Journal of Medical Entomology, 53(2), 2016, 315–320
doi: 10.1093/jme/tjv242
Advance Access Publication Date: 7 January 2016
Research article

Molecular Biology/Genomics

Knockdown Resistance (*kdr*) Mutations in Indian *Anopheles stephensi* (Diptera: Culicidae) Populations

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Received 5 October 2015; Accepted 9 December 2015

Abstract

Knockdown resistance (*kdr*) in insects resulting from mutation(s) in the voltage-gated sodium channel (VGSC) gene is one of the mechanisms of resistance against DDT and the pyrethroid group of insecticides. Earlier, we reported the presence of two classic *kdr* mutations, i.e., L1014F and L1014S in *Anopheles stephensi* Liston, a major Indian malaria vector affecting mainly urban areas. This report presents the distribution of these alleles in different *An. stephensi* populations. Seven populations of *An. stephensi* from six states of India were screened for the presence of two alternative *kdr* mutations L1014F and L1014S using allele-specific polymerase chain reaction assays. We recorded the presence of both *kdr* mutations in northern Indian populations (Alwar and Gurgaon), with the preponderance of L1014S, whereas only L1014F was present in Raipur (central India) and Chennai (southern India). None of the *kdr* mutations were found in Ranchi in eastern India and in Mangaluru and Mysuru in southern India. This study provides evidence for a focal pattern of distribution of *kdr* alleles in India.

Key words: *Anopheles stephensi*, voltage-gated sodium channel, knockdown resistance, insecticide resistance

Anopheles stephensi Liston is one of the major vectors of malaria in India, Pakistan, and the Middle East (Dash et al. 2007). Its distribution extends beyond the west of India to Afghanistan, Iran, Iraq, Bahrain, Oman, and Saudi Arabia, and in the east to Bangladesh, South China, and Myanmar (Rao 1984). Recently, this vector has been reported to invade Djibouti located in the horn of Africa, where it was found to harbor the *Plasmodium falciparum* circumsporozoite protein (Faulde et al. 2014).

Vector control is an essential component of malaria control program, which relies majorly on the use of chemical insecticides. Indoor residual spraying (IRS) is a major vector control strategy for rural areas, and antilarval measures for urban areas. Close to 60% of the high-risk areas are targeted through IRS, in which three insecticide groups are currently being used, i.e., DDT, malathion, and synthetic pyrethroids (National Vector Borne Disease Control Programme [NVBDCP] 2012). For urban malaria control, temephos and fenthion are being used as larvicides. Pyrethroids serve as an alternative to DDT (World Health Organization [WHO] 1989) and are extensively being used for impregnation in long-lasting insecticidal mosquito nets (LLIN), with the government targeting coverage of 80% of population under the risk area (WHO 2009, NVBDCP 2012).

DDT and pyrethroids act on the insect's voltage-gated sodium channel (VGSC) proteins found in nerve cell membranes by altering the gating kinetics that leads to paralysis and eventual death of the insect. Knockdown resistance (*kdr*) against DDT and pyrethroids is one of the resistance mechanisms in insects including anophelines, which confers reduced neuronal sensitivity against these insecticides, leading to the development of cross-resistance to all synthetic pyrethroids (Soderlund 2008). Knockdown resistance mutations in the VGSC are documented in conferring resistance against DDT and pyrethroids (Reimer et al. 2008, Davies et al. 2009, Ramphul et al. 2009), several of which are found occurring across the arthropod phyla (Davies et al. 2007a, Soderlund 2008). In anophelines, several *kdr* mutations are reported viz. L1014F, L1014S, L1014C, L1014W, N1013S, N1575Y, and V1010L (Silva et al. 2014) of which two classic *kdr* mutations—L1014F (Martinez-Torres et al. 1998) and L1014S (Ranson et al. 2000)—are most prevalent and are known to be associated with DDT and pyrethroid resistance.

