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Prevalence and genetic diversity of *Plasmodium falciparum* in patients attending regional health center in Daloa, Côte d'Ivoire

Mathurin Koffi[1*], Innocent Allepo Abe[1,2], Bernardin Ahouty Ahouty[1,2], Dieudonné Silué[3], Thomas Konan Konan[1] and Abiba Sanogo Tidou[1]

[1]Université Jean Lorougnon Guédé, UFR Environnement, Laboratoire de Biodiversité et Gestion durable des Ecosystèmes Tropicaux, Unité de Recherche en Génétique et Epidémiologie Moléculaire (URGEM), BP 150 Daloa, Côte d'Ivoire.
[2]Université Félix Houphouët-Boigny, UFR Biosciences, Laboratoire de génétique, 22 BP 582 Abidjan 22, Côte d'Ivoire.
[3]Université Félix Houphouët-Boigny, UFR Biosciences, Laboratoire de zoologie et Biologie animale, 22 BP 582 Abidjan 22, Côte d'Ivoire.

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Malaria is a parasitic disease defined as a major problem of public health in Côte d'Ivoire. One of the constraints of the control of this disease might be the genetic diversity of the main pathogen, Plasmodium falciparum. This study aims to evaluate the genetic diversity of Plasmodium falciparum in individuals at the regional health center in Daloa, Côte d'Ivoire. A total of 380 blood samples were collected from patients, from June to October, 2015, based on malaria clinical signs and symptoms. Whole DNA was extracted from blood samples tested positive by microscopic examination for P. falciparum. A nested PCR targeting the msp-1(block 2) allelic families was implemented for the parasite genetic diversity. The associations between malaria infection and socio-environment factors were estimated. Out of the 380 examined patients, 262 were tested positive by microscopic examination representing a prevalence of 68.95%. Genotyping from 160 sample randomly chosen allowed to identify 35 alleles comprising 13 K1, 12 Mad 20 and 10 Ro33 type. Mixed infections were significantly correlated with the parasite load. The number of parasite strains hosted by a patient was significantly linked to the level of anemia and the parasite density. The multiplicity of the infection (MOI) decreased significantly with the age. Genetic variability of strains is a very important parameter to be taken into account in the epidemiology of malaria. The present results should draw the attention of decision-makers to develop a better monitoring strategy for the elimination of malaria in an endemic country such as Côte d'Ivoire.

Key words: *Plasmodium falciparum*, malaria, infection rate, genetic diversity, Côte d'Ivoire.

INTRODUCTION

Malaria, a disease caused mainly by *Plasmodium falciparum*, is a major public health concern in sub-Saharan Africa (Menard et al., 2013). All age groups are affected but the most vulnerable are children aged five years and below, pregnant women and people living with HIV/AIDS (Kiggundu et al., 2013). Many studies have shown that malaria is influenced by the environment such as climate change and is often linked to developmental

projects of hydro-agricultures whose implementation disregards their impacts on malaria transmission (Koudou et al., 2007).

Malaria incidence is based on epidemiological patterns and varies from a year season to another and from one area to another. In Côte d'Ivoire, a country located in sub-equatorial area, malaria circulates into continuous mode with seasonal peaks. It is the leading cause of morbidity with an incidence of 105 per 1000 in the general population (Yavo et al., 2019) and the first cause of consultation in hospitals. In 2013, 63,000 deaths were recorded in children under 5 years old (WHO, 2014). The professional work absence and losses of agricultural revenue due to malaria are enormous (Silue et al., 2006). Faced with this alarming situation, the Ivorian government created a malaria national control program leading to several activities in favor of the fight against the disease (Kimou, 2010). This program recommended until 2003, chloroquine as first line drug and sulfadoxine-pyrimethamine (SP) in second line treatment of uncomplicated malaria. The rise in resistance found in respect of these drugs has led WHO to recommend the use of combination therapy containing artemisinin derivatives (ACT) which are Artemether-lumefantrine and amodiaquine-artesunate (WHO, 2015). Despite all these efforts, which helped to reduce around 47% of disease burden and mortality due to *P. falciparum* between 2000 and 2013 (WHO, 2014), much remains to be done and parasite resistance to drugs remains a critical issue for sustainable control or elimination of the disease because of the parasite antigenic diversity in response to the drug pressure (Khaminsou et al., 2011).

