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Survey of *Vibrio cholerae* O1 and its survival over the winter in marine water of Port of Osaka

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SUMMARY

The survey of *Vibrio cholerae* O1 in marine area was carried out in the Port of Osaka, Japan in 1987–2001, and 51 *V. cholerae* O1 strains were isolated. All strains were identified to be of El Tor biotype, Ogawa serotype and classic Ubon Kappa-phage type, and were cholera toxin (CT)-negative and CT gene-negative. In order to clarify certain ecological aspects of *V. cholerae* O1 in the marine environment of the temperate zone, we performed molecular analysis of the isolated strains using pulsed-field gel electrophoresis (PFGE) with *Not*I and *Sfi*I restriction enzymes. We found the indistinguishable strains by DNA analysis using PFGE with strains passed for 1 year, and also found the closely related strains with that passed for 3 and 12 years. Those results indicated that *V. cholerae* O1 can survive over one winter at least, and that it survives in marine water for a long time by undergoing continuous mutation.

INTRODUCTION

Cholera is an important diarrhoeal disease, caused by the action of a cholera toxin (CT) produced mainly by *Vibrio cholerae* O1 [1]. Pathogenicity of *V. cholerae* O1 is based on the presence or absence of CT production (CT-positive or -negative strains). In the natural environment, CT-positive *V. cholerae* O1 has been generally isolated from environmental waters in cholera-endemic countries [2] and CT-negative *V. cholerae* O1 has been isolated from environmental waters in countries that are not endemic for cholera, such as USA [3] and Japan [4]. However, it introduced CT-positive *V. cholerae* may colonize surface waters in nonendemic countries. McCarthy and Khambaty [5] reported that CT-positive *V. cholerae* O1 was isolated from ballast water in a cargo ship that had navigated through endemic areas. Their findings may indicate that CT-positive *V. cholerae* O1 colonizes marine water in nonendemic areas via ballast water. Marine water was also contaminated with *V. cholerae* O1 after an outbreak of cholera in nonendemic areas [3]. Based on the above findings, we conducted a survey of *V. cholerae* O1 in marine water in Japan.

In temperate climates with significant seasonal changes, it is important to know whether *V. cholerae* survives in winter. Several studies indicated that *V. cholerae* has been isolated from estuarine environments, such as the surface of live copepods in natural water [6] and estuarine sediment [7]. Although *V. cholerae* O1 becomes viable but nonculturable (VNC) at a low temperature [8] and in the natural environment [2], it is not clear whether the bacterium can survive over the winter in the natural marine

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(*a*)

Year	No. of isolates	Month											
		1	2	3	4	5	6	7	8	9	10	11	12
1987	8									—			
1989	30								—	—	—		
1992	7					—			—				
1998	1							—					
2000	3								—	—			
2001	2								—		—		

(*b*)

