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Original Article

The Absence of Sporozoites in *Anopheles* Mosquitoes in the Malaria Pre-elimination Area

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Abstract

Introduction: The existence of sporozoites in the salivary gland of *Anopheles* is a prominent factor in the transmission and spread of malaria. Malaria control programs must be comprehensive including the treatment of patients, management of the vector population, and efforts to prevent human-vector contact. The present study aimed to track the presence of sporozoite in the *Anopheles* mosquito population in a malaria pre-elimination area and evaluate the implementation of a malaria elimination program.

Methods: This cross-sectional study was conducted in a malaria pre-elimination area over a period of one year. Mosquitos were caught using human bait and in resting sites close to cattle pens. Sporozoites were detected microscopically and circumsporozoite protein (CSP) was tested using polymerase chain reaction technique.

Results: A total of 212 *Anopheles* mosquitoes were caught. *Anopheles maculatus, Anopheles aconitus, Anopheles subpictus, Anopheles barbirostris, Anopheles vagus*, and *Anopheles kochi* species were identified. Salivary gland of 111 mosquitoes were dissected and CSP was detected in 101 specimens. Neither type of test found any sporozoites in the captured *Anopheles*.

Conclusion: The absence of sporozoites in *Anopheles* in the pre-elimination area is a good sign of the ongoing malaria control program. Breaking the life cycle of the parasite that causes it by minimizing contact with vectors is an effective method that should be continuously improved.

Keywords: Circumsporozoite protein, Sporozoite, Elimination, Vector, Menoreh hills

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Introduction

The number of malaria cases in Central Java province of Indonesia has declined from 1140 in 2016 to 273 cases in the third quarter of 2020. The annual parasitic incidence (API) also decreased from 0.03 in 2016 to 0.008% in 2020. This achievement far exceeded the API target of 0.06% in 2020. The proportion of indigenous cases is getting smaller. In 2019, only 0.5% of indigenous cases occurred, and in 2020, all cases were categorized as imported malaria (1).

Malaria cannot be separated from the presence of the *Anopheles* vector. Data on the diversity of mosquitoes are prominent for consideration of vector-borne disease control programs (2). Each mosquito species has unique behavior and habitat that is different from other species (3). In endemic areas of malaria, data and information regarding the presence, characteristics, and bionomics of the *Anopheles* mosquito vector should be provided in as much detail as possible (2,4,5). All the related data are needed to prevent malaria transmission by female *Anopheles* mosquitoes. The nature and behavior of the *Anopheles* cannot be ignored in malaria control (6).

The *h-Plasmodium* parasite that causes malaria requires two hosts in its life cycle. One of them is the *Anopheles* mosquito as the sexual cycle host (7). With mating between male and female, gametocytes will produce a final stage of sporozoites. The circumsporozoite protein (CSP) is detected in lamina basalis when sporozoites reach the salivary glands (8). The survey of the presence of sporozoites in *Anopheles* mosquitoes in malaria-endemic areas is of great importance. It not only detects the presence of sporozoites but also acts as an early warning against the potential recurrence of malaria and evaluates the implementation of the elimination program. The purpose of this study was to trace the presence of sporozoites in the *Anopheles* mosquito population in malaria pre-elimination areas as an evaluation of the malaria elimination program.

Materials and Methods

Materials

The testing specimens were obtained from female

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Anopheles mosquitoes caught in the study site over a period of 12 months, from September 2020 to September 2021. Mosquitoes caught were divided into two groups. The salivary glands of the 111 mosquitos were dissected and the other 101 were stored in the freezer at −20 C. The frozen specimens were tested for CSP by polymerase chain reaction (PCR) technique.

Methods

Study Design and Site

This cross-sectional study was conducted in the villages of Jatirejo and Ngadirejo, which are located in the Kaligesing sub-district, Purworejo District, Indonesia. Both villages belong to a malaria pre-elimination area located in the Menoreh hills.

Anopheles Collection

The collection of the mosquitoes was performed by the use of an aspirator. The technical team consisted of an entomologist and village malaria workers at the Kaligesing Health Center. Mosquito collection was carried out from September 2020 to September 2021 in two ways, using human bait around the cage and resting place in the livestock pens.

