Detection of the Cadang-Cadang Associated RNA in African Oil Palm and Buri Palm

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ABSTRACT

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A low-molecular-weight RNA with the same apparent electrophoretic mobility in 3.3% polyacrylamide gels as the diagnostic viroid-like RNA associated with the cadang-cadang disease of coconut (ccRNA-1) has been detected in the African oil palm (*Elaeis guineensis* Jacq.) and buri palm (*Corypha elata* Roxb.). Palms with the RNA displayed cadang-cadang-like symptoms in the field and came from within the area of distribution of the disease. A ^{3}H-labeled DNA probe complementary to ccRNA-1 was used to show that these two species contained a low-molecular-weight RNA apparently with a nucleotide sequence the same as that of ccRNA-1. African oil palm and buri palm are therefore shown to be naturally infected hosts of cadang-cadang disease. These techniques failed to detect ccRNA-1 in specimens of three other palm species.

Additional key words: nucleic acid extraction and analysis, molecular hybridization assay, viroid.

Although a viroid etiology now seems probable for cadang-cadang disease of coconut palm (*Cocos nucifera* L.) (4, 8,9,12) very little is known of its epidemiology. Natural spread occurs (6,14), but the source of inoculum and mode of transmission is unknown. Host range studies of pathogens are important for identifying possible reservoirs of inoculum, and we are attempting to determine whether species other than coconut palm are natural hosts of cadang-cadang.

Two anomalous RNA species with unique nucleotide sequence and predominantly circular structure are associated with the disease in coconut (8,10,11). One of them (ccRNA-1) is regarded as a diagnostic marker of the disease and although it is readily detectable by gel electrophoretic analysis in extracts of coconut palm (8) this technique has not unequivocally demonstrated the presence of ccRNA-1 in other palm species which we suspect are natural hosts. We report a modification of the extraction technique which allows the detection of a ccRNA-1-like component in the African oil palm and the buri palm. Furthermore, we have used a complementary DNA probe (2,3,11) to detect ccRNA-1-specific nucleotide sequences in those species.

MATERIALS AND METHODS

Source of palm material. Leaflets were collected from the second or third youngest fully opened fronds of palms growing within the area of distribution of cadang-cadang. Species sampled were: *Cocos nucifera* L., *Elaeis guineensis* Jacq., and *Phoenix dactylifera* L. (coconut, African oil palm, and date palm, respectively) from Albay Research Center, Albay, The Philippines; *Corypha elata* Roxb. (buri palm) from San Fernando, Pasacao, Camarines Sur, The Philippines; *Livistonia rotundifolia* Mart. (anahaw palm) from Binogsacan, Guinobatan, Albay; and *Caryota cumingii* Ladd. (fish tail palm) from San Isidro, Santo Domingo, Albay. Samples were either processed immediately after collection or stored overnight at 4 C before processing.

Nucleic acid extraction. Leaflet samples (50–100 gm) were blended in three volumes (w/v) of 0.1 M Na_2SO_3, sometimes containing 1% diethylpyrocarbonate, and the nucleic acids were purified from the crude extracts by one of the methods given below, depending on the analysis to be performed.

Method 1: for gel electrophoretic analysis. Coconut palm leaflet extracts were processed by the polyethylene glycol (PEG) procedure, as previously described (12). The method was modified for oil palm extracts by adding polyvinyl pyrrolidone (insoluble form; Calbiochem, Los Angeles, CA 90063) to 2%, stirring the mixture for 30 min on ice before clarification, extracting with an equal volume of chloroform for 5–15 min before the PEG step. Buri palm extracts were treated as for the oil palm, but after sedimentation of the PEG-insoluble material by centrifugation, the supernatant was treated with 20% ammonium sulfate for 30 min at

TABLE 1. Detection of ccRNA-1 specific nucleotide sequences in nucleic acids of oil and buri palm with leaf spot symptoms resembling those of cadang-cadang disease in coconut palm.