Some authors have shown that this genetic diversity can vary from one region to another within the same country because of climate variability (Oyebola et al., 2014). Thus, in the context of control and emphasizing the elimination of malaria, it would be important to document the genetic diversity and characteristics of *P. falciparum* strains at regional level regarding particular epidemiological facies. To our knowledge, based on literature, no study on the genetic diversity of *P. falciparum* has been undertaken in the Haut-Sassandra region, in Western part of Côte d'Ivoire. This study aims to fill this gap by assessing the impact of malaria infection and the genetic diversity of *P. falciparum* based on the msp-1 gene polymorphism in patients attending the regional health center in Daloa.

MATERIALS AND METHODS

Study site and design

This cross-sectional study was conducted at the biggest hospital in the Haut-Sassandra region, located in Daloa State, in the Central-West of Côte d'Ivoire from June to October 2015. This hospital was chosen as sampling site because it receives patients throughout the region and shows good distribution of malaria cases in the region and presents consistently high annual incidence of malaria recorded. In addition, hospital records show that 8,841 (79.89%) cases of malaria were recorded in pediatrics and general medicine services out of 11,066 examined in 2014.

Sampling and microscopy test

The patients of any age attending the hospital with suggestive symptoms of uncomplicated malaria (axillary temperature ≥37.5°C or history of fever 72 h preceding hospital visit) and permanently residing in one of the departments of Haut-Sassandra region were eligible to participate. Only patients with absence of other diseases and willing to participate were included. Inform consent from participants or guardians and assent for children were signed for ethical purpose.

The socioeconomic status of patient family, the immediate neighborhood environment, age and sex of the patient, the use of mosquito nets and level of education were recorded. The clinical signs of malaria such as fever, headache, general fatigue, anemia and vomiting were also recorded. 5.0 mL of peripheral blood was collected from symptomatic subjects by a lab technician and microscopic examination was implemented to determine the parasite load after thick blood staining standard method with 10% Giemsa (Rogier et al., 2009). The hemoglobin level was also determined by complete blood count with an automatic hemographer (Kotepui et al., 2016).

Molecular genotyping

DNA was extracted from collected peripheral blood using the commercial kit QIAGEN (QIAamp DNA Blood Mini Kit) according to the manufacturer's instructions. The polymorphic Block 2 region of msp-1 gene was amplified by nested PCR using for the first reaction, primer pairs of conserved sequence spanning the msp-1 gene block-2 region (Snounou, 2002; Ghoshal et al., 2018). The product generated in this reaction served as a template in the second reaction, performed with a primer pair allowing allelic variant identification of the K1, Mad20 and Ro33 of msp-1 gene block 2 families. Primary PCR was done in 25 µl of final volume containing 5 µl of DNA, 1 µl of each primer (10 pM), 2.5 µl of 10x buffer including $Mg2^{+}$, 2.5 µl of deoxynucleoside triphosphate (dNTPs) (400µM), 0.2 µl of Taq Polymerase (5 units) and 12.8 µl of sterile water. The DNA amplification conditions are 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 58°C for 2 min and 72°C for 2 min and final extension at 72°C for 10 min. The Nested PCR was performed in 25 µl of final volume with 1 µl of each primer (10 pM), 2.5 µl of 10x buffer including Mg^{2+}, 2.5 µl of deoxynucleoside triphosphate (dNTPs) (400 µM), 0.2 µl of Taq Polymerase (5 units) and 12.8 µl of sterile water, in addition to 5 µl of the primary PCR product as template. PCR conditions were then 94°C for 5 min, followed by 94°C for 1 min, 58°C for 2 min, 2°C for 2 min and final extension at 72°C for 10 min. The PCR products obtained were migrated on 2% agarose gel during 40 min at 100 volts followed by ethidium bromide staining for 10 min. DNA fragments were visualized with a gel viewer under UV light. The multiplicity of infection (MOI) which is the average number of *P. falciparum*

*Corresponding author. E-mail: m9koffi@yahoo.fr. Tel: 00 225 40 18 56 02.

parasite genotypes carried by an infected individual were determined and used in evaluating the endemic level of malaria transmission.

