii* during pregnancy, which, in turn, stimulates the immune response that may cause the fetus to be lost or deformed.(2) The host's responses to infection, inflammation, and trauma are controlled by pro-inflammatory cytokines, which can make disease worse in co-disease states.(3) While its biological activities overlap on a large scale, IL-1β is developed during early pregnancy by autotrophic cells at the interface between the fetus and the mother and is involved in trophoblastic invasion and tissue repair.(4) Whereas, IL-6 is a powerful vascular cytokine that stimulates endothelial cell proliferation. In vitro, the behavior of the female reproductive system and pregnancy tissues secreted by groups of fallen cells are regulated.(5) It also plays a major role in erection process or as anti-inflammatory. In helper T cells 17 (Th17), while suppressing the production and regulatory function of T cells (Treg) in vitro,(6) it is also known to enhance the differentiation between the germinal center B cells and the follicular helper T cells, and thus are critical for the production of antibodies anti-high affinity.(7) The sinusoidal polymorphism of the nucleotide in the IL-6 gene interferes with its transitional regulation, such as the -174G/C polymorphism.(8) *T. gondii* inhibits IL-1 controlled by inflammation, a typical multivariate complex consisting of caspase-1, an ASC (protein-like protein transformer for apoptosis-associated protein) and acellular sensor, which can be either an oligonucleotide domain as NLR receptor or a receptor that appears for AIM2.(9) The problem of eradication of parasites, reactivation of pathogens, toxic effects and emerging drug resistance in parasites makes

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long-term drug treatment ineffective.[10] Therefore, the production of successful vaccines against toxoplasmosis is important in order to fight against the parasite. There is no vaccine for humans. Toxovax® (Intervet, B.V), is the only vaccine on the market to prevent toxoplasmosis.[11] The genetic variation in a single locus does not give an adequate explanation for the inter-individual changes in the host's immune responses that lead to different clinical manifestations.[12] In order to reduce the risk to public health and livestock development, it is crucial to stablish novel control and preventive toxoplasmosis strategies.

Currently, the commercial vaccine, based on live attenuated tachyzoites of *T. gondii* (strain S48), is available for veterinary use in a small number of countries to minimize the incidence of abortion in cattle.[13] Several experiments have been carried out to test multi-component candidate vaccines combining GRA2 or GRA5 with other possible toxoplasmosis genes[14] and different adjuvant formulations, for example: alum (Th2 inducer), IL-12 (Th1 inducer),[15] adjuvants commonly used in subcutaneous injection like Freund's complete adjuvant and Freund's incomplete adjuvant, liposomes. It is possible that genetic variation co-exists with parasitic infections, therefore, this study was designed to analyze the association of IL-6 and IL-1β genetic polymorphism with the risk of recurrent miscarriage associated with toxoplasmosis, leading to, increased gene expression that affects regulation of the immune response. These cytokines can be a target for vaccines or used as adjuvants in the future.

Materials and Methods

Cases and control

One hundred and twenty-five miscarriage women were enrolled in the study, in addition to 50 healthy pregnant women who didn't have history of miscarriage as a control group. Fifty recurrent miscarriage women with toxoplasmosis were diagnosed by ELISA test from total miscarriage cases.

Matched patients for ethnicity and age were also included in the study according to a pre-prepared questionnaire. The inclusion criteria were: woman age 20-45 years, number of miscarriages and children number. The exclusion criteria were autoimmune dysfunction, genetic anomalies, inflammatory disease, and other systemic disorders.

The women's blood samples were collected in the period between January to July, 2019, from women who attended delivery's and children hospital in Al Muthanna Governorate, Iraq.

Sample collection

Blood samples were collected by venipuncture from women experiencing one or more miscarriages on her first hospital visit and from healthy women as control. Then the blood was stored at 4°C until analysis. Five milliliters of blood were obtained by venipuncture, transported to the laboratory, allowed to clot and later centrifuged at 1,500 rpm for 10 min. The sera were aspirated after centrifugation and stored in tubes at -70°C until tested.