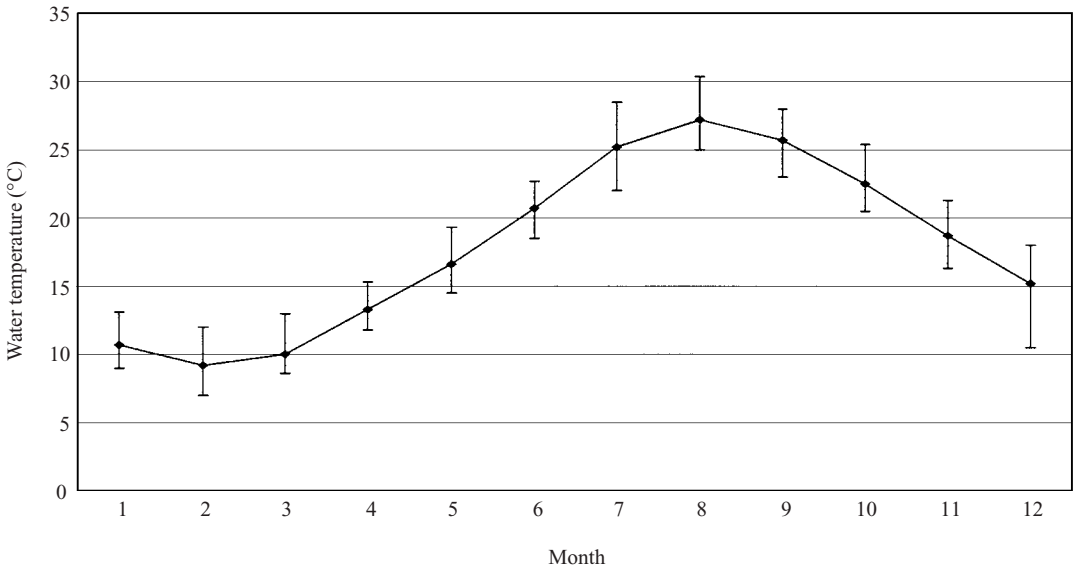

Fig. 1. (*a*) Isolated *V. cholerae* O1 and periods of isolation from the Port of Osaka. *V. cholerae* O1 was isolated from the Port of Osaka in 1987, 1989, 1992, 1998, 2000 and 2001, and these isolation periods are shown. (*b*) Marine water temperature at the time of sampling from the Port of Osaka. The marine water temperatures shown are the averages of water temperatures of approximately 10 sampling points in the Port of Osaka from 1991–1995.

environment. Therefore, in order to determine whether *V. cholerae* O1 can survive over the winter, we examined the phenotype and restriction fragment length polymorphism (RFLP) pattern of the bacterial isolates from the Port of Osaka, Japan over the course of several years.

MATERIALS AND METHODS

Sample collection and isolation of suspect *V. cholerae* O1 colonies

One litre marine water samples were collected monthly in 1987–2001 at a 1 m depth from approximately 10 points in the Port of Osaka and placed in a sterilized bottle. The samples were kept at room temperature and processed within 1 h. The marine water temperature was measured at a 1 m depth of those points. In order to isolate *V. cholerae* O1, 500 ml of each marine water sample was inoculated into 250 ml of 3% w/v polypeptone (pH 9·2; alkaline peptone water; APW). The enrichment cultures in APW were incubated at 37 °C for 6 h, and approximately 10 loopfuls of pellicle were inoculated into 10 ml of non-NaCl alkaline peptone water (polypeptone 1% w/v, pH 8·6) and incubated at 37 °C for 15 h. One loopful of pellicle was streaked on thiosulphate citrate bile-salt sucrose (TCBS; Eiken Chemical Co., Ltd., Tokyo, Japan) agar and incubated at

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37 °C overnight. Suspect colonies on TCBS agar were subjected to the slide agglutination test using a polyvalent O1 rabbit antiserum and monoclonal antibodies with a Vibrio Cholerae Ad Seiken kit (Denka Seiken Co., Ltd., Tokyo, Japan).

Biochemical characterization and Kappa-phage typing of *V. cholerae* O1

The O1-antigen-positive strains were characterized by conventional biochemical tests such as IMViC, amino acid and sugar assimilation tests. Biotype determination was based on haemolysis of sheep erythrocytes, Voges-Proskauer reaction, and sensitivity to classical phage IV (Mukerjee), polymyxin B 50 IU and colistin 50 IU. The strains were identified based on Bergey's Manual of Systematic Bacteriology [9]. The Kappa-phage type was determined by the lysogenicity of the test strains against the phage or the production of Kappa-phage using the method described by Takeya et al. [10, 11] with modifications.

Detection of CT and CT subunit A gene (*ctxA*)

The CT production of all strains was investigated by reversed passive latex agglutination (RPLA) with a CT assay kit (VET-RPLA, Denka Seiken, Tokyo, Japan) according to the manufacturer's instructions after their culture in casamino acid-yeast extract broth (CAYE Medium; Nissui Co., Ltd., Tokyo, Japan) supplemented with 90 μg/ml lincomycin (CAYE-L). The *ctxA* gene was examined by PCR assay using the method of Miyagi et al. [12].