Species Identification

Mosquitoes were killed by chloroform and female Anopheles were identified microscopically using the identification key available in Indonesia (9). The identified mosquitoes were put in a petri dish with a cotton pad and covered with moistened filter paper to keep the mosquito body fresh.

Salivary Gland Dissection

The legs and wings of the mosquitoes were removed. The mosquito body was placed on the slide with the head on the right. NaCl solution (0.9%) was dropped next to the mosquito. The chest was pierced with a needle. The neck was cut and pulled slowly until the salivary gland was excised. The salivary gland was placed on drops of NaCl 0.9% solution and covered with a coverslip. The coverslip was gently pressed until the salivary glands ruptured. The presence of sporozoites was determined using a microscope (10).

PCR Testing Method

The blood of the patient confirmed positive for *P. falciparum* with a high parasitemia living in Abepura, Papua Province, which is the highest malaria-endemic area in Indonesia was used as the positive control. Mosquitoes caught at the study site which were confirmed negative by salivary gland dissection for detection of sporozoites were used as negative controls. DNA isolation was carried out using InstaGene Matrix (Bio-Rad Laboratories, USA). DNA was amplified using Bio-Rad Thermal Cycler-100. Primers were produced by Macrogen Humanizing Genomics (Synapse, Singapore) with base sequences 5'ACAATCAAGGTAATGGACAAGG3' (Forward) and 5'GGATTAATAATGGTATTATCCTTCT3' (Reverse) (11). The reaction mixture contained 2x Taq Plus PCR Master Mix (Tiangen KT205). Detection of CSP was done by Gel Red nucleic acid stain (Biotium Inc) using a Consort-Electrophoresis system and the emerging band was read using a small illuminator. The propagation distance of the orange-colored spot on the sample was compared with the spot on the marker. The circumsporozoite protein (CSP) gene weight measurement was read using GelAnalyzer 19.1 software. As a comparison, a gene weight bank is used between 70–285 base pairs (12) or in the range of 265 base pairs (13).

Results

Kaligesing sub-district is one of the malaria-endemic areas in Purworejo district. Local cases still occurred until 2018. Before the increase in cases in Bener district, Purworejo Regency would reach malaria elimination status if there were no local cases until October 2021. Unfortunately, in June 2021, there was a large increase in cases in Wadas village and its surroundings in Bener district where the goal of malaria elimination has not been achieved.

The number of *Anopheles* caught was 212. Six species were identified in the two villages over a period of one year from September 2020 to September 2021. The number of mosquitoes collected by human bait was higher compared to those collected in the resting places around cattle pens (Table 1). The majority of the mosquitoes caught belonged to *A. vagus* species, while *A. aconitus* was the rarest species (Figure 1).

The number of mosquitoes caught was fluctuating, with the mean being 16 mosquitoes per month. There was an increase in the *Anopheles* population in the rainy season compared to the dry season. Most of the mosquitoes were obtained in February. The number of Mosquitoes began to decline drastically in March and decreased to the lowest in June. The lack of *Anopheles* catches was anticipated by increasing the number of catch points. But the increase of catch point number was not directly proportional to

Table 1. Summary of the Survey Results

Data of survey	Jatirejo village	Ngadirejo village
Catch point (N)	334	318
Mosquito caught (N)	72	140
Human bait (N)	41	85
Resting place (N)	31	55
Species	*A. maculatus, A. aconitus, A. subpictus*	*A. vagus, A. barbirostris, A. kochi*
Salivary gland dissection (N)	39	72
PCR for CSP (N)	33	68

the number of *Anopheles* mosquitoes caught. The lowest catch of Anopheles was in June which had the most catch point (Figure 2).

The dominant species was *A. vagus*. The monthly number of mosquitoes did not differ significantly throughout the year. All species caught were found to have relatively the same population throughout the year. The number of *Anopheles* was higher in the rainy season and it decreased in the dry season (Figure 3).

The scarcity of the *Anopheles* population underlies catching by human bait and resting place around the cage. It is done to attract the interest of the zoo-anthropophilic *Anopheles*. The proportion of mosquitoes caught using human bait and in resting places was 59.4% and 40.6%, respectively. Man-hour density (MHD) ranged from 0.009 to 0.025. The highest and lowest MHD belonged to *A. barbirostris* and *A. aconitus*, respectively (Table 2).

