International Journal on Infectious Disease and Epidemiology

Temporal Changes in Genetic Diversity of *Plasmodium Falciparum* Populations in Isolates from Asymptomatic Children in Brazzaville after the Introduction of ACT

Research Article

Volume 2 Issue 1- 2021

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Article History

Received: December 13, 2020 Accepted: January 08, 2021 Published: January 12, 2021

Abstract: Asymptomatic infections and genetic diversity of *Plasmodium falciparum* play a major role in the natural acquisition of immunity to malaria. This study investigated the genetic diversity of *msp1, msp2* and *glurp* in *P. falciparum* isolate from patients in Republic of Congo. The use of ACT selected different populations of *P. falciparum* between 2010 and 2018. This change in the parasites needs to be monitored to prevent a re-increase of malaria prevalence despite the use of ACT.

Keywords: Asymptomatic malaria; Genetic diversity; Molecular markers; *Pfmsp1; Pfmsp2; Pfglurp;* Brazzaville

Abbreviations: ACT: Artemisinin-Based Combination Therapy; P: *Plasmodium*; *Pfmsp1*: *P. Falciparum* Merozoite Surface Protein 1; *Pfmsp2*: *P. Falciparum* Merozoite Surface Protein 2; *Pfglurp*: *P. Falciparum* Glutamate-Rich Protein.

Introduction

Malaria is the most lethal parasitic infection and constitutes one of the major public health issues in the world, especially in sub-Saharan Africa where this disease is endemic and the transmission perennial [1]. The world malaria report registered 229 million cases and 409,000 deaths in 2019 [2]. *Plasmodium falciparum* is the most virulent malaria species and is responsible for more than 80% of all cases [2]. Asymptomatic malaria infection is defined by the presence [microscopic or submicroscopic] of malaria parasites in the human body without symptoms of uncomplicated or complicated malaria disease. Asymptomatic people are considered a great reservoir for malaria parasites and an important factor of multiplication and spread. These cases are often missed or underestimated because the control programs are focused on the passive and active study of symptomatic cases.

Genetic diversity of *P. falciparum* plays a major role in the natural acquisition of immunity to malaria infections and is also a concern to the development and deployment of control measures. In people living in malaria-endemic regions, immunity to *P. falciparum* is acquired as a result of natural exposure to multiple infections by different parasite strains over many years [3,4]. Asexual blood stages antigens, such as Merozoites Surface Protein-1 [MSP-1], merozoites surface protein-2 [MSP-2] and Glutamate-Rich Protein [GLURP] are considered prime candidates for the development of malaria vaccines and are also suitable markers for the identification of genetically distinct *P. falciparum* parasite subpopulations [3,5]. Block 2 of *P. falciparum msp1* may be divided as the most polymorphic block with three [3] allelic families K1, Ro33 and Mad20. The *P. falciparum msp2* can be divided into two [2] allelic families FC27 and 3D7. The *P. falciparum glurp* is constituted by only one allelic family but remains highly polymorphic [6]. Several studies have reported that the frequencies of *Pfmsp1*, *Pfmsp2* and *Pfglurp* allelic variants vary in different geographical areas, even in neighboring villages [4,7]. *P. falciparum* subpopulations vary also with clinical status or the presence of sickle cell trait [7-10].

The Republic of Congo is located in central Africa where malaria is endemic, and the transmission is perennial. During 2016, WHO estimated that almost a quarter of the Congolese population was at risk for malaria but only 171,847 cases were confirmed, and all confirmed cases were due to *P. falciparum* [11]. To control effectively malaria in the Republic of Congo, many malarial control measures have been deployed since the 2000s, including Artemisinin-Based Combination Therapy [ACT], Intermittent Preventive Treatment [IPT] and Long-