Preparation of DNA for pulsed-field gel electrophoresis (PFGE)

The RFLP pattern analysis for *V. cholerae* O1 was performed by PFGE. Agarose plugs containing DNA for PFGE were prepared using CHEF Bacterial Genomic DNA Plug kits (Bio-Rad Laboratories, Hercules) according to the manufacturer's instructions with modifications. In brief, bacterial cells were grown with rotating at 37 °C in heart infusion broth (Difco, Detroit, USA) to the concentration of McFarland 2. Bacterial cells were then inoculated into agarose blocks, and bacterial genomic DNA was extracted with lysozyme and proteinase K solution included in the kit. Bacterial DNA in agarose plugs was then digested with *Not*I and *Sfi*I restriction enzymes (TaKaRa Shuzo Co., Ltd., Otsu, Japan) at 37 and 50 °C overnight, respectively.

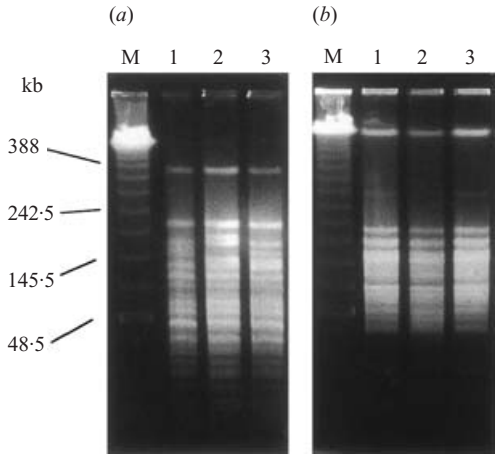

Fig. 2. (*a*) RFLP patterns of *Not*I-digested DNA of representative 12 *V. cholerae* O1 strains isolated from the Port of Osaka in 1989. Twelve *V. cholerae* O1 strains were classified into three groups based on RFLP patterns obtained following *Not*I digestion. Lane M, 48·5 kb lambda ladder size marker; lane 1, OQ102 isolated on 11 July (pattern A). It is the representative strain of 10 strains showing an 'indistinguishable' pattern; lane 2, OQ103 isolated on 13 July (pattern B); lane 3, OQ108 isolated on 14 August (pattern C). (*b*) Representative RFLP patterns of *Sfi*I-digested DNA of 10 strains showing the pattern A by *Not*I restriction. Ten *V. cholerae* O1 strains showing the pattern A following *Not*I restriction (Fig. 2*a*) were cleaved by the *Sfi*I restriction enzyme. Representative RFLP patterns of *Sfi*I-digested DNA are shown. Lane M, 48·5 kb lambda ladder size marker; lane 1, OQ102 isolated on 11 July; lane 2, OQ114 isolated on 4 September; lane 3, OQ125 isolated on 11 September.

PFGE

Electrophoresis was carried out by the contour-clamped homogeneous electric field method on a CHEF-DR II system (Bio-Rad Laboratories, Richmond, Calif.) in gels consisting of 1% agarose (Pulsed Field Certified Agarose; Bio-Rad Laboratories, Hercules) and 0·5 × TBE buffer (44·5 mM Tris, 44·5 mM boric acid, 1 mM EDTA, pH 8·3) at 14 °C for 24 h at 150 V for both *Not*I and *Sfi*I with the following pulse times: *Not*I, 3–40 sec; *Sfi*I, 3–60 sec. The gel was stained with the SYBR Green I nucleic acid gel stain (BMA, Rockland, USA) for 30 min and photographed with a UV transilluminator (302 nm). RFLP patterns were visually analysed and decided based on the criteria for bacterial strain typing of Tenover et al. [13].

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Table 1. *Fragment differences of* Not*I and* Sfi*I-RFLP pattern of* V. cholerae *O1 strains used in this study*

a. Difference of Not I - RFLP

Year of isolation	Strains											
1989	OQ102 (pattern A)	0										
	OQ103 (pattern B)	4	0									
	OQ108 (pattern C)	2	2	0								
1992	OQ133	6	2	4	0							
	OQ134	4	0	2	2	0						
	OQ137	4	0	2	2	0	0					
	OQ138	4	0	2	2	0	0	0				
	OQ135	4	0	2	2	0	0	0	0			
	OQ136	4	0	2	2	0	0	0	0	0		
2000	OQ140	2	6	4	NC	NC	NC	NC	NC	NC	0	
2001	OQ143	2	6	4	NC	NC	NC	NC	NC	NC	0	0
	Strains	OQ102 (A)	OQ103 (B)	OQ108 (C)	OQ133	OQ134	OQ137	OQ138	OQ135	OQ136	OQ140	OQ143