Species	Symptoms and tree no.	Percent of cDNA hybridized[a]
No RNA	...	2
Cocos nucifera (coconut)	Healthy	10
	With cadang-cadang	59
Elaeis guineensis (oil)	Healthy # 1	8
	# 2	13
	Diseased # 1	50
	# 2	56
Corypha elata (buri)	Healthy # 1	6
	# 2	2
	# 3	2
	# 4	2
	Diseased # 1	41
	# 2	58
	# 3	41
	# 4	51

[a]The equivalent of the nucleic acids extracted from 0.5 gm of leaf tissue was hybridized with cDNA for 112 hr. Values have had the 'no RNA' value subtracted, and show the percentage of input ^{3}H-cDNA which forms hybrids.

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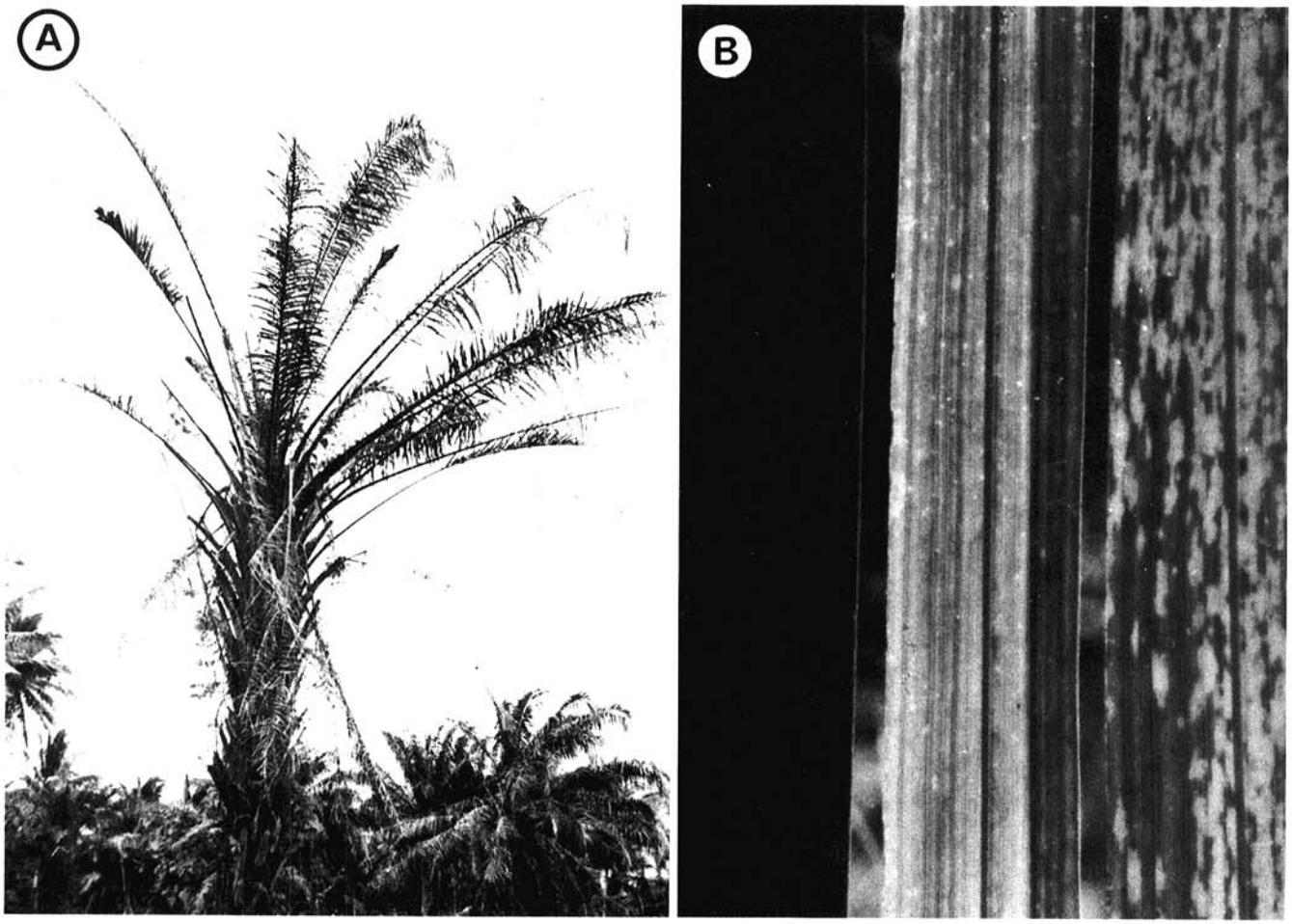

Fig. 1. Diseased oil palm. **A,** Reduced brittle pinnae on short fronds. **B,** Symptoms on leaflets from frond 2 (center) and frond 4 (right) of a diseased palm, compared with normal (left). The small yellow spots enlarge into non-necrotic orange spots as fronds age.

