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Distribution and molecular characterization of *Helicotylenchus multicinctus* (Tylenchida: Hoplolaimidae) from banana roots in Namakkal region, Tamil Nadu

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Abstract

Banana (*Musa acuminata*) is grown in tropical regions, cultivated in the delta regions of Tamil Nadu, India. Plant-parasitic nematodes (PPNs) are recognized as one of the major threats to a wide variety of host which affects the banana root system, leading to impaired plant growth and production. In this study, the population density of three different nematode species namely, *H. multicinctus*, *M. incognita* and *P. coffeae* were identified in Namakkal district of Tamil Nadu. Of these, the spiral nematode, *H. multicinctus* was found maximum of 72.7% at Kanavaipatty followed by Siluvampatty (63.6%) and Kondarasampalayam (62.5%) villages when compared to other species thus significantly influenced its production. The genus *Helicotylenchus* contains cosmopolitan, ubiquitous plant-parasitic nematodes with some species capable of causing significant economic damage to the agricultural crops. Consequently, the species identification is essential for the development of effective management strategies to enhance the banana production. *Helicotylenchus spp.* was found higher in the root samples obtained from this region and morphological data indicates the presence of *H. multicinctus*. Molecular characterization was carried out to identify the nematode species and phylogenetic analysis of the 18S rDNA segment revealed the identified species is *H. multicinctus* and it was morphologically similar but genetically some alterations in the nucleotides.

Keywords: *Musa acuminata*, Prevalence, Morphology, *H. multicinctus*, phylogeny

Introduction

Plant-parasitic nematodes are the most successful group of invertebrates that can live freely in the soil, feeding parasitically on plant roots. It is one of the most important groups of organisms limiting the production and causing severe economic losses to agricultural crops in the developing countries (Stirling and Pattison, 2008: Ravichandra, 2014) [29]. This can affect almost every part of the plant including roots, stems, leaves, fruits and seeds (Krif *et al*., 2020; Mandal *et al*., 2021) [22]. This infestation results in billions dollars loss in crops annually, and all the crops around the world are susceptible to at least one species of nematode parasites (Bozbuga *et al*., 2018) [5]. This seems to be the absorption of water nutrients through colonizes in the roots and inhibit the translocation of minerals. Such alterations can affect the shoot-to-root ratio, leading to poor growth performance of banana plantains (Gullino *et al*., 2019) [16]. The economic importance of these nematodes largely depends on the maintenance of farming system, physical and chemical barriers, which limiting the banana production.

The genus *Helicotylenchus* (Tylenchida: Hoplolaimidae) are among the most ubiquitous plant-parasitic nematodes widely distributed throughout the world. The most abundant nematode of this species may cause severe root damage on plantains after *R. similis* (Brentu *et al*., 2004) [6]. *Helicotylenchus spp*. has a wide range of host including fruit crops, vegetables, agronomic crops, ornamental plants, forages, turf grasses, weeds, and plants in natural habitats. With the exception of *H. multicinctus*, spiral nematodes are not considered as economically important pests on most of the hosts.

Helicotylenchus multicinctus is considered the most damaging nematodes on banana plantain and regarded as the main parasitic nematode species in the moderate temperature and rainfall (Mc Sorley and Parrado, 1986) [25]. However, *H. multicinctus* feeds within the outer layer of the root cortex, progressive root deterioration, uptake of water and nutrients which leads to toppling of plants (Daneel *et al*., 2015 [9]; Lara *et al*., 2016 [21]; Tzortzakakis *et al*., 2017) [43]. In addition, bunch maturation and size reduction, with the production losses between 19% and 34% at 2 to 3 years after planting (Selvaraj *et al*., 2014) [11].

In India, *H. multicinctus* has been recorded on banana in epidemic proportions in Andhra Pradesh (Sundararaju, (2006) [41] and occurs widespread in banana plantations in West Bengal (Khan and Hasan, 2010) [18], Tripura (Mukherjee *et al*., 1994) [26, 27] and Orissa (Ray and Parija, 1987) [30]. *H. multicinctus* is considered to be an end oparasite which is able to complete its life cycle within the cortex of the roots (Das *et al*., 2014) [11]. An adequate knowledge of the biology, host-parasite relationships, population dynamics of a target nematode species and its interactions with the environment is necessary for the

nematode management strategies (Chawla and Mittal, 1995; Adam *et al*., 2007; Riga *et al*., 2007) [8].