b. Difference of Sfi I - RFLP

Year of isolation	Strains											
1989	OQ102 (pattern A)	0										
	OQ103 (pattern B)	NC	0									
	OQ114	0	NC	0								
	OQ125	0	NC	0	0							
1992	OQ134	NC	4	NC	NC	0						
	OQ137	NC	3	NC	NC	3	0					
	OQ138	NC	4	NC	NC	5	2	0				
	OQ135	NC	3	NC	NC	3	0	2	0			
	OQ136	NC	5	NC	NC	6	3	3	3	0		
2000	OQ140	2	NC	NC	NC	NC	NC	NC	NC	NC	0	
2001	OQ143	2	NC	NC	NC	NC	NC	NC	NC	NC	0	0
	Strains	OQ102 (A)	OQ103 (B)	OQ114	OQ125	OQ134	OQ137	OQ138	OQ135	OQ136	OQ140	OQ143

The category with criteria [13] was decided by typical numbers of fragment differences compared with RFLP pattern as follows: indistinguishable, 0; closely related, 2–3; possibly related, 4–6; different, $\geqslant 7$. 'NC' could not compare because they were not carried out on the same gel.

RESULTS

A total of 51 *V. cholerae* O1 strains were isolated from marine water of the Port of Osaka in 1987, 1989, 1992, 1998, 2000 and 2001 (Fig. 1*a*). All isolates were identified to be of El Tor biotype, Ogawa serotype, and classic Ubon Kappa-phage type. All of the isolates were determined to be negative for CT production by RPLA and for the *ctxA* gene by PCR. The

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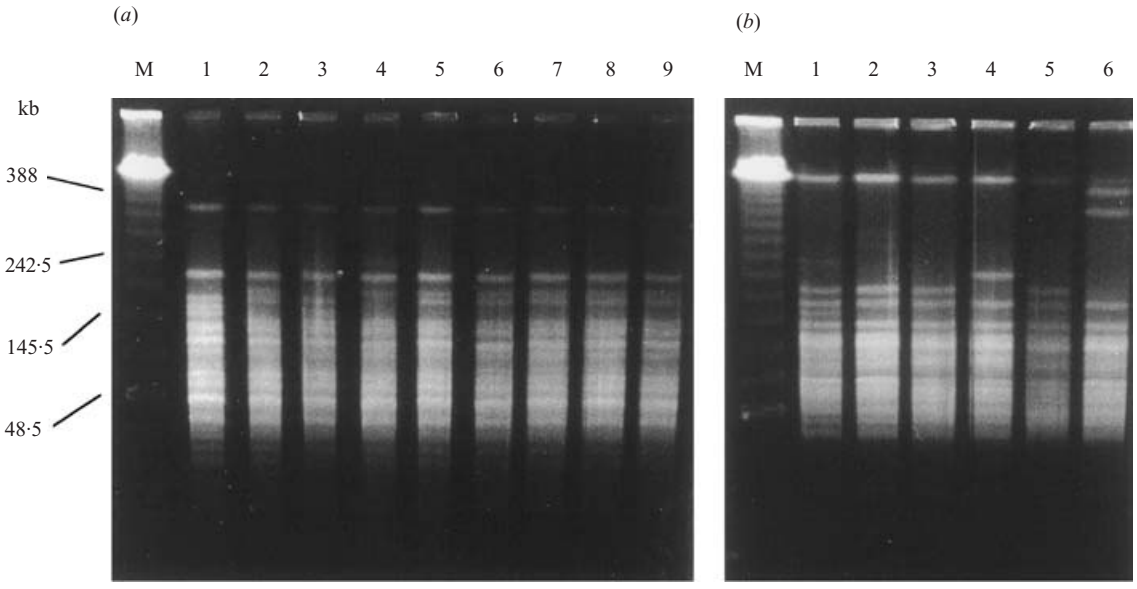