Statistical analysis of data

All statistical analyses as well as the calculation of odds-ratio (OR) were performed with R software 3.3.1 (Computer, 2016). Risk factors were studied by calculating the OR. The Fisher's exact test permitted to establish the relationship between msp-1 gene allele families and the clinical characteristics of malaria infection while the Kruskal-Wallis test compared median (MOI) and hemoglobin levels (Hgb) between the different groups. The difference was considered statistically significant for a p-value less than 0.05. For molecular analysis, the statistical unit was the allele and the variables studied. The different allelic families (K1, Mad20 or Ro33) of the msp-1 gene were characterized by the number of their sequence.

RESULTS

Malaria infection profile

262 (68.95%) of the 380 patients examined were confirmed by microscopy to be infected by *P. falciparum* with a predominance in children under 5 years (52.10%) representing 75.57% of cases (198/262) (Table 1). Malaria was found to be associated with various factors: the level of education, use of insecticide, mosquito nets, the immediate environment of dwellings and patient age were significantly correlated with parasite infection ($p < 0.05$) (Table 1). The prevalence of individuals who declared no use of the Insecticide Treated Nets (ITNs) is about 40.53% and are 3.5 times more infected than those who use ITN. People living in a damp areas presented a 2.16 times higher risk than those living in a dry environment (OR = 2.16 [1.37, 3.41]). We also observed that children under five are about 2 times more likely to contract malaria (OR= 1.99 [1.19 ,3.35]).

Allelic profile and polymorphism of msp-1 gene

160 of the infected samples were randomly chosen for genotype purpose. All three allelic families (K1, Mad20 and Ro33) of the msp-1 gene were observed in the samples genotyped and show a significant polymorphism (Figure 1). 35 alleles of msp-1 gene were identified in samples genotyped for an average of 11.7 alleles per allelic family. K1 family was the most polymorphic with 13(37.14%) followed by Mad20 family with 12 alleles (34.28%). Allele size ranged from 100 to 600 base pairs (bp). The MOI defined as the average number of strains hosted by a patient was 3.54 (566/160) in this study.

Analysis of genotype profiles showed that 126/160 (78.75%) had K1 allotypic infections, 103/160 (64.38%) Mad20 allotypic infections and 140/160 (87.5%) Ro33 allotypic infections. 24 patients were infected with only Ro33 family while 2 patients were infected only by Mad20 family. All possible mix allelic families' combinations of msp-1 gene were observed with a high proportion of trimorphic allelic infections (K1 + Mad20 + Ro33) (Figure 2).

Genetic diversity of *Plasmodium falciparum* and malaria clinical phenotype

Three objectively measurable key parameters related to malaria (axillary temperature, hemoglobin level and parasite density) were used to analyze the relationship between allelic family of msp-1 gene of *P. falciparum* and clinical phenotype. These analyses showed that parasite load was significantly associated with allotypic patterns of msp-1 gene (coexistence of two alleles from different families) and MOI variation ($p < 0.001$). Individuals with parasitaemia from 50,001 to 100,000 trophozoïtes/µl have a high MOI (Table 2). The parametric kruskal-wallis's test showed that the multiplicity of infection (MOI) significantly decreased with age ($p = 0.004$) with children under 5 hosting an average 3.69 different parasite genotypes while the value of this parameter was 2.35 in adults (≥ 15 years) (Figure 3).

DISCUSSION

Malaria overall prevalence of 68.95% was observed in the study population with a high infection rate in children under 5 (72.52%). This high prevalence might be the fact that the study was conducted in the rainy season (period from June to October) favorable to the proliferation of anopheles, increasing biting rate and causing a strong transmission of parasite (Koudou et al., 2007). Protective immunity against malaria infection is acquired with age, and exposure times are key reasons for high prevalence observed in children under 5 with developing immune system which constitute true Gametocyte reservoirs regarding epidemiological chain. A study carried out in villages from the central region of Côte d'Ivoire led to similar results (Ouattara et al., 2014). Another study carried out in the central south region of the country in 2010 and 2011 presented a prevalence of 46 and 56 % respectively (Bassa et al., 2016), indicating that infection prevalence is function of period, epidemiological facies and vegetation that promote and maintain anopheles pressure (Raso et al., 2012). The correlation between malaria infection and factors such as the use of treated insecticide nets, education level, age and residential environment demonstrate the role of these factors in malaria epidemiology. Indeed, a significantly highest risk was observed in patients with low levels of education.