Lasting Insecticide Nets [LLINs] for all pregnant women [12]. Before the change of policy, studies described the genetic diversity of *msp1* and *msp2* of *P. falciparum* populations from symptomatic patients collected in Brazzaville [13,14]. Less than ten[10] genotypes were found for each allelic family of *msp1* [8, 6 and 1 for K1, Mad20 and Ro33 respectively] although ten genotypes were found for each allelic family of *Pfmsp2* [3D7 and FC27] [13]. The mean of the Multiplicity of Infection [MOI] was 2-2.17 for *Pfmsp1* and 1.7 for *Pfmsp2* [13,14]. After the change of policy, only the genetic diversity of *Pfmsp2* was analyzed also on isolates from symptomatic patients in the Republic of Congo [10,15,16]. One study compared the genetic diversity of *Pfmsp2* in isolates between protected or asymptomatic individuals and unprotected or symptomatic patients with samples collected in 2010 [15]. The genetic diversity was higher in the protected group with 7 and 6 alleles although there were only 2 and 3 alleles for FC27 and 3D7 family respectively. However, the multiplicity of infection was higher in the unprotected group [1.43] than in the protected group [1.29] in 2015. Since the implementation of ACT, no studies assessed the polymorphism of *Pfmsp1*. Few data on *P. falciparum* diversity are available for isolates collected from asymptomatic patients.

This study aimed to investigate the genetic diversity of *msp1, msp2* and *glurp* in *Plasmodium falciparum* isolates from asymptomatic patients from Brazzaville in the Republic of Congo four and thirteen years after the implementation of ACT.

Methods and Materials

Ethics approval and participation consent

This study was reviewed and approved by the Ethical Committee of the Health Research Ministry of the Republic of the Congo [no 007/DGRST/CERSSA and 008/MRSIT/IRSSA/CERSSA]. We used archived material from previous studies preserved for research purposes which were collected from the study populations composed of minors with the consent of legal tutor, respectively.

Sample collection and diagnosis

Samples used for this work were collected and used for previous studies. Transversal passages were done in health centers to collect peripheral blood from asymptomatic children under 15 years in 2010 and 2018 in Brazzaville in the south of the Republic of the Congo. In this study, only positive samples were considered. The diagnosis of *P. falciparum* infection was done by blood smears. A total of 33 and 37 isolates infected by *Plasmodium falciparum* were collected.

DNA extraction

DNA was extracted with the DNA Blood Omega Bio-Tek E.Z.N.A® method [Omega Bio-Tek, USA] as previously described [17]. In short, 250 µl of blood, 25 µl of protease K [20mg/ml] and 250 µl of lysis buffer were mixed and heated to 71°C for 45 minutes, and then 260 µl of isopropanol was added. This mixture was transferred to a column and centrifuged at 8000 g for 1 minute. The column was washed twice, and DNA was eluted with 100 µl of sterile water preheated to 70°C. DNA aliquots were kept at -20°C until use.

Genetic diversity

The *P. falciparum* genes of merozoites surface protein 1 and 2 [*msp1* and *msp2*] and the glutamate-rich protein [*glurp*] sequences were amplified. Electrophoresis on agarose gel was done to determine the size of each fragment. The reaction mixture and conditions for the thermal cycler and electrophoresis were done according to the WWARN protocols. Primers used are described in table 1. The polymorphism in each allelic family was analyzed; assuming that one band represented one amplified PCR fragment derived from a single copy of *P. falciparum msp1, msp2* or *glurp* genes. Alleles in each family were considered the same if fragment size were within 20 bp interval. The minimum number of genotypes per isolate was estimated to be the highest number of fragments identified for either *msp1, msp2* or *glurp*.

Statistical analysis

Data were recorded in Excel [Office 2010]. Statistical analysis was carried out with the software R version 3.2.1. Ages were expressed as medians and Interquartile Ranges [IQR]. The *msp1, msp2* or *glurp* allele frequency was calculated as the proportion of allele found for the allelic family out of the alleles detected in isolates. The Multiplicity of Infection [MOI] was defined as the minimum number of *P. falciparum* genotypes per infected subject and estimated by dividing the number of amplified PCR fragments reflecting parasite genotypes by the number of positive samples. The Chi-square test was used to compare categorical variables among groups. The nonparametric Kruskal Wallis, Student t-test and Fisher's exact test were used for group comparisons, as appropriate. Significance was assumed at $p<0.05$.

Table 1: Primers and size range of amplicons.