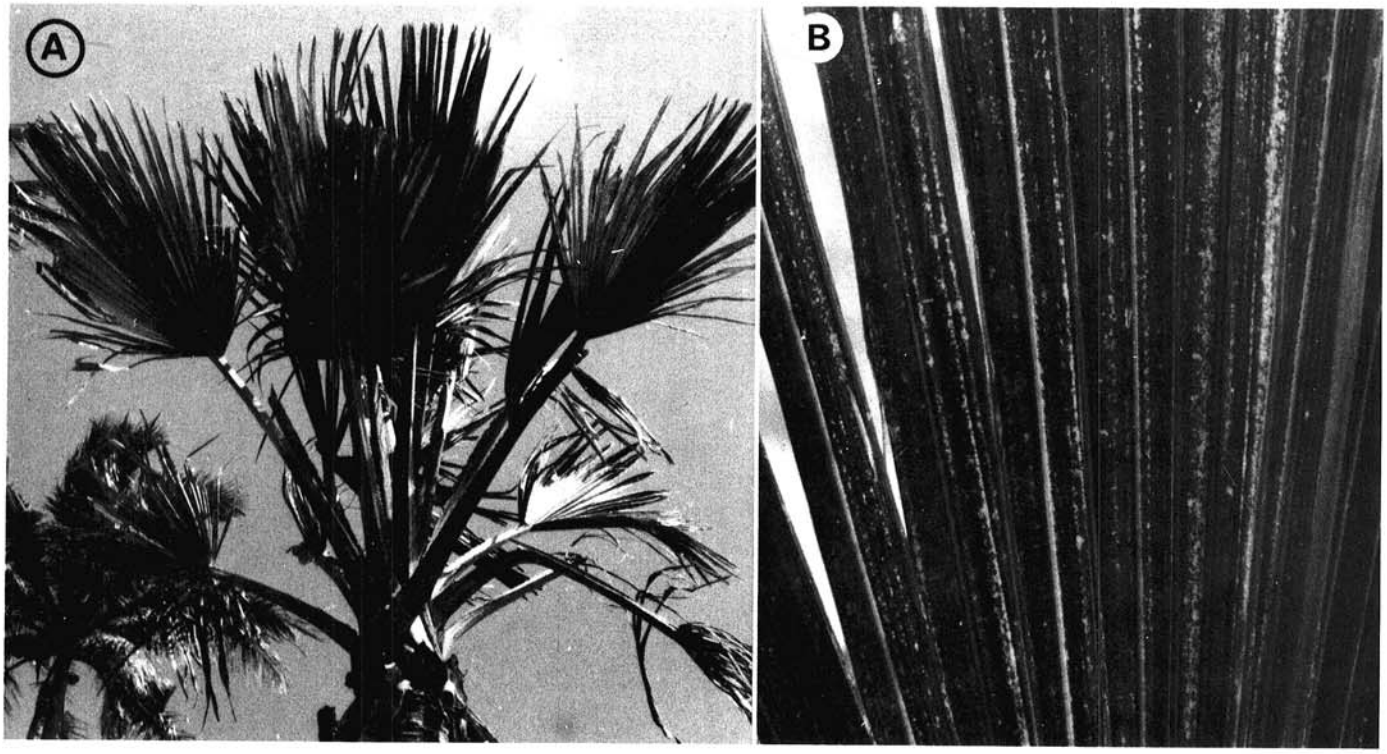

Fig. 2. Diseased buri palm. **A,** Reduced crown and pinnae. **B,** Yellow and orange leaf spotting on frond 6.