This means that highly education people have good knowledge of preventive measures and better practice of prescribed antimalarial treatments. This argument is supported by a study conducted in western Kenya where

Table 1. Bivariate analysis of factors associated with the prevalence of malaria.

Participant characteristics	Infected n (%)	Uninfected n (%)	Wald X^2	p-value	OR	Wald 95% CI
Study sample	262 (68.95)	118 (31.05)				
Use of ITN						
Yes (ref.)	108 (28.42)	84 (22.10)				
No	154 (40.53)	34(8.95)	29.224	< 0.001	3.52	2.21- 5.63
Education						
Superior (ref.)	13 (3.42)	18 (4.74)				
Secondary	56 (14.73)	21 (5.53)	9.083	0.003	3.69	1.54 - 8.83
Primary	53 (13.95)	22 (5.79)	7.706	0.006	3.34	1.40 -7.96
No education	140 (36.84)	57 (15)	10.297	0.001	3.40	1.56 - 7.40
Housing						
Drained (ref.)	70 (18.42)	52 (13.68)				
Wet	192 (50.53)	66 (17.37)	11.236	< 0.001	2.16	1.37- 3.41
Age groups (years)						
≥15 (ref.)	45 (11.84)	34 (8.95)				
5 -14	19 (5)	9 (2.37)	1.021	0.312	1.60	0.64 - 3.96
0 – 4	198 (52.10)	75 (19.74)	6.944	0.008	1.99	1.19 - 3.35
Gender						
Female (ref.)	128 (33.7)	68 (17.9)				
Male	134 (35.3)	50 (13.1)	2.507	0.113	1.42	0.92 - 2.21

X^2 : Wald test statistic, OR : Odd ratio, 95% CI : Wald confidence interval, n : number of individual.

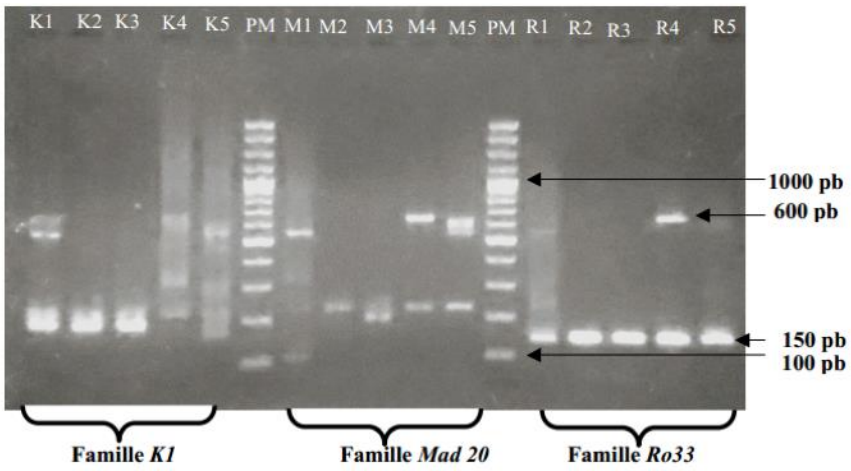

Figure 1. Genotypic profile of five samples obtained with allelic families of the *MSP-1* gene. Lane K1 to K5 = individuals tested with K1 primer; Lane M1 to M5 = individuals tested with Mad20 primer and Lane R1 to R5 = individuals tested with Ro33 primer; PM = 100 bp DNA ladder.

families with mother educated up to secondary school level had lower chances of contracting malaria (Essendi et al., 2019). Although most infections are due to the environmental issue and patient's age, factors such as genetic characteristics of the parasite could play a key role in the establishment of malaria infections and its