DDT resistance was first reported in *An. stephensi* larvae in 1955 in Erode, a town in Tamil Nadu, south of India (Rajagopalan et al. 1956). It was subsequently detected in adult mosquitoes (WHO. 1986) and confirmed in other parts of the country (Kumari et al. 1998). Studies on knockdown resistance in Indian *Anopheles* populations by Singh et al. (2011) revealed the presence of two classic *kdr* mutations L1014F and L1014S in *An. stephensi* (Singh et al. 2011) and *Anopheles culicifacies* Giles (Singh et al. 2009, 2010), although at low frequencies (Dykes et al. 2015), and L1014F in *Anopheles subpictus* Grassi (Singh et al. 2015). The spread of such *kdr* alleles is deleterious to the expanding use of DDT and pyrethroids in public health and agricultural sectors across the country. It is therefore important to try and curb their spread before they become fixed in the population. Knowledge of the prevalence and distribution of *kdr* alleles in this *An. stephensi* is important for insecticide resistance management. This study provides an insight to the existing *kdr* alleles, their level of presence in the Indian *An. stephensi* population, and the focal pattern of distribution exhibited by them.

Materials and Methods

Study Sites

Anopheles stephensi were collected from Gurgaon city (32° 42′ N, 75° 9′ E) of Haryana state, villages Kishangarh and Umren (27° 26′–29′ N and 76° 31′–35′ E) of Alwar district (Rajasthan), Mangaluru city (12.87° N, 74.88° E) of Karnataka, Mysuru city (12.26° N, 76.6° E) of Karnataka, Raipur city (21.25° N, 81.63° E) of Chhattisgarh, villages Karsidih and Giridih (23.35° N, 85.33° E) of Ranchi district (Jharkhand), and Chennai city (13° 5′ N, 80° 16′ E) of Tamil Nadu. The years of collection have been shown in Table 1.

Mosquito Collection

Adult female *An. stephensi* were collected from human dwellings and cattle sheds in rural areas (Ranchi and Alwar) during 06:00–08:00 a.m. using an aspirator and flash torch. From urban settings (Gurgaon, Raipur, Mangaluru, Mysuru, and Chennai) immatures (larvae and pupae) were collected from breeding sites such as cemented tanks, fountain tanks, building construction sites, cisterns etc., with the help of a dipper. At least 50 breeding sites were checked. Immatures were brought to the laboratory and reared till their emergence into adults. Rearing of larvae was done in enamel trays containing water, and supplemented with fish-food flakes and yeast powder as food. Upon pupation, pupae were transferred to a plastic cup containing water and kept inside a cloth cage measuring 30 by 30 by 30 cm^3 for their emergence into adults. The emergent mosquitoes were given access to water-soaked resin and wet cotton pad. The temperature and relative humidity (RH) of insectary was maintained at 27 ± 2°C and 70–75%, respectively. Adult mosquitoes were identified morphologically using Christophers' key (Christopher 1933) and were preserved individually in a micro centrifuge tube containing silica gel.

Insecticide Susceptibility Tests

Insecticide susceptibility tests of mosquitoes were carried out using WHO's standard susceptibility test. Briefly, 4–6-d-old and sugar-fed adult females, which emerged from field-collected larvae (F_0) or F_1 progeny from adult collection, were kept in a holding tube (a maximum of 20 mosquitoes per tube) and were transferred to exposure tubes lined with insecticide impregnated and control paper supplied by Vector control and Research unit, Universiti Sains Malaysia. Tests were carried out exposing mosquitoes to 4% DDT-, 0.05% deltamethrin-, and 0.75% permethrin-impregnated papers keeping appropriate controls. On 1 h of exposure to the insecticide papers, mosquitoes were transferred back to holding tubes and allowed for recovery. Mosquitoes were given access to 10% glucose solution soaked in cotton pad during recovery. All the experiments were carried out in a room with a constant temperature of 25 ± 1°C and 75% RH. Mortalities were counted after 24 h of recovery period, and corrected mortalities were calculated using Abbott's formula.