Genes	PCR/Allelic Family	Primers
Pfmsp1	PCR1	5' CTA GAA GCT TTA GAA GAT GCA GTA TTG 3' 5' AAA TAG TAT TCT AAT TCA AGT GGA TCA 3'
	PCR2/Mad20	5' AAA TGA AGG AAC AAG TGG AAC AGC TGT TAC 3' 5' ATC TGA AGG ATT TGT ACG TCT TGA ATT ACC 3'
	PCR2/Ro33	5' TAA AGG ATG GAG CAA ATA CTC AAG TTG TTG 3' 5' CAT CTG AAG GAT TTG CAG CAC CTG GAG ATC 3'
Pfmsp2	PCR1	5' AAA TGA AGG AAC AAG TGG AAC AGC TGT TAC 3' 5' ATC TGA AGG ATT TGT ACG TCT TGA ATT ACC 3'
	PCR2/3D7	5' AAT ACT AAG AGT GTA GGT GCA A/GAT GCT CCA3' 5' TTT TAT TTG GTG CAT TGC CAG AAC TTG AAC 3'
	PCR2/FC27	5' ATG AAG GTA ATT AAA ACA TTG TCT ATT ATA 3' 5' CTT TGT TAC CAT CGG TAC ATT CTT 3'
Pfglurp	PCR1	5' TGA AAT TGA AGA TGT TCA CAC TGA AC 3' 5' GTG GAA TTG CTT TTT CTT CAA CAC TAA 3'
	PCR2	5' TGT TCA CAC TGA ACA ATT AGA TTT AGA TCA 3' 5' GTG GAA TTG CTT TTT CTT CAA CAC TA 3'

Results

Plasmodium falciparum merozoites surface protein 1 [*Pfmsp1*]

Approximately 90.90% [n=30] and 64.86% [n=24] of isolates could be amplified for *Pfmsp1* for 2010 and 2018 respectively.

The overall MOI associated with the polymorphism of *Pfmsp1* decreased slowly from 2.7 to 2.04 between 2010 and 2018 *[p=0.14]*. The MOI associated with the Mad 20 family was 1.83 and 1.14 for 2010 and 2018 respectively *[p=0.12]*. The MOI associated with the Ro33 family was 2.6 and 1.89 for 2010 and 2018 respectively *[p=0.33]*. The frequencies of isolates with multiple allelic types were not significantly different during the time [Table 2] *[p>0.05]*. In 2010, 7 isolates carried the Mad20 and Ro33 types although 4 isolates were found with both types in 2018 *[p=0.74]*.

We investigated the polymorphism of the allelic families Ro33 and Mad20 of *Pfmsp1*. The proportion of the family Ro33 was predominant in the two years: 74.71% in 2010 vs 80.95% in 2018 and the proportion of Mad20 was 25.29 and 19.05 for the same years. No difference was observed in the proportion of Mad20 and Ro33 [*p*=0.57].

Between 2010 and 2018, the number of alleles decreased from 28 to 16 [about 43% decline] for Ro33 and from 15 to 5 [about 67% decline] for Mad20. The band sizes were 100–480 bp for 2010 and 100-220 for 2018 for the Mad20 type; 100–950 bp for 2010 and 100-420 for 2018 for the Ro33 type [Figure 1]. Only 10 alleles of Ro33 and 3 alleles of Mad 20 were common in the two years.

Plasmodium falciparum merozoite surface protein 2 [*Pfmsp2*]

Approximately 63.64% [n=21] and 78.38% [n=29] of isolates could be amplified for *Pfmsp2* for 2010 and 2018 respectively.

The overall MOI associated with the polymorphism of *Pfmsp2* was similar [1.95 to 1.93] between 2010 and 2018 *[p=0.94]*. The MOI associated with the 3D7 family increased from 1 to 1.22 between 2010 and 2018 respectively *[p=0.04]*. The MOI associated with the Fc27 family was 1.72 and 1.43 for 2010 and 2018 respectively [p=0.34]. The frequencies of isolates with multiple allelic types were not significantly different during that time [table 2] *[p>0.05]*. In 2010, 7 isolates carried the Fc27 and 3D7 types whereas 12 isolates were found with both types in 2018 *[p=0.85]*.

We investigated the polymorphism of the allelic families 3D7 and Fc27 of *Pfmsp2*. The proportion of the family Fc27 was predominant in the two years: 74.36% in 2010 vs 65.38% in 2018 and the proportion of 3D7 was 25.64 and 34.62 for the same years. No difference was observed in the proportion of 3D7 and Fc27 *[p=0.49]*.