Materials and Methods

Sample collection

The study was carried out for a period of 3 months from October 2020 to December 2020 in North-Western zone (Namakkal) located in the central part of Tamil Nadu, India. For the extraction of nematode DNA, a total of 30 banana plants were examined for nematode infestation in 3 different villages in Namakkal district of Tamil Nadu, which includes Kondarasampalayam (11.24° N, 77.92° E), Siluvampatty (11.26° N, 78.11° E), and Kanavaipatty (11.16° N, 78.17° E) (Fig.1). The roots were collected randomly from nematode-infested plants, transferred to polypropylene bags, and stored at 5°C. Each root samples were individually analyzed for its morphological features by the method of Uzma *et al*. (2015) [44] and Brucher *et al*. (2019) [7].

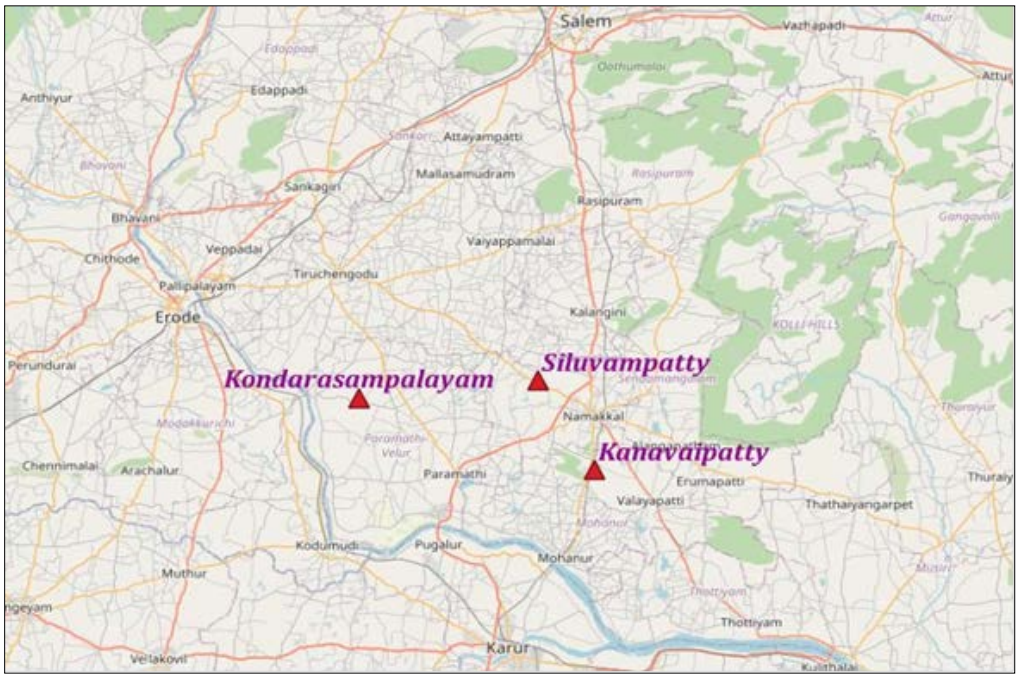

Fig 1: Field locations in Namakkal district of Tamil Nadu, India. Regions are in Red (▲) represent the *H. multicinctus* populations present in the respective regions marked by QGIS mapping.

Distribution of Nematodes

Nematode distribution was calculated as a frequency of occurrence {(Number of samples containing a species/number of samples collected)* 100}. The population density (PD) of nematode occurrence was counted as the number of samples in which each species was detected / the total number of samples, while the Prominence value (Pv) was calculated as population density X (√ frequency of occurrence) as described by De Waele *et al*. (1998) [2, 6] and Dawabah *et al*. (2020) [12].

Isolation of genomic DNA

Nematode DNA was extracted from the infected roots followed by the method of Alagesan *et al*. (2019) [3] with minor modifications. For the microscopic observation of nematodes, the infested root tissues were crushed using mortar and pestle and placed in a 1.5 mL micro centrifuge tube. The DNA was extracted from the homogenized tissues by using the QIA amp DNA mini kit (Qiagen, Valencia, CA, USA) according to the method and a final volume of 30 µL DNA extract was eluted and stored at -20°C for future use.