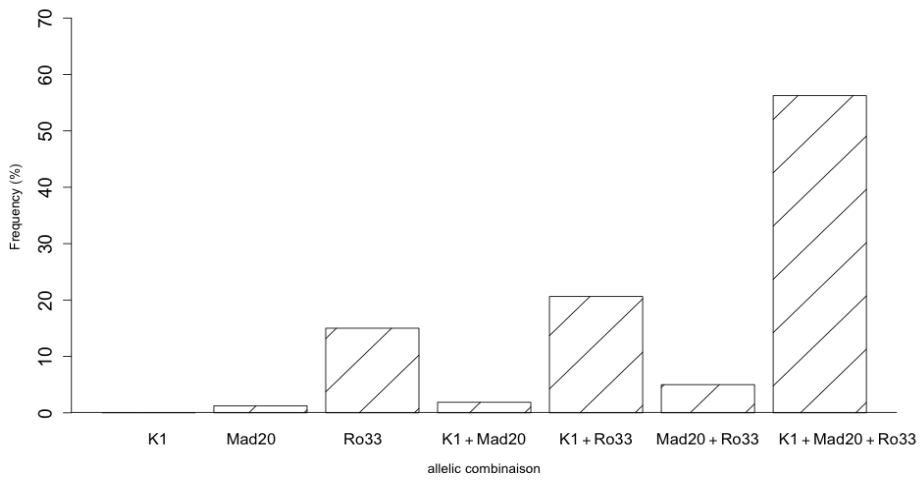

Figure 2. Distribution of allelic families combinations of the *MSP-1* gene.

Table 2. Relation between parasitaemia, msp-1gene allelic groups and MOI.

msp-1 block 2 allelic type	Parasitaemia (trophozoite/ µl) Number of cases (%)				Total	*p-value*
	1- 5000	5001-10000	10001-50000	50001-100000		
K1+MAD20	1 (0.75)	0 (0)	1 (0.75)	1 (0.75)	3 (2.24)	
K1+RO33	24 (17.9)	1 (0.75)	3 (2.24)	5 (3.73)	33 (24.6)	0.0024 [a]
MAD20 + RO33	4 (2.98)	1 (0.75)	1 (0.75)	2 (1.49)	8 (6)	
K1+MAD20+ RO33	25 (18.66)	9 (6.72)	19 (14.18)	37 (27.61)	90 (67.2)	
median MOI (IQR)	2 (2-4)	3 (2-4)	3 (3-4)	4 (3.25-6)		< 0.001[b]
Total	54 (40.30)	11 (8.21)	24 (17.91)	45 (33.58)	134(100)	

[a] Fishers 'exact test, [b] kruskal-wallis test, IQR: Interquartile Interval.

emergence (Yazdani et al., 2006). The current study showed a significant genetic diversity of *P. falciparum* strains in patients with 35 different allelic forms identified. This large genetic polymorphism of msp-1 gene observed in the population could be due to drug pressure that forces the parasite to mutate to accommodate rapid expression of new genotypes and better adapted to its environment (Ahmedou-Salem et al., 2014). This allelic polymorphism is similar to that obtained in Burkina Faso (Soulama et al., 2009) with 41 alleles and differs from that obtained in Gabon (Bouyou-Akotet et al, 2015) with 25 alleles and in Mauritania (Ahmedou-Salem et al., 2014) with 27 alleles. The polymorphic variation of *P. falciparum* msp-1 gene could also be related to climate variations in the different epidemiological facies (Oyebola et al., 2014) and represent a constraint for vaccine development (Khaminsou et al., 2011).

All the expected family profiles of msp-1 gene are found in this study with a predominance of Ro33 family. This indicates that the *P. falciparum* strains circulating in the Haut-Sassandra region belong mostly to the Ro33 family with Ro33-150 strain being the type mostly represented. This distribution of Ro33 alleles was supported by previous studies conducted in Uganda (Kiwuwa et al., 2013).

Mixed infections are significantly associated with high parasite loads in addition to high prevalence observed in the triple combinations (K1 + Mad20 +Ro33). We noticed that mixed infection is an important factor in the occurrence of high parasitaemia. This could be due to the multiplicative capacity of allotypes K1, Mad20 and Ro33 meiotic recombination occurring during sexual phase in the mosquito vector. Indeed, if two gametes fused from different clones of the parasite (in case of high parasitaemia in the source patient), that assortment of different chromosomes produces new allelic combinations and therefore a genetic polymorphism (Su et al., 2007).