Serological Test

Sample analysis was carried out by Enzyme-linked immunosorbent assay (ELISA). Anti-toxoplasma IgG and IgM were performed using the Biotech, USA, kit following the manufacturer´s instructions. Negative and positive controls and calibrator were ready to use. At first, 1:21 dilution of specimens was prepared by adding 10 µL of the sample to 200 µL of sample diluent and mixed well. Secondly, 100 µL of diluted sera, calibrator and controls were dispensed into the appropriate wells. As reagent blank, 100 µL of sample diluent in 1A well position were dispensed. After that, the holder was tapped to remove air bubbles from the liquid and mixed well. An incubation for 20 min at room temperature was followed and liquid was removed from all wells. After that, wells were washed three times with 300 µL of 1X wash buffer. Then, 100 µL of enzyme conjugate were dispensed to each well and incubated for 20 min at room temperature. Enzyme conjugated was removed from all wells that were washed three times with 300 µL of 1X wash buffer and 100 µL of 3,3',5,5'Tetramethylbenzidine (TMB). Then the substrate was dispensed and incubated for 10 min at room temperature; at last 100 µL of stop solution were added. All samples and standards were run in duplicates; the average value was considered after reading optical density (OD) at 450 nm using ELISA reader within 15 min. A dual wavelength is recommended with reference filter of 600-650 nm. Each standard OD (Y-axis) versus the corresponding standard concentration (X-axis) were used to construct the standard curve that was drawn on linear graph paper manually to obtain the best linear/linear curve to give the most accurate results.

Table 1. Primer sequences, annealing temperatures, and amplicon size for amplification of IL-6 and IL-1β.

Gene	Specific primer sequences (5'- 3')	Tm (˚C)	Product Size (bp)
IL-6	F: CAGAAGAACTCAGATGACTG	58	431
	R: GTGGGGCTGATTGGAAACC		
IL-1B	F: GTTGTCATCAGACTTTGACC	431	291
	R: TTCAGTTCATATGGACCAGA		

Tm: temperature.

DNA isolation and cytokine genotyping

Genomic DNA was isolated from ethylene-diamine-tetraacetic acid (EDTA)-treated blood from 2-mL peripheral blood volumes using the Miniprep DNA extraction kit (Favorgen, Europe) according the commercial method. The obtained DNA was diluted in 100 µL of elution buffer and stored at -20°C until further molecular analyses. To determine the occurrence of IL-6 and IL-1β polymorphism from blood samples of toxoplasmosis miscarriage and healthy women, polymerase chain reaction (PCR) was performed to detect IL-6 -174G/C (rs1800795) and IL-1β +3954 G/A (rs1143634) single nucleotide polymorphisms (SNPs), using qPCR kit (Favorgen, Europe). DNA was amplified using the forward and reverse primers shown in Table 1. The conditions for amplification were achieved as follows: initial denaturation at 95°C for 4 min; 35 cycles of denaturation at 95°C for 45s, annealing at 60°C for 30s and extension at 72°C for 60s; a single final extension step at 72°C for 10 min.[16,17] PCR products were sent for sequencing using ABI3730XL, an automated DNA sequencer by Macrogen Corporation-Korea; the reference fragments of these genes were compared, using BLASTN program for alignment of two (or more) sequences.

Statistical Analysis

All the statistical analyses were performed using the statistical package for the social sciences (SPSS) software version 13.0 for Microsoft windows. The genotype and alleles frequencies for SNPs were calculated directly by counting method. Hardy-Weinberg equilibrium (HWE) for SNP was investigated via the use of the online calculator of Michael H. Court (2005-2008). If the P-value was more than 0.05, the population was consistent with HWE.

The odds ratio (OR) was estimated for evaluating the risk related to genotypes and alleles; it was calculated by chi-square and Fischer's exact probability via utilizing the statistical software epidemiological (WINPEPI) version 11.65. Also, p-values were statistically significant when less than (0.05).

Genotype distributions of cytokine gene polymorphisms were compared between cases and controls by chi-square test. Statistical analysis was performed to determine odd ratio (OR) and 95% confidence intervals (95% CI) associated with recurrent pregnancy loss, using Finch TV version 1.4 to display DNA sequences.

Ethical approval

This study protocol was approved by the Ethics Committee of the Children's Health Memorial Institute on Humans and a written consent from each participant has been obtained No.1466 in 22/5/2019.

Results and Discussion

Serotyping methods based on polymorphic polypeptides have the potential to become the choice for typing *T. gondii* in humans and animals. Detection of specific anti-Toxoplasma immunoglobulin (IgM and IgG) discriminates chronic from reactivated infection. Present results revealed infection with *T. gondii* in 50 miscarriage women with spontaneous abortion, due to their seropositivity to anti-*T. gondii* IgM and IgG antibodies. Cytokines are essential for the normal development of pregnancy, any imbalance in the amount or location of expression can influence trophoblastic and endometrial reactions leading to pregnancy complications.[16] Further investigations focused on genome-wide association studies are needed to better characterize the SNPs of the cytokine genes in the toxoplasma virulence effect.[7] Logistic regression analysis was used to estimate genotype-toxoplasmosis associated miscarriage risk under four genetic models (recessive, dominant, codominant and over dominant). In the present study, results indicated in Table 2 showed the equivalence of percentage between alleles frequency of IL-6 in both G and C (it was 50%,

Table 2. Distribution of the allele frequency of IL-6 rs1800795 G/C, IL-1β rs1143634 G/A in study groups.