Polymerase Chain Reaction

The PCR was performed as described by Subbotin *et al*. (2015) [39, 40]. For the amplification of the 18S rRNA gene of *Helicotylenchus* species, genus specific ITS primers of PRATTW81-5'-GTAGGTGAACCTGCTGCTG-3'and AB28-5'- ATATGCTTAAGTTCAGCGGGT-3' were used for this study. PCR amplification was performed in a final volume of 25 µL, with each reaction mixture containing 1 µL of DNA template, 10.5 µL of nuclease-free water, 12.5 µL of 1.5 mM $Mgcl_2$ (Taq 2X Master mix, HS, Takara) and 0.5 µL of each primer at 10 pmol. Target amplification was carried out by the following PCR steps: 35 cycles of three steps each, comprising initial denaturation at 95°C for 30 sec, primer annealing at 61°C for 35 sec, and product extension at 72°C for 30 secs. At the end of the amplification, a final extension step was achieved at 72°C for 5 min. A total of 10 µL of the PCR products from each PCR reaction was electrophoresized 1% (w/v) agarose gel containing 5 mg/ml of Ethidium bromide in a 1X TAE buffer (pH 8.4). 100-bp DNA ladder (Sigma-Aldrich, Bangalore) was used as a Marker. Gel electrophoresis was performed at 100 V, and it was characterized by the Gel Documentation system (Bio Rad, California, USA).

Sequence Analyses

The amplified products were sequenced and obtained sequences were analyzed by basic local alignment search tool (BLAST) served at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/). Species identification was performed using the lowest expected value (E-value) of the BLAST output, and the percentage of homology was analyzed. Multiple sequence alignment was prepared by MEGA X software. Molecular evolutionary relationships through neighbor-joining method and bootstrap values were derived by MEGA X software.

Results and Discussion

The nematode distribution pattern was assessed from 30 household gardens of *Musa* in the Namakkal district of Tamil Nadu (Fig.1). The nematode infestation was identified by the signs and symptoms observed on the roots of the banana (Fig.2). The most frequently occurring plant-parasitic nematodes associated with banana were *Pratylenchus coffeae*, *Helicotylenchus multicinctus*, *Meloidogyne incognita*, *Rotylenchus reniformis* and *Radopholus similis* in Tamil Nadu, India. Likewise, four endoparasitic nematodes species *viz*., *H. multicinctus*, *R. reniformis*, *R. similis*, and *P. coffeae* were reported by Roy *et al*. (2014) [33] in Kerala. Tiwari *et al*. (2000) [42] also reported that all the endoparasitic nematodes occurred in cent percent at Kerala except *P. coffeae*. These findings corroborate with our results revealed that *H. multicinctus* was widely distributed and causes severe infection when compared to *P. coffeae* and *M. incognita*.

Fig 2: Signs and symptoms of *H. multicinctus* nematodes causing reddish brown and necrotic lesions on banana roots.

The spiral nematode, *H. multicinctus* was recorded first time in the Namakkal district in *Musa* and it might be due to moderate temperature, average rainfall, and dry soil which is more suitable for increasing the nematode population. These findings agree with those of Gowen *et al*. (2005) [14, 15], who reported that *H. multicinctus* is often the major parasitic nematode, on bananas where temperature and rainfall conditions are optimum for the crop. On the contrary, Nath *et al*. (1998) [26, 27] suggested that nematode populations were increased when the low temperature and dry soil conditions in December due to almost all the entire soil population migrated into the roots. This result indicates that the environmental factors which influence the soil population themselves, not in root population. According to that the population of *H. multicinctus* was higher depends on its host.