The number of specimens used for microscopic examination was higher compared to specimens used for CSP detection. Considering *A. aconitus*, all of them were used for CSP testing because only three mosquitoes were caught. No sporozoite was found microscopically using the salivary gland dissection. Likewise, in the CSP test, no positive samples were found (Figure 4).

The positive control band (C2) was on 182 base pairs on the electrophoresis agarose gel. No band on the negative control (C1) and sample testing.

Discussion

Malaria cases in Kaligesing did not spread from endemic villages to neighboring villages. There is no record of the domestic movement of malaria sufferers from Jatirejo village to Ngadirejo village before the case emerged. Imported cases in Jatirejo village were only reported at the

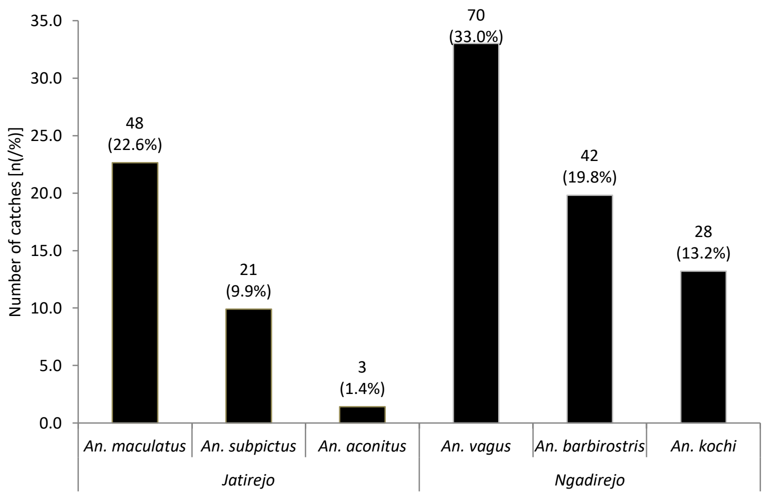

Figure 1. The Proportion of Mosquitoes Caught by Species

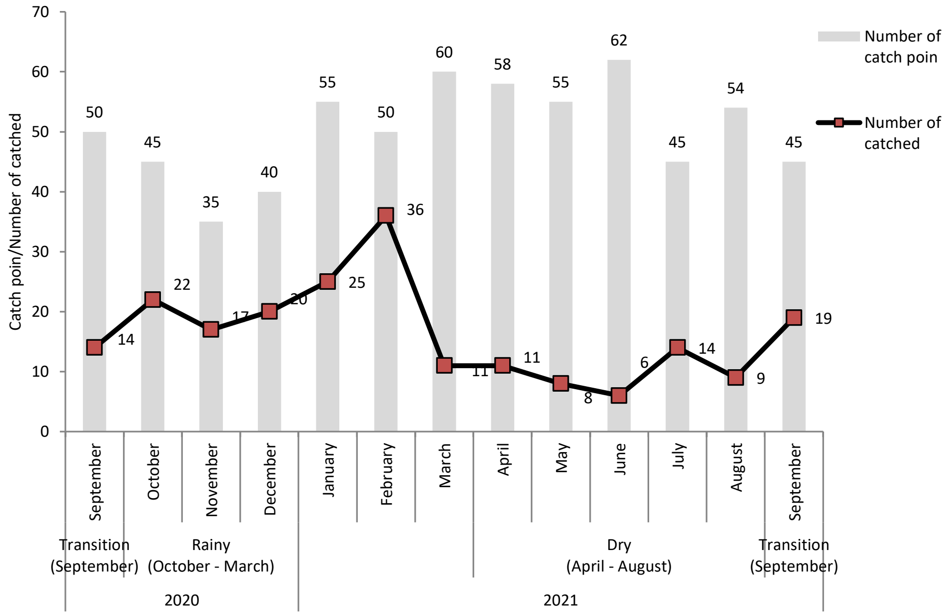

Figure 2. Fluctuations in the Number of Mosquitoes Caught Based on the Number of Catch Points