Gene	Allele frequency No.(%)		OR (CI 95%)	p-value
	Toxoplasmosis cases (50)	Control (50)		
IL-6 -174 G/C	G:25 (50) ref	G:34 (68)	1 (0.41 -2.428)	1
rs1800795	C:25 (50)	C: 16 (32)	2.12(0.94-4.78)	0.0691
IL-1β+3954	G: 32 (60) ref	G:42 (82)	1 (0.52- 1.91)	1
G/A rs1143634	A:18 (33.3)	A: 8 (9.5)	0.338 (0.13- 0.87)	0.025*

ref: base found in the reference genome.

respectively) for infected miscarriage women, while in the control group, G allele was 68% and C allele, 32%. These findings deal with the previous study about GC heterozygosity associates with the disease incidence in TR patients.[16] For IL-1β, the allele frequency for G and A in toxoplasmosis were 32 (60%) and 18 (33.3%), respectively. In a previous study, G allele in the site of polymorphism (rs1800795), had more incidence of variant genotypes in miscarriage women with toxoplasmosis.[17]

Recently, the polymorphic IL-6 -174 G>C was also detected associated with altered levels of encoded cellular expression.[14] In this study, three genotypes were observed in IL-6 -174 G>C (GG, CG, and CC) with frequency of (32%), (40%), (28%), respectively in relation to control group (Table 3). These results showed that the heterozygous GC were significantly (P<0.01) associated with toxoplasmosis and an increased risk of toxoplasmosis in the coded form, as this heterozygous was three times more dangerous than the rest of the genotypes OR (3.18), 95% CI (1.22- 8.30) for GC and CC genotypes in the dominant model, P <0.050; this deals with the previous study of GC heterozygosity associated with disease incidence in TR patients.[14] This result contrasts with a previous study on rs1800795 which showed a high significant of (C/C) genotype leading to a negative regulatory domain (225 to 164). Moreover, it is contained within a sequence that carries partial nucleotide homogeneity with the Smad4 binding component and the C allele may bind to Smad4 more effectively and thus inhibit IL-6 transcription.[18]

To date, for IL-6 -174G>C SNP, the C allele has been documented to generate new binding sites for transcription factors for NF1 and Smad4 that were not reported in the presence of the G allele.[16]

These results don't agree with[19] who observed that CC carriers, among recurrent spontaneous abortion (RSA) patients with polycystic ovary syndrome (PCOS), were 10% compared to controls (3%) and the GG genotype in RSA women with PCOS was significantly different (60%) compared to control subjects; while the present study agrees with the GC genotype (30%) they reported for RSA patients with PCOS.

Results of sequencing illustrated that the guanine nucleotide was substituted by cytosine in the site of guanine in toxoplasmosis miscarriage women and no displacement or deletion occurred in adjacent bases, as shown in Figure 1.

On the other hand, the polymorphism and amplification product for IL-1β gene agree with[20] who stated that the amplification of the IL-1β gene rs 1143634 showed the amplified product was 249 bp, despite using self-designed nested PCR assays. Several epidemiological studies have found rates of toxoplasmosis in miscarriage cases; in this regard, several factors can be proposed, including genetic make-up variation, age group, target DNA region, and other risk factors such as genetic predisposition to miscarriage and immunodeficiency, which may influence the prevalence of toxoplasmosis.[21] Results in Figure 2 showed the guanine nucleotide was substituted by adenine at the site of polymorphism (rs 11146343) in toxoplasmosis women, after sequencing PCR product according to the sanger method. IL-1β plays a significant role in reproductive physiology and has been implicated in ovulation, fertilization, and embryo implantation as a critical regulatory factor.[22] IL-1β increases the expression of adhesion molecules such as ICAM-1 on the surfaces of endothelial cells and others.[22]

Results in Table 2 showed the percentage of G allele was higher than A allele. The frequency of A and G allele in toxoplasmosis miscarriage women was 33.3% and 60%, respectively; while in healthy women it was 9.5% and 82%, respectively. Similar results were obtained in other studies where the incidence of TR patients exhibited identical genotype distributions.[20]