The morphological structure of the spiral nematode, *Helicotylenchus spp*. lip region is hemispherical with slightly offset. The stylet region was well developed and stylet knobs are prominent, wide, appearing anteriorly flattened or concave. Although, nematode excretory pore close to pharyngo-intestinal junction and vulva a depressed transverse slit at 70% of the body length (Fig.3). Similar observations of the nematode morphology were reported by Riascos-Ortiz *et al*. (2020) [31], who found that the nematodes having a hemispherical, stylet knobs are flattened anteriorly and rounded posteriorly, a rounded tail and functional spermatheca which is present in male. This Namakkal population of *Helicotylenchus spp*. was identified as *H. multicinctus* according to their morphological features which are greatly described by Subbotin *et al*. (2011) [39, 40]. Khan *et al*. (2016) [18] reported that the morphology and morphometrics of *H. multicinctus* from nematode population in banana from India showed minor variations in stylet knobs, smaller stylet and no variations in lip region. Thus this study further confirms and supports observations of Marias (2009) [24]. Khan *et al*. (2016) [18] and Marais (2001) [24] described that the population of *H. multicinctus* from Gujarat and Union Island showed relatively longer females and males with greater morphometric values. This information will be helpful for understanding variations in natural populations, difference between closely related species and identification of nematodes in species level.

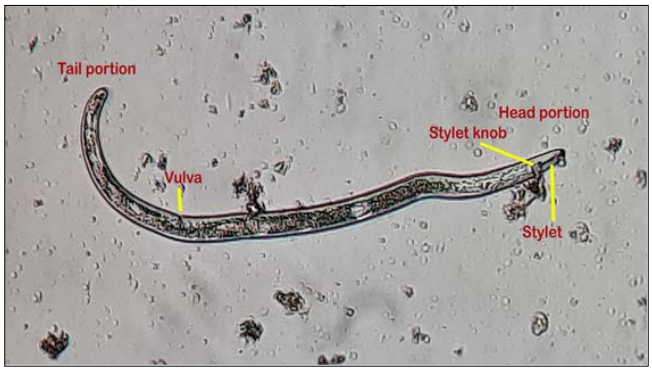

Fig 3: Light microscope images of *H. multicinctus* nematode extracted from roots

The present study revealed that *H. multicinctus* was the most frequently occurring nematodes in 3 different villages of Namakkal district and it was identified based on the morphological studies. The presence of spiral nematode, *H. multicinctus* was found to be 72.7% at Kanavaipatty followed by Siluvampatty (63.6%) and Kondarasampalayam (62.5%) villages in *Musa* (Table.1). Similarly, the maximum infestation of *M. incognita* and *P. coffeae* was found 54.5% and 37.3% at Siluvampatty, respectively. Moreover, the overall population of *H. multicinctus* was much higher than the *M. incognita* and *P. coffeae*, which produces necrotic lesions on the cortical tissues and injuries on the epidermis layer of the banana roots in the specific region (Fig.2). According to Gowen (2000) [14, 15] and Araya and Moens (2005) reported that *Helicotylenchus spp*. is an ecto and endoparasitic nematode that induces necrotic lesions on the root surface. Subbotin *et al*. (2011) [39, 40] also reported that the genus *Helicotylenchus* is distributed globally and is associated with the root system of diverse groups of plants. However, Olaniyi (2014) [18] and Sayed Abdul Rahman *et al*. (2014) [35] reported that *H. multicinctus* and *H. dihystera* causing significant reductions in crop yield in Nigeria. Every indication of these cosmopolitan nematodes is widespread, abundant and has a wide host range in India.

The population density (PD) of *H. multicinctus* was a maximum of 144.75 at Kanavaipatty followed by 137.57 at Siluvampatty villages. However, *M. incognita* was found the maximum PD at Siluvampatty (134.16) followed by Kondarasampalayam (97) and Kanavaipatty (92). Similarly, PD of *P. coffeae* was recorded at 89 and 88.33 of Kondarasampalayam and Siluvampatty villages, respectively. Our results are consistent with earlier reports in Hawaii (Wang and Hooks, 2009) [46] and Brazil (Riascos-Ortiz *et al*., 2020) [31], who suggested that *H. multicinctus* were the most abundant nematodes on banana than those of *Meloidogyne spp*., *Pratylenchus spp*., and *R. similis*. These nematode genera encountered in this study are consistent

with those found earlier in India by Seenivasan (2017) [11, 36, 37] California by Subbotin *et al*. (2011) [39, 40] Ethiopia by Addis *et al*. (2006) [2], Poland by Rybarczyk-Mydłowska *et al*. (2019) [34] and Nigeria by Daramola *et al*. (2020) [10].