Between 2010 and 2018, the number of alleles increased from 8 to 15 [about 87.5% rise] for Fc27 and from 6 to 9 [about 50% rise] for 3D7. The band sizes were 390–560 bp for 2010 and 250-460 for 2018 for the 3D7 type; 480–680 bp for 2010 and 100-710 for 2018 for the Fc27 type [Figure 2]. Only one allele of 3D7 was common in the two years. No common alleles were observed.

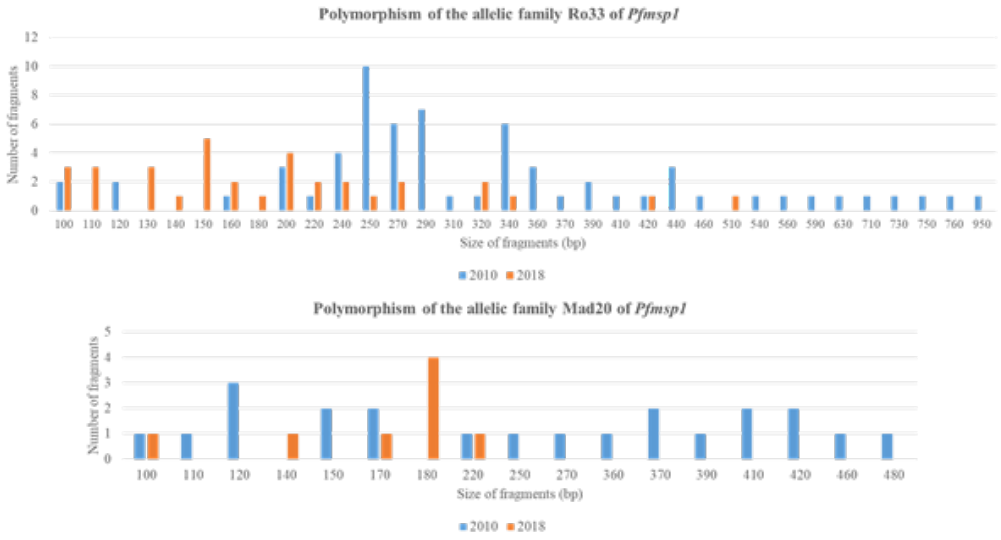

Figure 1: Polymorphism of *Pfmsp1*.

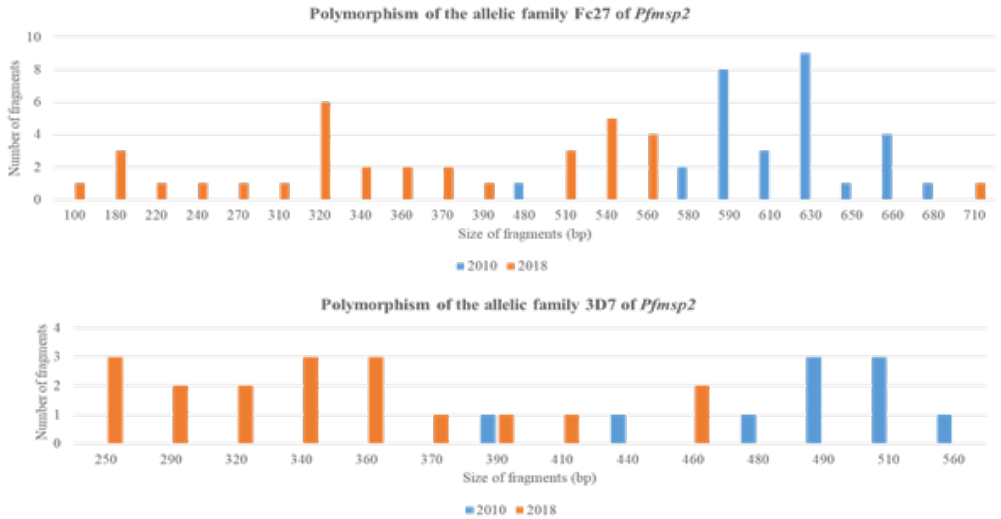

Figure 2: Polymorphism of *Pfmsp2*.

Plasmodium falciparum glutamate rich protein [*Pfglurp*]

Approximately 81.82% [n=27] and 70.27% [n=26] of isolates could be amplified for *Pfglurp* for 2010 and 2018 respectively.