DNA Isolation

Prior to DNA isolation, the lower one-third of the mosquito's abdomen was removed to eliminate contamination of DNA originating from sperms stored in spermatheca. Genomic DNA from individual mosquitoes was isolated following Livak's method (Livak 1984), eluted in 200 µl TE (Tris-EDTA) buffer, and stored at 4°C until use.

kdr Genotyping and DNA Sequencing

kdr genotyping of *An. stephensi* was carried out using polymerase chain reaction (PCR)-based assays as described by Singh et al. (2011). Two allele-specific PCR assays, i.e., PCR-F and PCR-L/S, were carried out for detection of *kdr* alleles where the former PCR discriminates 1014F from all other alleles (wild and 1014S) and the latter discriminates 1014S from L1014. The primers used for PCR-F were 0.50 µM of St-PheR (5′-GAT CGG AAA GTA AGT TAC TTA CGg CA-3′), 0.25 µM of St-L/SR and 0.25 µM of St-F (5′-GAT TGT GTT CCG TGT GCT GT-3′), and for PCR-L/S, primers used were 0.50 µM each of St-F, St-L/SR (5′-GCG GGC AGG GCG GCG GGG GCG GGG CCC GAT CGG AAA GTA AGT TAC TTA CGt CT-3′), and St-SerR (5′-CGA TCG GAA AGT AAG TTA CTT ACG AtT G-3′). Each PCR reaction (15 µl) contained 1× buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.375 units of AmpliTaq Gold taq polymerase (Applied Biosystems), and primers. The thermal cycling conditions for both ASPCR were one cycle at 95°C for 5 min, followed by 35 cycles each at 95°C for 30 s, 55°C for 30 s, and 72°C

Table 1. Distribution of *kdr* alleles in different field populations of *An. stephensi*

Locality	Year	*n*	Genotypes (frequency)						Allelic frequencies			HWE parameters		
			L/L	L/F	L/S	F/S	F/F	S/S	L1014	L1014F	L1014S	H_O	H_E	*p*
Alwar (Rajasthan)	2012	217	133 (0.613)	11 (0.051)	87 (0.401)	6 (0.028)	0	22 (0.101)	0.703	0.033	0.264	0.401	0.436	0.410
Gurgaon (Haryana)	2011	32	17 (0.53)	4 (0.12)	1 (0.03)	0	0	10 (0.31)	0.61	0.06	0.33	0.156	0.525	0.000
Raipur (Chhattisgarh)	2012	44	17 (0.386)	25 (0.568)	0	0	2 (0.045)	0	0.670	0.329	0.000	0.568	0.446	0.092
Ranchi (Jharkhand)	2012	84	84 (1.0)	0	0	0	0	0	1.000	0.000	0.000	0.000	0.000	1.000
Mangaluru (Karnataka)	2012	129	129 (1.000)	0	0	0	0	0	1.000	0.000	0.000	0.000	0.000	1.000
Mysuru (Karnataka)	2011	33	33 (1.000)	0	0	0	0	0	1.000	0.000	0.000	0.000	0.000	1.000
Chennai (Tamil Nadu)	2012	101	101 (1.000)	0	0	0	0	0	1.000	0.000	0.000	0.000	0.000	1.000
	2015	99	91 (0.919)	8 (0.081)	0	0	1 (0.010)	0	0.949	0.051	0.000	0.081	0.964	0.213

for 45 s, and a final extension step at 72°C for 7 min. Positive and negative controls were used for all PCR assays.