For molecular characterization, species-specific primer sets were performed on three isolates of *Helicotylenchus spp*. recovered from three different locations of banana root samples. The primer sets of PRATTW81/AB28 were used to amplify the 18S rDNA sequences of *Helicotylenchus* species amplified a product size of 1031 base pairs (bp) (Fig.4). The amplified sequences generated for the *Helicotylenchus* species were compared with available homologous sequences from the NCBI database, and together with sequences from NCBI, they were used to identify homology between the species. The sequencing and phylogenetic relationship between populations identified in this study were clustered together with other *Helicotylenchus* species from GenBank database.

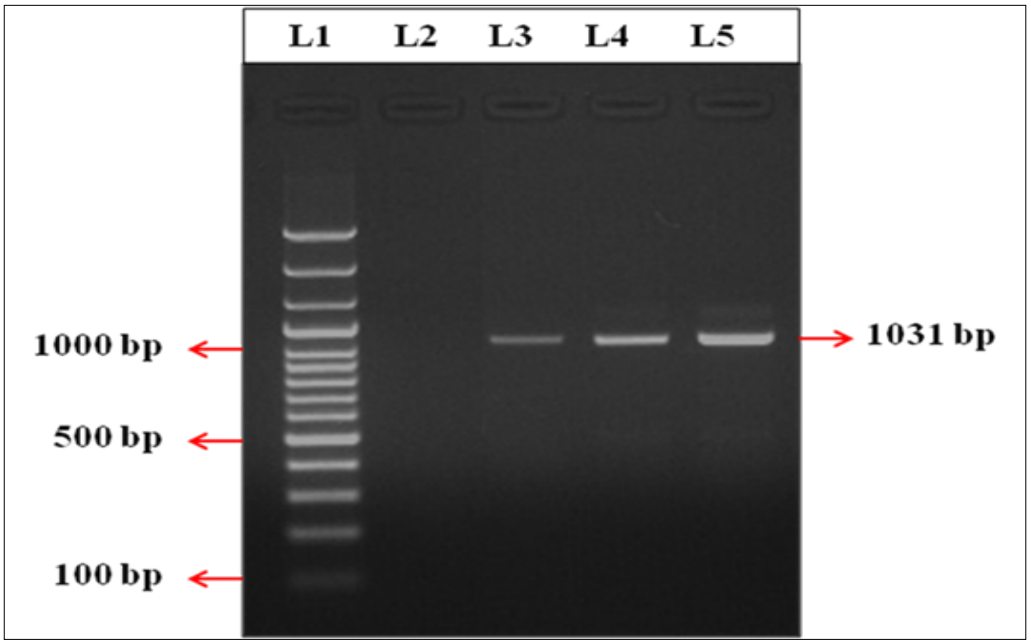

Fig 4: Agarose gel electrophoresis pattern of amplified genomic DNA of *H. multicinctus*. A product of 1031 bp indicates an individual positive for being *H. multicinctus*.
Lanes L1-100 bp DNA ladder, L2-Negative control, L3-HM-01,L4-HM-02, L5-HM03.

Table 1: Community analyses of *H. multicinctus* from 30 locations of banana growing region (Namakkal) in Tamil Nadu.

Sample location	Nematode species	No. of samples containing nematodes	Total no of Nematodes	Frequency distribution (%)	Population Density (PD)	Prominence Value (Pv)
Kondarasampalayam (8)	*P. coffeae*	2	178	25	89	445
	M. incognita	3	291	37.5	97	594
	H. multicinctus	5	424	62.5	84.8	670.40
Siluvampatty (11)	*P. coffeae*	3	265	27.3	88.33	461.52
	M. incognita	6	805	54.5	134.16	990.42
	H. multicinctus	7	963	63.6	137.57	1097.12
Kanavaipatty (11)	*M. incognita*	4	368	36.4	92	555.06
	H.multicinctus	8	1158	72.7	144.75	1234.20