0 C. Insoluble material was recovered by centrifugation and extracted as for the coconut and oil palms (12).

Method 2: total nucleic acids for molecular hybridization analysis (MHA). Aliquots (50-ml) of extract were mixed with 0.5 gm of sodium dodecylsulfate (SDS) and stirred with an equal volume of 90% phenol containing 0.1% 8-hydroxyquinoline for 3–5 hr. After centrifugation, the aqueous phase was mixed with three volumes of ethanol to precipitate nucleic acids. The precipitate was collected by centrifugation, resuspended in 10 ml of 1% SDS and 10 ml of phenol and shaken for 10–15 min before separation of the aqueous phase and ethanol precipitation of nucleic acids. The phenol-SDS extraction and ethanol precipitation were repeated once more. Then the total nucleic acids were resuspended in sterile distilled water and mixed with an equal volume of 4 M LiCl. After 18 hr at 4 C, LiCl-soluble RNA was separated by centrifugation at 10,000 *g* for 15 min, and recovered by ethanol precipitation. This was further purified by dissolving in 2 × SSC (SSC buffer = 0.15 M NaCl, 0.015 M Na-citrate) and precipitating the RNAs with one half volume of 1% cetyltrimethylammonium bromide (7). The precipitate was washed and dried.

Gel electrophoresis. Polyacrylamide and agarose gel electrophoreses were as previously described (8,11).

Fractionation of nucleic acids on sucrose density gradients. Nucleic acids (method 2) dissolved in 0.2 ml of sterile distilled water were fractionated by centrifugation on 10–35% linear sucrose density gradients containing 0.1×SSC. Gradients were centrifuged for 16 hr at 37,000 rpm in the Spinco SW41 rotor. Fractions (2 ml) were collected, 50 μg of yeast RNA was added to each, and nucleic acids were precipitated with three volumes of ethanol. Fractions were dissolved in 50 μl of hybridization buffer (0.18 M NaCl, 0.05% SDS, 0.01 M Tris-HCl, pH 7.0 [2]).

Molecular hybridization analysis (MHA). ^{3}H-labelled DNA complementary to ccRNA-1 (cDNA) was synthesized using S_1-nuclease-cleaved, polyadenylated ccRNA-1 as the template for avian myeloblastosis virus reverse transcriptase (11).

Hybridizations were done as previously described (11). Nucleic acids were dissolved in 25–40 μl of hybridization buffer and cDNA (1,000–1,500 cpm) was added. Solutions were covered with paraffin oil, heated at 100 C for 3 min, then incubated at 65 C for periods up to 112 hr. Percentage of hybridization was determined by removing a 25-μl aliquot, and mixing it with 300 μl of the S_1 nuclease digestion medium containing 0.3 M NaCl, 0.03 M sodium acetate buffer, 1 mM $ZnSO_4$, and 5% glycerol, pH 4.6. Aliquots of 150 μl were incubated with two units (13) of S_1 nuclease for 40 min at 45 C and the acid-insoluble radioactivity was compared with that of another 150-μl aliquot not treated with S_1 nuclease (1,2).

Blank values (without RNA) were subtracted and percentage of hybridization was expressed as the ratio of S_1-resistant radioactivity to total cDNA present after hybridization. Homologous hybridization kinetics in this system typically showed a Rot½ of between 1×10^{-3} and 7×10^{-3} mol·sec·L^{-1}, and a maximum of between 40 and 60% of the cDNA hybridized to the homologous RNA (see Table 1).

RESULTS

Symptoms. The two diseased African oil palms sampled for the study were producing no inflorescences, showed broken pinnae, and number and size of fronds were reduced. Large orange spots were seen on pinnae of older fronds, whereas the youngest opened fronds were generally chlorotic,and developed smaller spots (Fig. 1).

The diseased buri palms characteristically showed reduced frond size compared with adjacent normal palms, and large pale green to yellow spots on the third to fourth youngest opened frond (Fig. 2). Because inflorescences are produced only once in the life of the palm, effects on inflorescences could not be used diagnostically as with coconut palm (9). Nevertheless, observations suggest that diseased palms eventually die without producing the typical final "fruiting" stage. Specimens of three other species of palm (*C. cumingii, L. rotundifolia,* and *P. dactylifera*) showed reduced frond size, spotting of pinnae, and unfruitfulness. Spots were yellow, smaller than those seen on oil or buri palms, but similar in size to those seen on diseased coconut palms.