At least 10 DNA isolates from each locality, which were negative for L1014F and L1014S alleles, were sequenced for the partial S4–S6 segment of the domain 2 of the VGSC for verification of results and to screen the presence of any other existing alternative mutation. PCR products were amplified using primers VGS1F (5′-CTG AAT TTA CTC ATT TCC ATC-3′) and VGS1R (5′-CGA AAT TGG ACA AAA GCA AGG-3′) following the PCR conditions described by Singh et al. (2011). The PCR product was sequenced using primer VGS1F and internal primers VGS2R (5′-GAT ATG GTG CGA GCG AAT TT-3′) and VGS2F (5′-ATC CGT TTG CCC AAA CTA CA-3′). Sequence chromatograms were analyzed and edited using software Finch TV and aligned using ClustalW implemented in Mega 5 (Tamura et al. 2013).

Genetic Analysis

The observed and expected heterozygotes of *kdr* alleles and test of significance were calculated using Arlequin ver 3.5 software (Excoffier et al. 2005).

Results

Insecticide susceptibility tests carried out on three populations viz. Alwar, Ranchi, and Chennai showed that the populations are susceptible to synthetic pyrethroids but resistant to DDT. Results are shown in Table 2.

The result of *kdr* genotyping of mosquitoes for different *kdr* alleles for the seven field populations, their allelic frequencies, and Hardy–Weinberg equilibrium (HWE) parameters are provided in Table 1. It was observed that of the seven field populations studied, *kdr* allele 1014F was present in Gurgaon, Alwar, Raipur, and Chennai with allelic frequencies 0.06, 0.033, 0.329, and 0.051, respectively. No mutations were found in Chennai during 2012; however, we recorded a low frequency of 1014F mutation in 2015. The 1014F allele was absent in the remaining field populations. The *kdr* allele 1014S was present in Alwar and Gurgaon populations with relatively high frequencies (0.264 and 0.33, respectively) and in association with very low frequency of 1014F mutation (0.033 and 0.06, respectively). Genotypes in all populations were in HWE except for Gurgaon, which may be due to its small sample size. The compliance of HWE in other populations indicates random mating and rules out any possibility of genotyping error and presence of null allele. Distribution of allelic frequencies of different L1014 alleles in the field populations is presented in Fig. 1.

DNA sequencing of samples negative for *kdr* allele through PCR confirmed the absence of mutation.

Discussion

At present, vector control in India relies mainly on the use of chemical insecticides for IRS in rural areas and antilarval measures and fogging in urban areas besides the use of insecticide-treated mosquito nets. Currently, DDT, malathion, and synthetic pyrethroids are used for IRS, and organophosphates (temephos and fenthion) for larval control. The use of synthetic pyrethroid-treated LLINs is currently being promoted in areas with high API (annual parasite incidence per thousand population). The environmental burden of insecticides is high, as >40 insecticide formulations are currently registered with Central Insecticide Board and Registration Committee ([CIBRC] 2015) for the control of household and agricultural pests, contributing to the development of resistance in malaria vectors. *Anopheles stephensi* is reported to be resistant to the commonly used insecticides in public health in the past, particularly DDT, dieldrin (Subbarao 1980), malathion (Tikar et al. 2011), and BHC (Singh et al. 2014). However, *An. stephensi* is still susceptible to pyrethroids, which has emerged as a potential and preferred insecticide in public health and agricultural sectors due to their high knockdown action, low residual effect, and low mammalian toxicity. A prevention or delay in resistance against this precious group of insecticide relies on the rational use of the insecticide and an understanding of insecticide resistance management, which is crucial.

Anopheles stephensi is widely distributed in urban area in India, as it breeds in cemented structures which are abundantly found as overhead tanks, cisterns, fountain tanks, and uncovered water storage cemented underground tanks. Profuse breeding has been recorded in active building construction sites, which has been attributed as a cause of epidemics in a metropolitan city (Covell 1928). The control of this vector in urban areas relies on the use of antilarval methods, legislative measures, and personal protection in the form of mosquito nets or repellents. Pyrethroids are an important group of insecticides that are being used for the impregnation of mosquito nets and in commercially available mosquito-killing devices, such as coils, evaporators, and mats, which are common in urban area for protection against mosquito nuisance, besides space spray and fogging. Therefore, emergence of pyrethroid resistance in the coming years may hamper vector control program, as synthetic pyrethroids would be rendered ineffective.