The MOI associated with the polymorphism of *Pfglurp* was similar [1.76 to 1.57] between 2010 and 2018 *[p=0.53]*. In 2010, isolates with only one allele of *Pfglurp* was major; in 2018, the number of isolates with two alleles was 5-fold higher than previously observed [Table 2] *[p>0.01]*.

Between 2010 and 2018, the number of alleles increased from 19 to 22 [about 15.79% rise]. The band sizes were 150–850 bp for 2010 and 170-870 for 2018 [Figure 3]. Only 9 alleles were common in the two years [Figure 3].

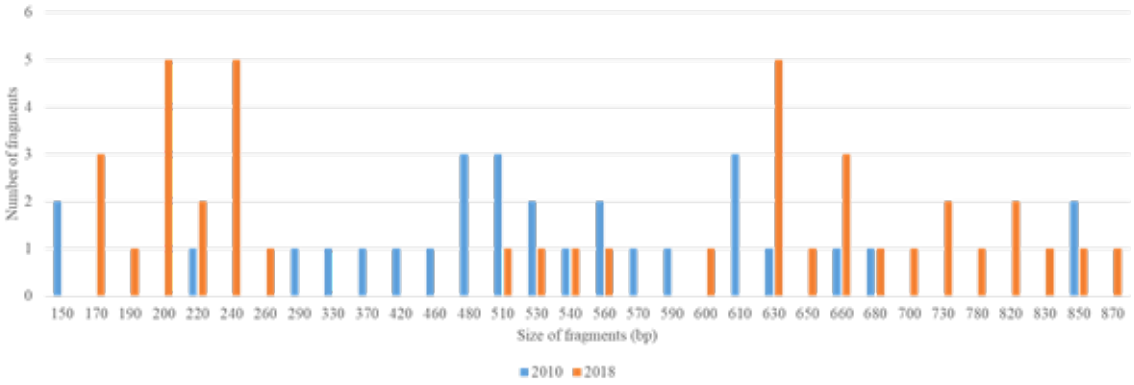

Figure 3: Polymorphism of *Pfglurp*.

Table 2: Minimal number of allele per sample.

	Allelic Family	Number of Alleles Per Sample	2010 n/N	2018 n/N	*p*
Pfmsp1	Mad20	1	6/11	6/7	0.85
		2	3/11	1/7	
		4	1/11	0/7	
		5	1/11	0/7	
	Ro33	1	7/25	11/18	0.1
		2	8/25	2/18	
		3	7/25	2/18	
		4	0/25	1/18	
		5	3/25	2/18	
Pfmsp2	FC27	1	9/18	14/23	0.8
		2	6/18	7/23	
		3	2/18	2/23	
		4	1/18	0/23	
	3D7	1	10/10	14/18	0.27
		2	0/10	4/18	
Pfglurp		1	10/17	11/26	5.37*10^{e}-3
		2	3/17	15/26	
		3	3/17	0/26	
		4	1/17	0/26	

Table 3: Proportion of allelic family of each gene per year.

	Pfmsp1 N (%)		*Pfmsp2* N (%)	
	MAD20	RO33	FC27	3D7
2010	22(25,29)	65(74,71)	29(74,36)	10(25,64)
2018	8(19,05)	34(80,95)	34(65,38)	18(34,62)
p	0,57		0,49	

Discussion

Among the most studied markers of *Plasmodium falciparum* diversity are *Pfmsp1, Pfmsp2* and *Pfglurp*. These genes are highly polymorphic and thus constitute great markers to distinguish *P. falciparum* populations during infections. This work aimed to determine the evolution of the genetic diversity of these markers in isolates collected from asymptomatic children aged 0 to 15 years in Brazzaville in 2010 and 2018. This study is among the few that address allelic diversity in asymptomatic carrier individuals in Congo.

In this study, not all isolates gens could be genotyped. The use of low-quality stored DNA or primers, not sensitive enough could explain these success percentages.