Assay for ccRNA-1 by polyacrylamide gel electrophoresis. A RNA with the same mobility as ccRNA-1 was detected by polyacrylamide gel electrophoretic analysis of the nucleic acids from oil and buri palms (Fig. 3). Analysis of nucleic acids from the other three species of palm extracted by the oil palm technique showed no ccRNA-1-like band.

The presence of a band similar in mobility to ccRNA-1 was not sufficient evidence in itself for the unequivocal identification of cadang-cadang. We therefore adopted MHA to determine whether these palm species contained ccRNA-1 specific nucleotide sequences.

Molecular hybridization analysis. Table 1 shows the percentage homology detected between cDNA and the total nucleic acids extracted from coconut, oil, and buri palms. The oil and buri palms with the symptoms already described showed approximately the maximum percent hybridization attainable with this system. Buri and oil palm therefore contain at least half and probably all of the nucleotide sequences of ccRNA-1. Extracts of *C. cumingii, L. rotundifolia,* and *P. dactylifera* with the unusual spotting contained no detectable ccRNA-1.

To determine the minimum size of the RNA which contained the homologous sequences, nucleic acids were fractionated on sucrose density gradients, and fractions were allowed to hybridize with cDNA. Fig. 4 shows that maximum homology was observed in the

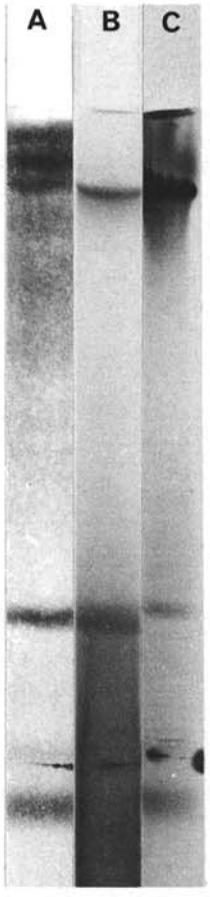

Fig. 3. Polyacrylamide (3.3%) gel electrophoretic analysis of diseased **A,** oil and **B,** buri palm showing bands with mobility similar to that of ccRNA-1 (1) from cadang-cadang diseased **C,** coconut palm. Nucleic acids were extracted from each species by method 1, subjected to electrophoresis in Tris-borate-EDTA buffered gels, and stained with toluidine blue (8,12). The band just ahead of the bromophenol blue tracking dye (marked with ink) is 4S RNA. Nucleic acids from normal palms show no band in the ccRNA-1 region.