Anopheles stephensi, although reported to be resistant to DDT, is still susceptible to pyrethroids. We did not detect pyrethroid resistance in Alwar and Chennai even in the presence of *kdr* mutation, indicating that *kdr* alone is not sufficient to produce resistance phenotype against discriminating dose. An earlier report on laboratory selection of resistance against deltamethrin has shown selection of 1014F *kdr* trait (Gayathri et al. 2006), indicating the positive role of *kdr* mutation against deltamethrin. However, the role of L1014-*kdr* in conferring resistance against DDT and pyrethroids has been established in a plethora of insect species (Silva et al. 2014) and is manifested in homozygous condition being recessive or incomplete recessive (Chandre et al. 2000, Davies et al. 2007b).

This is the first report on the distribution of *kdr* alleles in Indian *An. stephensi* populations. Earlier, we reported the presence of two alternative *kdr* mutations i.e., L1014F and L1014S, in just one

Table 2. Susceptibility status of *An. stephensi* against DDT and pyrethroids

Locality	DDT 4%		PER 0.75%		DEL 0.05%	
	N	Corrected mortality	*N*	Corrected mortality	*N*	Corrected mortality
Alwar	40	10%	70	98.7%	50	100%
Ranchi	40	20%	40	92.5%	40	100%
Chennai	39	66.7%		ND	52	100%

Abbreviations used: PER—permethrin, DEL—deltamethrin, ND—not done.