Fig. 3. (*a*) Comparison of RFLP patterns among the three groups of *V. cholerae* O1 strains isolated in 1989 and six strains isolated in 1992. The NotI-RFLP patterns of the three groups of *V. cholerae* O1 isolated in 1989 were compared with those of six *V. cholerae* O1 strains isolated from same area in 1992 using *Not*I restriction enzyme. Lane M, 48·5 kb lambda ladder size marker; lane 1, OQ102 isolated in 1989 (pattern A); lane 2, OQ103 isolated in 1989 (pattern B); lane 3, OQ108 isolated in 1989 (pattern C); lane 4–9, OQ133, OQ134, OQ137, OQ138, OQ135 and OQ136 isolated from the Port of Osaka in 1992, respectively. (*b*) Comparison of the RFLP pattern of *Sfi*I-digested DNA among strains isolated in 1989 and 1992. Six *V. cholerae* O1 strains isolated in 1989 and 1992 show the 'indistinguishable' RFLP pattern determined using the *Not*I restriction enzyme with Figure 3*a* and they were cleaved by the *Sfi*I restriction enzyme, and the RFLP patterns of *Sfi*I-digested DNA are shown. Lane M, 48·5 kb lambda ladder size marker; lane 1, OQ103 isolated in 1989; lane 2–6, OQ134, OQ137, OQ138, OQ135 and OQ136 isolated in 1992, respectively.

isolates were detected from July to October except for one strain that was detected in April 1992 (Fig. 1*a*). The marine water temperature was 22–27 °C on the average when the samples were collected from the Port of Osaka (Fig. 1*b*). *V. cholerae* O1 was not isolated at a low water temperature in autumn and winter.

In order to confirm how many clones exist in 1 year, 12 strains isolated from the Port of Osaka from July to September in 1989 were investigated by PFGE. The strains were classified into three groups based on the patterns of *Not*I restriction (Fig. 2*a*; Table 1*a*). The groups were tentatively named as follows; pattern A for 10 strains including OQ102, pattern B for one strain (OQ103), and pattern C for the remaining strain (OQ108). The 10 strains with the pattern A by *Not*I restriction were further examined using the *Sfi*I restriction enzyme and were determined to be 'indistinguishable' (Fig. 2*b*; Table 1*b*) according to the criteria of Tenover et al. [13]. These data indicate that the clones of pattern A predominantly existed in the port in 1989 and a few minor clones of pattern B and C also existed.

In order to clarify whether *V. cholerae* O1 can survive over the winter, NotI-RFLP patterns of six strains isolated from the same area in 1992 were compared with those of pattern A, B and C. The RFLP pattern of five of the six strains (Fig. 3*a*, lanes 5–9; Table 1*a*) was determined to be 'indistinguishable' with the pattern B (Fig. 3*a*, lane 2; Table 1*a*). To confirm whether these isolates are of the same clone, the SfiI-RFLP pattern of the five strains were compared with that of the pattern B. The SfiI-RFLP patterns of two strains (Fig. 3*b*, lanes 3 and 5; Table 1*b*) showed a pattern that is 'closely related' with that of pattern B clone (Fig. 3*b*, lane 1; Table 1*b*), and the other strains showed 'possibly related' patterns (Fig. 3*b*, lanes 2, 4 and 6; Table 1*b*) according to the criteria of Tenover et al. [13]. These data may indicate that the pattern B clone survived over three winters by undergoing some mutations. To investigate the 1 year survival of *V. cholerae* O1, two isolates collected in 2000 and 2001 were examined for their RFLP patterns based on digestions with *Not*I and *Sfi*I. The patterns of these isolates were completely the same following the digestions (Fig. 4*a*,

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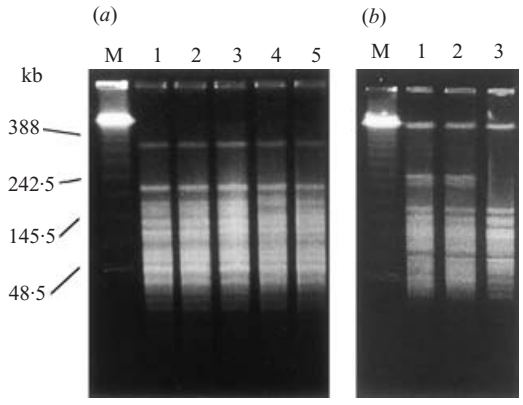