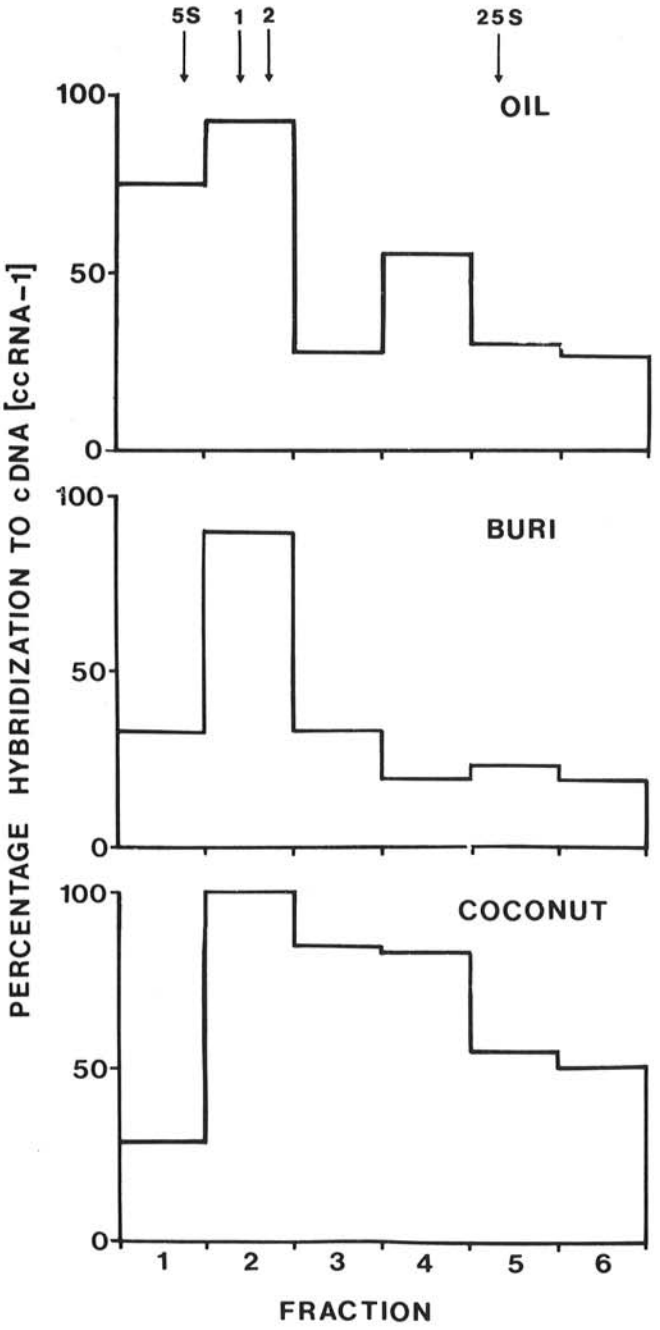

Fig. 4. Molecular hybridization analysis of sucrose density gradient fractions of palm nucleic acids. Diseased oil and buri palm total nucleic acids extracted from 1.75 gm of leaf tissue (method 2) were fractionated simultaneously with cadang-cadang diseased coconut palm marker nucleic acids extracted by method 1. The UV absorbance profile for coconut was as observed previously (8). The positions of ccRNA-1 and -2 and 5S and 25S RNA are shown. No UV-absorbing peaks in the position of ccRNA-1 or -2 were detectable in oil or buri palm extracts. Percentage hybridization has been calculated taking the maximum homologous hybridization value in fraction 2 as 100% (actual value 54%).

low-molecular-weight zone predominantly in the region where ccRNA-1 and -2 would band. Homology lower in the gradient with the coconut nucleic acids is probably due to the incomplete separation of nucleic acids on sucrose gradients and the consequent detection of trace amounts of ccRNA-1 and -2 at the high Rot values used (11). Previous studies (11) in which agarose gel electrophoresis was used indicated that ccRNA-1 and -2 sequences are not represented in higher molecular weight nucleic acids. Rot values could not be calculated for oil and buri palm because the concentrations of ccRNA-1 and -2 were too low for detection by UV absorption, but again homology low in the gradient probably results from incomplete separation of nucleic acid species.

When agarose gel electrophoresis was used to fractionate the nucleic acids of oil and coconut palms, maximum homology was again observed over the ccRNA-1 and -2 regions.

DISCUSSION

Evidence is presented which shows that oil palm and buri palm are naturally infected with cadang-cadang, whereas three other palm species with unexplained leaf spotting contained no detectable ccRNA-1. Symtomatology has led other workers to conclude that oil and buri palm are probably affected by cadang-cadang (5). Our detection of RNA with an electrophoretic mobility resembling that of ccRNA-1 in both oil and buri palm, together with evidence from MHA that diseased specimens of the two palm species contain an RNA of similar size and essentially the same nucleotide sequence as ccRNA-1, prove that the diagnostic ccRNA-1 occurs in these two species.

The diseased specimens of both species were mature palms growing within the area of incidence of cadang-cadang. The susceptibility of oil palm to natural infection in particular indicates the potential hazard that cadang-cadang presents to this economically important crop.

Because we have only tested palms growing in the field, the failure to detect ccRNA-1 in other species does not conclusively show that these palms are not hosts for the cadang-cadang disease. Experimental inoculations and the development of other nucleic acid extraction methods may be necessary to further check their susceptibility by the methods we have used here.

The potential value of MHA for identifying plants infected with viroid-like RNA and for which serological techniques are inappropriate has been demonstrated. This technique allows the detection of specific nucleotide sequences at very low concentration in unfractionated total nucleic acid extracts of diseased plants. It should be useful in extending studies on the host range of the cadang-cadang agent.

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