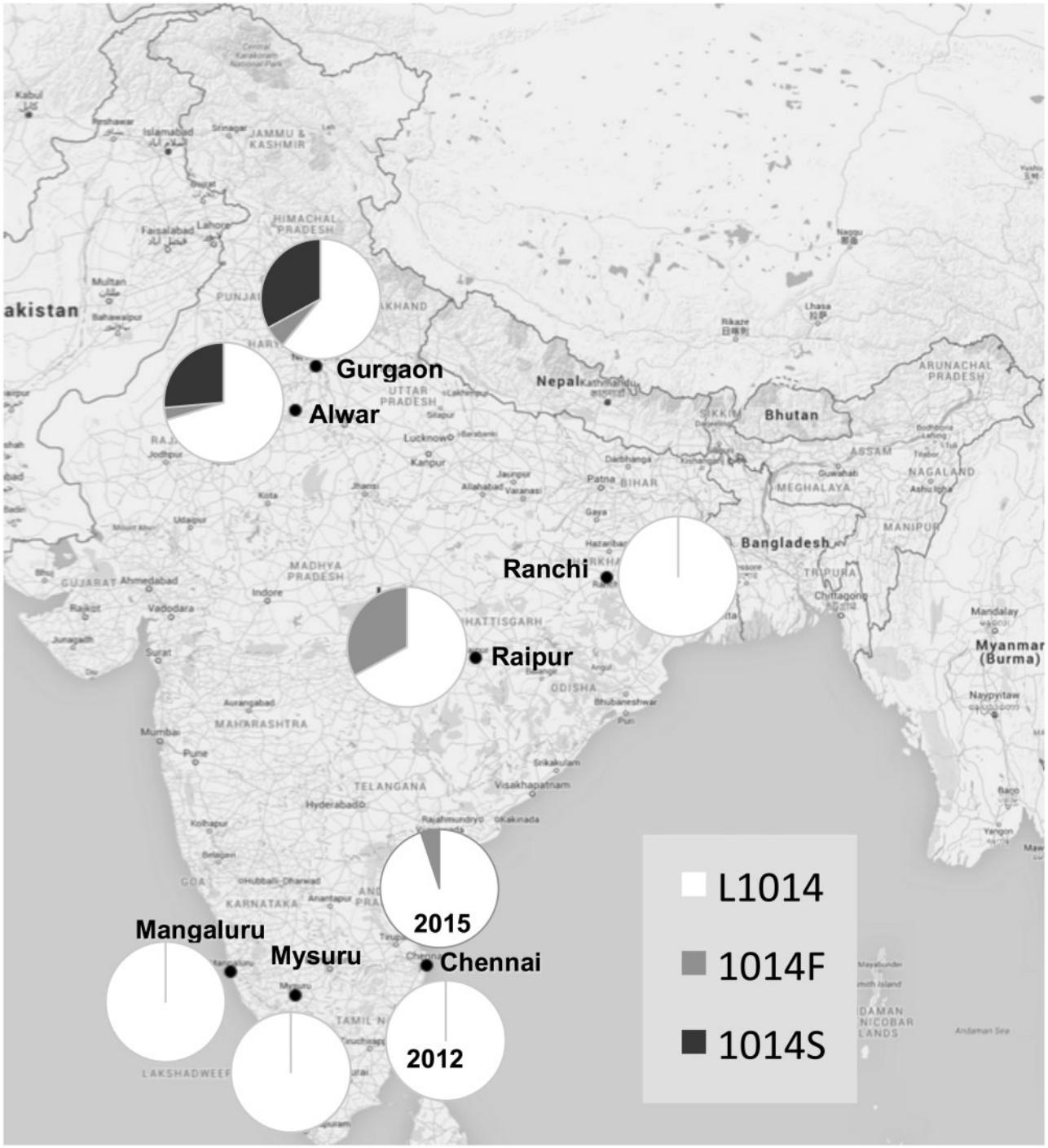

Fig. 1. Distribution of *kdr* alleles L1014, 1014F, and 1014S in Indian *An. stephensi* populations.

population in northern India (Singh et al. 2011). The focal presence of *kdr* alleles in this study warrants a close monitoring of the spread of such alleles, considering the presence of widespread resistance to DDT in India.

In *An. stephensi*, regional differences in the distribution pattern of *kdr* mutations and their allelic frequencies were noticed in the Indian populations in this study. Both *kdr* mutations, L1014F and L1014S, were found in north Indian populations—Alwar and Gurgaon, with L1014S frequencies of 26 and 33%, respectively. In central Indian population (Raipur), only L1014F was found to be present, with a frequency of 33%. In eastern and southern India, we did not record any *kdr* mutation except for Chennai where the presence of L1014F mutation is found at low frequency in 2015. Earlier record showed the presence of L1014F in a laboratory colony originating from Chennai, southern India, which was selected for deltamethrin resistance for several generations (Gayathri et al. 2006). The data suggest that *kdr* is focal in distribution in the case of *An. stephensi*.

With the *kdr* trait said to be incompletely recessive (Chandre et al. 2000) or recessive (Davies et al. 2007b), it may not be a major

insecticide resistance mechanism unless *kdr* alleles are present in high frequency so as to maintain sufficient *kdr*-homozygotes. We noted high frequency of 1014S in northern Indian populations and 1014F in a central Indian population (>25%), while very low frequency of *kdr* mutation or its absence was observed in other populations. Fixation of *kdr* alleles may play a discrete role in insecticide resistance in the long run as evident from the study in Kenya (Mathias et al. 2011). In considering efforts toward effective monitoring of insecticide resistance, knowledge of the frequency distribution of *kdr* alleles is essential to prevent their wide spreading and in effecting a delay in the imminent resistance that is gradually expanding against pyrethroids, an invaluable class of insecticide that remains for the fight against Malaria in India.

Acknowledgments

We are grateful to Mr. Uday Prakash for genotyping assistance and to Mr. Bhopal Ram, Mr. Shri Bhagwan, and Mr. N. S. Bhakuni for insectary and field assistance. C.L.D. was supported by Defence Research Development Establishment (DRDE) grant, and S.M. was supported by National Institutes of Health/National Institute of Allergy and Infectious Diseases grant U19AI089676. The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of funding agencies.

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