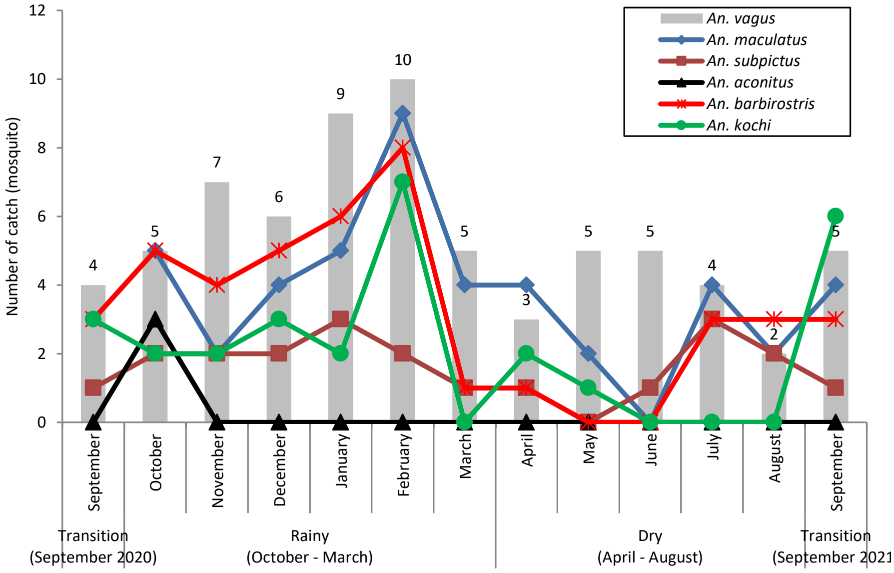

Figure 3. Monthly Number of Mosquito Caught Based on Species

Table 2. The Proportion of Catch, Density, and Sporozoites Testing

Species	Catching		Man-hour density (MHD)	Sporozoites Testing			
				Microscopically		CSP	
	Human bite	Resting		Positive	Negative	Positive	Negative
A. maculatus (n=48)	19	29	0.012	0	28	0	20
A. subpictus (n=21)	19	2	0.012	0	11	0	10
A. aconitus (n=3)	3	0	0.002	0	0	0	3
A. vagus (n=70)	30	40	0.019	0	37	0	33
A. barbirostris (n=42)	40	2	0.025	0	22	0	20
A. kochi (n=28)	15	13	0.009	0	13	0	15
Total	126	86	-	0	111	0	101

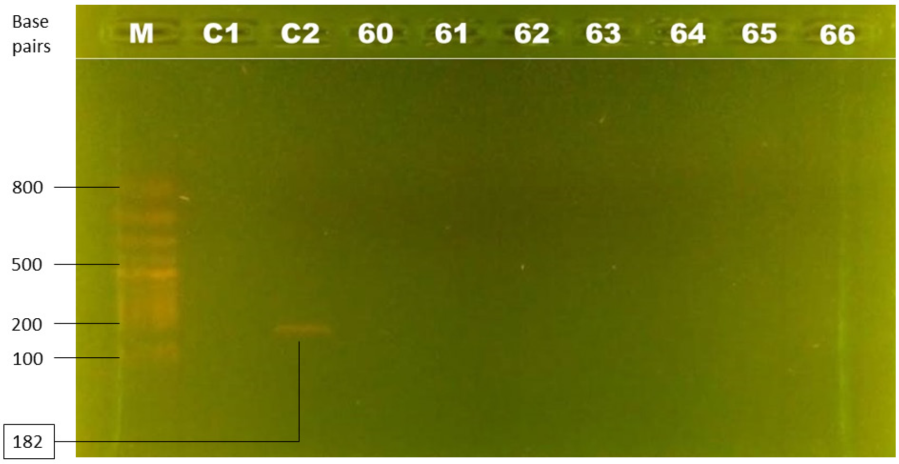

Figure 4. CSP Band on Agarose Gel Electrophoresis. M = marker, C1 = negative control, C2 = positive control

end of 2018 after local cases began to subside somewhat (1). Meanwhile, in 2018, 23 malaria cases were found in the RT.2 area of Kembangsoko Hamlet, Ngadirejo village (14). The emergence of this malaria case was unique because positive confirmed patients only gather in one location, not spreading throughout the village area. Before the incident, there was no history of patients traveling to high malaria-endemic areas, especially from transmigration areas outside Java. Patients also had no kinship with malaria sufferers in Jatirejo village and other endemic areas, so they have never traveled or stayed there.