The molecular analysis allowed us to explore the polymorphism of the Mad20 and Ro33 allelic families of *Pfmsp1*; FC27 and 3D7 from *Pfmsp2* and *Pfglurp*, which are associated with the genetic diversity of *Plasmodium falciparum*. Overall, we have demonstrated a decrease in the allelic diversity studied between 2010 and 2018 for *Pfmsp1* and an increase in allelic diversity for *Pfmsp2* and *Pfglurp*. A decrease in band

size of alleles found for *Pfmsp1* and *Pfmsp2* and an increase in band size of *Pfglurp* alleles were also observed. This could be the result of the pressure from the drug with the intensification of the fight against malaria, particularly that of ACTs as observed in Comoros [18]. In the Republic of Congo, less attention has been paid to studying the genetic diversity of *P. falciparum* among of asymptomatic parasites carriers. In 2015, almost 10 years after the introduction of ACTs, the multiplicity of infection found in our study was similar to that described previously for *Pfmsp1* [2.5] but greater than that described for *Pfmsp2* [1.59] in Congo for children who consulted the pediatrics department in Brazzaville after microscopic diagnosis [10,19]. These MOIs are greater than those described for these markers before the introduction of ACTs in the south of Brazzaville [13,14]. These genes appeared to be highly influenced by the treatment and other malaria measures in survival processes [20,21]. For *Pfmsp1* the number of subpopulations per infection did not change whereas the genetic diversity decrease. To adapt itself efficiently, changes are more expressed in *Pfmsp2* and *Pfglurp*, for which the parasite is increasing the number of subpopulations per infection and the genetic diversity of allelic family. In this work, new alleles of these markers were found with new bands size.

The isolates analyzed for this study carried multiple parasite populations, up to at least five allelic populations per gene considered. Although the polymorphism of the K1 allelic family has not been studied, the proportion of Ro33 is greater than that of Mad20 in isolates from individuals and asymptomatic for *Pfmsp1*; a similar observation is made for the FC27 family of *Pfmsp2* as previously described for isolates from symptomatic patients in Brazzaville [10,19]. However, the number of Ro33 and Mad20 alleles for *Pfmsp1* and Fc27 and 3D7 for *Pfmsp2* found here in asymptomatic patients is higher than that observed in symptomatic patients before and after the introduction of ACTs [8,10,13,16,19]. This observation can be extended to the numbers of fragments of different sizes. This difference could be explained by the fact that not all Plasmodium strains are equal in terms of induction of the pathology. One can easily imagine that many strains found in asymptomatic patients are not virulent, whereas, in symptomatic patients, a few strains emerge and induce febrile access. The proportions found in our study can also be compared to what has been described in patients consulting in northern Ethiopia and Eritrea [22]. The Republic of Congo is considered a highly endemic country for malaria with a high level of malaria transmission. Studies conducted in low transmission areas show that the genetic diversity of *Pfmsp1* and *Pfmsp2* is low contrary to what used to be observed in high transmission areas, where genetic diversity is also high [20,21,23,24].

This study is the first to explore the diversity of the *Pfglurp* marker in Congo. However, in comparison to isolates from patients consulting in northern Ethiopia, we observe that the number of *Pfglurp* alleles found here is higher than that described in malaria symptomatic patients [22]. This confirms the genetic diversity of parasitic populations depending on the geographical location, the level of transmission of the parasite and the presence of the clinical symptoms of the disease [7,25,26]. In this study, despite the significant decrease in populations per infected isolates, the number of alleles increased.

The absence of *Pfmsp1*-K1 allelic family is a limitation to explore the genetic diversity of this gene. Agarose gel electrophoresis of amplified products lacks sufficient sensitivity to resolve a very small size difference between different bands, which constitutes a limitation of the study. Alternative methods such as genotyping using capillary electrophoresis or genome sequencing are preferred and would offer a better resolution to distinguish parasite populations.

Conclusion

These results showed that the diversity of the *Pfmsp1* has diminished whereas the diversity of *Pfmsp2* and *Pfglurp* genes has increased over the years. This could be seen as a sign that *P. falciparum* is adapting to the pressure of established malaria control methods.

Acknowledgment

We thank the patients and the staff of the medical centers from whom the isolates were collected in Brazzaville in 2010 and 2018. We also thank all the people who contributed to the logistics of this study.

Funding

This study was supported by funds from the *Laboratoire National de Santé Public du Congo*, the Gabonese government, Total Gabon, the *Centre International de Recherches Médicales de Franceville*, the *Agence Universitaire de la Francophonie* and the Associations of African Universities.

Conflict of Interest

Author has no conflict of interest.

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