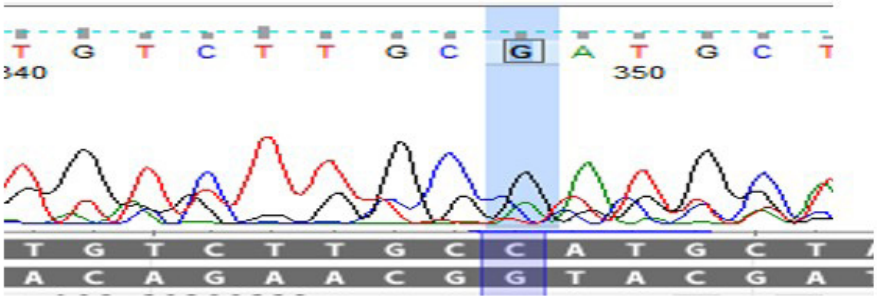

Fig. 1. IL-6 DNA sequence chromatogram of IL-6 (rs1800795C/G) showing the heterozygous genotype of each SNP in toxoplasmosis miscarriage women.

Table 3. Genotype frequencies of IL-6-174 G/C rs (1800795) in the study groups.

Gene	Genotype incidence rates, No. (%)		OR (CI 95%)	p-value
	Toxoplasmosis cases (50)	Control (50)		
IL-6 codominant	GG: 16 (32) ref	GG: 28 (56)		
	GC: 20 (40)	GC: 11(22)	3.18 (1.22-8.30)	0.0179*
	CC:14 (28)	CC: 11(22)	2.22(0.819-6.05)	0.116
Dominant	GG:16 (32) ref	GG: 28 (56)		
	GC-CC:34 (68)	GC-CC:22 (44)	0.36 (0.16 - 0.83)	0.0168
Recessive	GG-GC:34 (68) ref	GG-GC:39 (78)		
	CC: 14 (28)	CC: 11 (22)	0.6850 (0.27-1.70)	0.117
Over dominant	GG-CC:30 (60) ref	GG-CC:39 (78)		
	GC:20 (40)	GC:11 (22)	0.4 (0.20- 0.95)	0.0179

ref: base found in the reference genome. *Significant (P < 0.01).

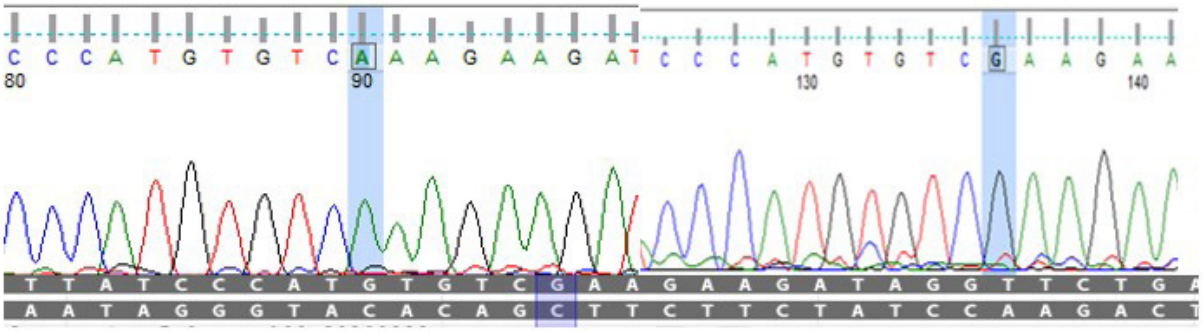

Fig. 2. IL-1β DNA sequence of IL-1β (rs1143634 G>A) showing the heterozygous genotype of each SNP in toxoplasmosis miscarriage women. The reference sequences of SNPs denoted with A.

Table 4. Genotype frequencies of IL-1β +3954 G/A rs1143634 in the study groups.

Gene	Genotype incidence rates, No. (%)		OR (CI 95%)	p-value
	Toxoplasmosis cases (50)	Control (50)		
IL 1B codominant	GG:35 (70)	GG: 27 (54)		
	GA: 13 (26)	GA: 1 (2)	10.02(1.23- 81.48)	0.031
	AA: 2 (4)	AA:22 (44)	0.07(0.01 -0.32)	0.0007*
Dominant	GG: 35 (70)	GG: 27 (54)		
	GA-AA: 15 (30)	GA-AA: 23 (46)	0.503 (0.22- 1.144)	0.101
Over dominant	GG-AA: 37 (71) ref	GG-AA: 49 (98)		
	GA: 13 (26)	GA: 1 (2)	17.21(2.15- 137.57)	0.0073**
Recessive	GG-GA:34(98)	GG-GA: 28 (56)		
	AA:6 (2)	AA:22 (44)	0.22(0.08 - 0.63)	0.0046*

ref: base found in the reference genome. **Highly significant (P<0.01).