In the same year, there was also a significant increase in malaria cases in the Gebang sub-district (15). The location is far away and not directly adjacent to Kaligesing sub-district, but it is still connected by the Menoreh hills. This condition rejected the hypothesis of the spread of malaria in the Kaligesing sub-district due to the patient's mobility from one endemic village to another. It seems that the mobility of *Anopheles* as a malaria vector has more potential to take the role in this area. The geographical position of the East side of Kaligesing sub-district, starting from Jatirejo village to Ngadirejo village, is directly adjacent to the Kulonprogo Regency area along the Menoreh hills. The Menoreh hills area is partly natural forest and partly land used for the production of cold climate crops such as tea, cocoa, and the like (16). The forest areas adjacent to residential areas also are potential breeding places for *A. aconitus, A. maculatus, A. vagus, A. subpictus, A. Barbirostris*, and *A. leucosphyrus* (17).

Anopheles vagus was the most common species, which is in line with a previous report (18). This report is consistent with the finding of the study conducted in Seram, Maluku (19). Several habitats such as rice fields (20), ponds (21), puddles of flooded soil, grassy fields with shrubs and shade plants that hold water, and even dug wells are breeding places for *A. vagus* (22). The ditch around the settlement (21) collects rainwater and household waste overgrown with tall grass is also favored by this species (23), especially those with a mud bottom (24)(25). Even in Simpang Empat village, Lengkiti, Ogan Komering Ulu, South Sumatra, this species is commonly found in puddles on roads (26), including the ground holes made by heavy vehicles which are filled with water (21,26).

Poorly maintained vacant land, which can hold freshwater with little acidity or neutral water, is also highly preferred by *A. vagus* (25,27). Apart from marshy areas in the lowlands (24), *A. vagus* can also be found in the highlands (28). Adult mosquitoes prefer to forage outside the home, especially around cattle pens (27) and about 10–60 m from settlements (25). Buffalo puddles should be watched out because they are favored for breeding (29). The proportion of *A. vagus* caught by human bait and in the resting places around cattle pens is 30:40. These results are in line with reports from the same location where a higher number of *A. vagus* were caught in resting place than using human bait (18). The highest number of mosquitoes caught does not guarantee that the MHD value is also the highest because the standard measurement only counts the number of mosquitoes caught using human bait (10), while in this survey, mosquitoes were also caught in resting places around cattle pens.

Anopheles barbirostris does not have the largest population in Ngadirejo village, but this species has the highest MHD. This species has also reportedly been found in forest and residential areas in 2015 (18). In Kupang NTT, this species dominates the *Anopheles* population and is mostly found outdoors between 9:00 pm and 04:00 am (4). Similar to other species, *A. barbirostris* also prefers buffalo puddles, ground holes near cowsheds, former ponds, rice fields, and lakes as breeding grounds (29). The proportion of mosquitoes caught by human bait and those caught in resting place in the cattle pens is relatively higher than *A. vagus,* so that *A. barbirostris* had the highest MHD value.

A high number of *A. kochi* were caught in Ngadirejo village. The breeding place of this species includes fish ponds that were not maintained well or used for raising fish any more (29). The fish ponds in the home field deserve attention because they are full of mosquito larvae. *A. kochi* was also found in fish ponds in Simpang Empat village, Lengkiti, Ogan Komering Ulu, South Sumatra (26).

Freshwater with low salinity is the preferred breeding place of *A. maculatus* (30). *A. maculatus* was also found in this survey, which is in line with a previous study conducted in Jatirejo village (31). The presence of the species *A. maculatus* has also been reported in Kokap Kulonprogo (32) and Borobudur Magelang (33) which are directly adjacent to Kaligesing. This species prefers to breed in water reservoirs that form on the banks of rivers and waterfalls (30). The study sites are located in a hilly area with deep ravines. Many rivers flowing from the highlands are in the form of waterfalls due to the steep distance between the ground surface levels. The larvae of *A. maculatus* was also found in ponds with a depth of 5–15 cm containing clear water with a substrate of mud and plants (30). Some residents of the two villages who have fish ponds but do not utilize them optimally can make them breeding grounds for mosquitoes during the rainy season. In addition, this species is found in dam water, puddles, and swamps at the foot of the hills (34). Although it is zoo-anthropophilic, the species prefers goat blood over humans (35). The population increases after the rainy season and prefers resting areas in cattle pens (36).