Fig. 4. (*a*) RFLP pattern of *Not*I-digested DNA of two strains isolated in 2000 and 2001. Two *V. cholerae* O1 strains isolated from the Port of Osaka in 2000 and 2001 were cleaved by the *Not*I restriction enzyme, and compared with the three groups of strains isolated in 1989 based on RFLP patterns. Lane M, 48·5 kb lambda ladder size marker; lane 1, OQ140 isolated in 2000; lane 2, OQ143 isolated in 2001; lane 3–5, OQ102 (pattern A), OQ103 (pattern B) and OQ108 (pattern C) isolated in 1992, respectively. (*b*) RFLP patterns of *Sfi*I-digested DNA of strains isolated in 2000, 2001 and 1989. Two *V. cholerae* O1 strains isolated from the Port of Osaka in 2000 and 2001 and one strain with the pattern A isolated from this port in 1989 were further cleaved by the *Sfi*I restriction enzyme. Lane M, 48·5 kb lambda ladder size marker; lane 1, OQ140 isolated in 2000; lane 2, OQ143 isolated in 2001; lane 3, OQ102 isolated in 1989.

lanes 1 and 2; Fig. 4*b*, lanes 1 and 2; Table 1). In addition, strains isolated in 2000 and 2001 were determined to be 'closely related' to the pattern A clone in terms of the RFLP patterns of both the *Not*I and *Sfi*I restriction enzymes (Fig. 4*a*, lane 3; Fig. 4*b*, lane 3; Table 1). These data may indicate that a clone of *V. cholerae* O1 survived over 12 years by undergoing minor mutations.

DISCUSSION

The survey carried out by Minami et al. [4] showed that *V. cholerae* O1 isolated from natural water in Osaka did not possess the CT gene and were of the classic Ubon type as determined by Kappa-phage typing. In this study, we confirmed that the 51 *V. cholerae* O1 isolates collected in 1987–2001 are of the classic Ubon Kappa-phage type, El Tor biotype and Ogawa serotype, and are CT-negative and CT-gene-negative. We demonstrated the survival of one *V. cholerae* O1 clone over one winter with no change in RFLP pattern and of another clone over 12 years with minor changes in RFLP pattern, suggesting minor mutations of their genome. These results suggest that *V. cholerae* O1 is able to survive for a long time in marine waters of the temperate zone such as Japan.

In this study, we did not explore how the bacterium survives over the winter. However, *V. cholerae* O1 has been isolated from the surface of copepods [6], shellfish [14], prawn [15] and estuarine sediments [7]. Furthermore, *V. cholerae* O1 becomes VNC at a low temperature [8] and in the natural environment [2], and the VNC *V. cholerae* can multiply and maintain its progeny in the mucilaginous sheath of the cyanobacterium, *Anabaena* sp. [16]. These previous reports may explain how the bacterium survives winter.

The CT gene has been found to be encoded by a filamentous bacteriophage called cholera-toxin phage (CTXΦ), which exists within *V. cholerae*, and CTXΦ can either replicate as a plasmid or integrate into the *V. cholerae* chromosome [17]. Taylor [18] suggested that the product of the *V. cholerae* pathogenicity island phage (VPIΦ) is the receptor of CTXΦ, and VPIΦ is transferred between *V. cholerae* strains [19]. In fact, the CT genes of both *V. cholerae* O139 and El Tor *V. cholerae* O1 have the same sequence [20]. Although isolates in this study were all CT-gene-negative and therefore nontoxigenic, the gene may be transferred to an existing bacterium in this port through CTXΦ and VPIΦ.

Water pollution with *V. cholerae* O1 in ports at nonendemic area may be caused, in general, by such as drains from contaminated river after an outbreak of cholera [3] and/or ballast water of a cargo ship travelling from endemic areas to nonendemic areas [5]. It was further suggested that *V. cholerae* O1 can survive in marine water for a long time by undergoing continuous mutation in this study. Thus, because of the potential for introduction and proliferation of CT-positive strains, it is reasonable to conduct periodic surveys for *V. cholerae* O1 in international ports, even in the temperate zone.

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