Phylogenetic analysis of 14 deduced nucleotide sequences of *Helicotylenchus* species in GenBank was used to compare the relationship between the identified species. These analyses revealed that the *Helicotylenchus* species were grouped into two major distinct clusters. Cluster I was divided into two different sub-clusters (Sub-cluster I and II) based on the homology between the species (Fig.5). Sub-cluster I revealed all the sequences are genetically identical to *H. dihystera* isolates and sub-cluster II are belonged to *H. multicinctus* isolate in Japan (AB602604.1) with a sequence similarity of 85% homology. Similarly, *H. multicinctus* sequences in Malaysia (FJ460169.1, FJ460170.1, FJ460172.1 and FJ460173.1), Brazil (MT239112.1), Namakkal, India (MZ706973.1) and Spain (MF401447.1) are considered to be various isolates of *H. multicinctus* and was grouped under cluster II with a sequence similarity of more than 90% (Fig.5). It was stated that the *Helicotylenchus* species were morphologically similar but genetically varied among the same species with some alterations in nucleotides. Holterman *et al*. (2009) [17, 45] and Van Megan *et al*. (2009) [44] reported that the phylogenetic analyses using 18S rRNA gene sequences of *Helicotylenchus* was supported as monophyletic and it formed a single clade but with bootstrap supports varying from strong to weak.

The result of the present study suggests that observed genetic diversity of *Helicotylenchus* is significantly higher than has been shown by morphological observations. The molecular analyses may clarify the identification of species within this complex genus. Molecular characterizations of *Helicotylenchus* species using analysis of the 18S rRNA gene sequences are more helpful for the diagnostics and identification of the spiral nematodes.

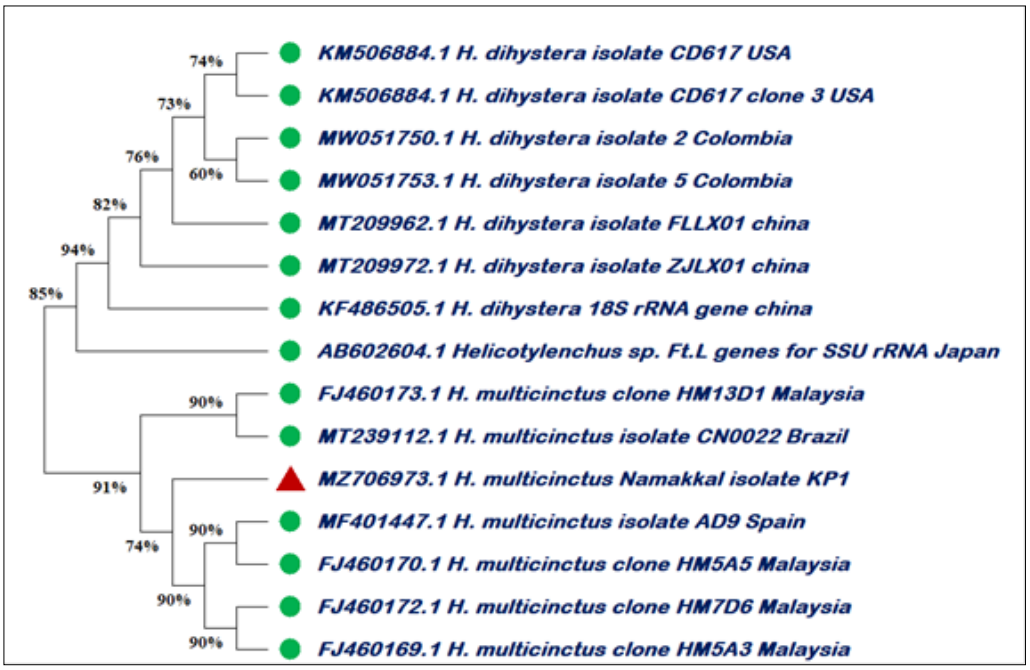

Fig 5: Phylogenetic relationships of the Namakkal isolate of *H. multicinctus* with other *Helicotylenchus* species based on 18S rRNA, reconstructed using neighbour-joining (NJ) method. Nodes show the percentage bootstrap values (out of 100).

Conclusion

H. multicinctus is considered the most perilous species of nematodes in banana and plantains. Based on our results, *H. multicinctus* are widely distributed in the Namakkal region, which may cause severe infestations on banana roots depending upon the population density. According to molecular characterization, the nematode species are further confirmed by conventional PCR, which indicated that the identified species are morphologically similar but genetically varied among the species from different populations. This can be controlled by the defense related enzymes for increasing the egregious production of banana in India.

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