Anopheles aconitus found in the survey has also been reported in the previous study (30). This species can breed in coastal areas and highlands including rice fields in the hills (37). In Jatirejo village, there is no rice field area at all, but the hilly area with a height of 226 m above sea level is still a possible breeding place for Anopheles. Another breeding area for *A. aconitus* is Menoreh Hill which is located in the sub-district of Borobudur Magelang (33). Catching this species by human bait is indeed results in the lowest number among other species. The low catch may be due to the absence of catching using cattle bait, even though it was reported *A. aconitus* prefers cattle feed (38).

The presence of *A. subpictus* has also been reported in Jatirejo village in previous studies (30). This species has the second largest population in Kupang, NTT, where it is often found outdoors from 22:00 to 23:00 (4). The same species is also commonly found in river areas in New Delhi, India (39). Puddles that have a muddy bottom flowing slowly, overgrown with aquatic plants and mosses are favored by this species. The species prefers lagoons and brackish water ponds with a depth of 20–25 cm (40,41). *A. subpictus* prefers mixed blood meals, including human and livestock (42).

A total of 16 *Anopheles* mosquitoes per month or 0.6 mosquitoes per night were caught by human bait outside the home and in resting cages. There has been no further study on the decline in the *Anopheles* population in Kaligesing. In the previous year's study conducted in Jatirejo village, only 15 mosquitoes were caught (31). In another report from other areas, 13 Anopheles were caught in one night in Bontosunggu village, Selayar Islands Regency (43), however, the vector density was certainly strongly influenced by local environmental conditions. *Anopheles* population showed an increase in the rainy season compared to the dry season. The number of mosquitoes increased in the rainy season, which can be easily understood because the falling rainwater fills the entire soil basin that lies in the open area to collect water. This accommodated water has the potential to become a breeding ground for *Anopheles* (22). This condition does not apply to areas that geographically have permanent breeding sites (44).

The absence of sporozoites in the *Anopheles* population in this malaria pre-elimination area is considered progress. This is likely related to good and fast handling of any suspected malaria sufferers found. Immediate treatment of patients found is expected to kill the parasites in the body so that the life cycle of *h-Plasmodium* can be broken. The implementation of malaria migration surveillance is the key to controlling the spread of malaria (45) in addition to insecticide-residual spraying (IRS) and the distribution of long-lasting insecticide-treated nets (46,47). If the condition is maintained, the *Anopheles* vector will never get the parasite again. In the end, malaria can be controlled and eliminated.

Conclusion

The absence of sporozoite in the *Anopheles* mosquito population in these two pre-malaria pre-elimination villages offers a new hope for the local community to be completely free from the threat of malaria. Good cooperation between the government and the community in controlling the *Anopheles* population and breaking the life cycle of the human *Plasmodium* parasite is the key to the success of the malaria elimination program.

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Authors' Contribution

Conceptualization: Didik Sumanto.
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Formal analysis: Didik Sumanto, Sayono Sayono.
Funding acquisition: Tri Dewi Kristini.
Investigation: Wahyu Handoyo, Tri Dewi Kristini.
Methodology: Didik Sumanto, Sayono Sayono.
Project administration: Tri Dewi Kristini.
Resources: Wahyu Handoyo, Tri Dewi Kristini.
Software: Didik Sumanto.
Supervision: Didik Sumanto.
Validation: All authors.
Visualization: Didik Sumanto, Sayono Sayono.
Writing–original draft: Didik Sumanto.
Writing–review editing: Sayono Sayono.

Competing Interests

The authors declared no conflict of interests.

Ethical Approval

This study was approved by the Commission of Health Research Ethics, Faculty of Public Health, Universitas Muhammadiyah Semarang.

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