Frequencies may increase among pregnant women, but not in toxoplasmosis in miscarriage.[23] On the other hand, a recent study showed that patients did not demonstrate any significant effect of these alleles on disease susceptibility.[6] The A allele could be a risk factor for increasing susceptibility to infection by toxoplasma, while G allele acts as a prophylactic. The prevalence rates of GG, GA, and AA genotypes at IL-1β rs 1143634 G>A were 35 (70%), 13 (26%), 2 (4%), respectively; while among the control subjects, the prevalence rates of the analyzed variants were 27 (54%), 1 (2%), 22 (44%), respectively as shown in Table 4. These results referred that the GA polymorphic heterozygous and AA polymorphic homozygous were significantly associated (p<0.01) with the susceptibility to toxoplasmosis and increased the risk to the infection with *T. gondii*, where the odd ratio was 10.02 (95% Cl=1.23-81.48) in toxoplasmosis versus healthy pregnant women in the codominant model. On the other hand, odd ratio for recessive GG vs GA+AA was 0.503 (95% Cl= 0.22-1.144) with no significant (p<0.01) in toxoplasmosis miscarriage women versus healthy pregnant women, while the odd ratio for recessive AA vs GA+GG was 0.22 (0.08-0.63) with a highly significant difference in patient women versus healthy pregnant women.

The nature of the genetic changes observed suggests an active relationship with congenital toxoplasmosis. This study indicated that A allele could be a risk factor leading to increased susceptibility for infection, while G allele acts as a preventive agent.

Despite the importance of IL-1β during the immune response to *T. gondii*, no significant difference of the allelic frequencies of this polymorphism between patients and controls were found. Another study suggested that the presence of mutated T allele in the gene with IL-1β (C/T) SNP has a protective function against the development of congenital toxoplasmosis.[21]

Conflict of interest

The authors declare that there is no conflict of interest.

Author's contributions

Nuha M. Mousa carried out the molecular study and participated in data analysis.

Hameed M. Jasim conceived and coordinate the study and helped to draft the manuscript.

All authors reviewed and approved the final version of this manuscript for publication.

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Asociación de polimorfismos de IL-1β +3954 G>A e IL-6-174 G/C en la toxoplasmosis congénita

Resumen

La toxoplasmosis es causada por la infección con el parásito protozoario *Toxoplasma gondii*, que tiene la capacidad de infectar a todos los animales de sangre caliente en todo el mundo. El propósito de esta investigación fue determinar la distribución de genotipos y alelos en mujeres con abortos espontáneos como resultado de la infección por *Toxoplasma gondii* asociada con polimorfismos de interleucina 1β e interleucina 6. Se inscribieron en este estudio un total de 125 mujeres con aborto espontáneo sospechosas de infección por toxoplasma y 50 embarazadas sanas, sin aborto espontáneo previo, como control. Los casos se examinaron para detectar IgM e IgG anti-toxoplasma mediante la prueba ELISA. Entre las 125 mujeres que sufrieron un aborto espontáneo, solo 50 fueron positivas a anticuerpos IgG e IgM anti-*Toxoplasma gondii*. El presente estudio se centró en analizar los genotipos de IL-6-174 G/C e IL-1β +3954 G>A para establecer las asociaciones entre polimorfismos genéticos e infección por *Toxoplasma gondii*. Los resultados mostraron que los genotipos alterados de IL-1β GA, AA fueron significativamente elevados en mujeres con aborto espontáneo con toxoplasmosis (P = 0,03), OR = 10 e intervalos de confianza del 95% (1,32-81,48); (P = 0,0007), OR = 0,07 e intervalo de confianza del 95% (0,01-0,32). El genotipo GC de IL-6 (G/C) parece estar altamente correlacionado con la infección (P = 0.01); OR = 3,18 e intervalo de confianza del 95%, (1,22- 8,30). En términos de heterogeneidad alélica, los alelos C fueron significativamente más comunes en los casos infectados que en los no infectados para la IL-6, mientras que el alelo A es común en los polimorfismos de nucleótido simple de IL-1β (P = 0.050). Además, este estudio demuestra que existe una asociación fuerte y altamente significativa entre dos formas de polimorfismos nucleótido simple y el mayor riesgo de toxoplasmosis. Se deben considerar los genotipos de estos polimorfismos al evaluar los efectos genéticos sobre la incidencia de la toxoplasmosis. Sin embargo, para mejorar la predicción de esta predisposición a la enfermedad, se justifica un estudio adicional basado en una cohorte más grande de pacientes.

Palabras clave: *Toxoplasma gondii*; Polimorfismo de Nucleótido Simple; genotipo.

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