The study showed that more than two thirds of patients (87.5%) were infected with more than one strain of *Plasmodium falciparum* with an average multiplicity of infection equal to 3.9. This indicates a hyper-endemicity of malaria in the Haut-Sassandra region (Rogier et al., 2009). The lack of single allelic type K1 infection found in this study may be due to high level of allelic

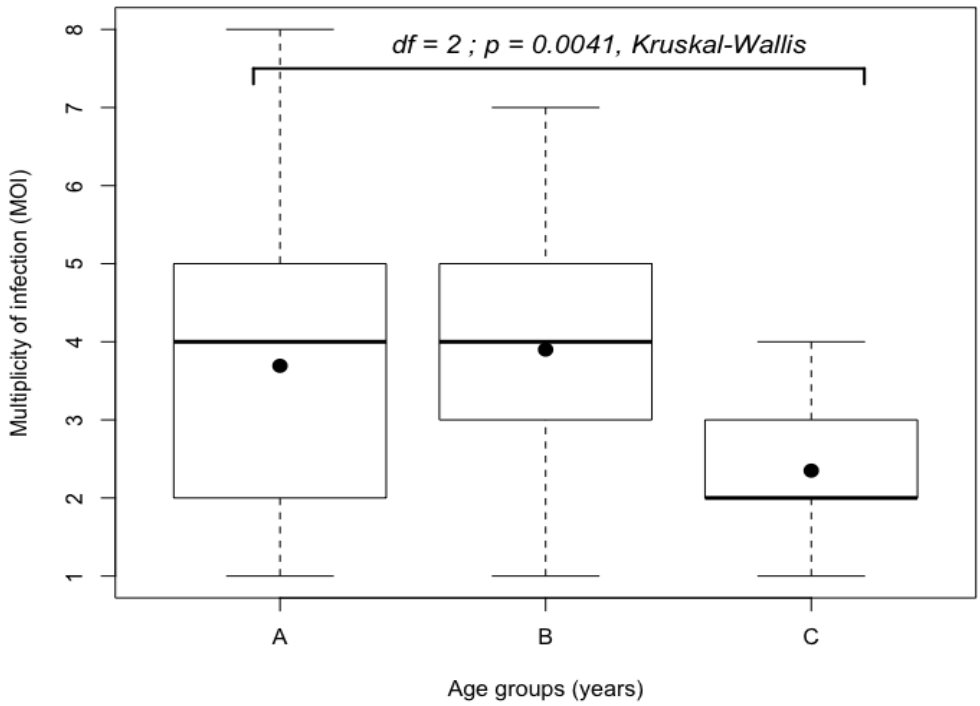

Figure 3. Variation of the multiplicity of infection between different age groups.
A= individuals aged less than 5 years, B = individuals aged 5 to 14 years, C = individuals aged 15 and over.

recombination. Unlike authors (Issouf et al., 2001) who showed that patient age had no influence on allelic number, our results showed a statistically significant reduction of MOI along with age. This relationship may be explained by the acquisition of an anti-parasitical immunity among adults living in endemic areas resulting in elimination of certain parasite strains (Hamid et al., 2013). This study also shows that the increase in the number of parasite genotypes hosted by the patient could be a cause of increase anemia (Mockenhaupt et al., 2003). High parasite densities also had an impact on occurrence of multiple strains due to resistance of the parasite to antimalarial drugs, which thus generates an increase in parasite strains. It is also a parasitological indicator of the level of acquired immunity against malaria (Mayengue et al., 2009). However, the fragment size polymorphism of *msp-1* is under positive natural selection and alleles may converge at the population level, with fragments of the same or similar size having different sequences. This leads to an underestimation of MOI and genetic diversity, and limits the generalizability of the results to other settings (Takala et al., 2006; Mohammed et al., 2018).

Conclusion

This is the first study in the Haut-Sassandra region in Côte d'Ivoire addressing the relation between genetic diversity of malaria parasite and disease parameters. We observed an important genetic diversity of *P. falciparum* strains circulating in the region with a high rate of mixed infections particularly in children under five. This multiplicity decreases with age of the patients. It appears from this study that the genetic variability of strains is a very important parameter to be considered in the epidemiology of malaria. Regarding our results and outlook, this study should get the attention of decision makers to develop better monitoring strategy for the elimination of malaria in the Haut-Sassandra region of Côte d'Ivoire.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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