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Title

Silane primers rather than heat treatment contribute to adhesive bonding between tri-n-butylborane resin and a machinable leucite-reinforced ceramic

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Sakai, Miyuki; Taira, Yohsuke; Sawase, Takashi

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Imported Dogs as Possible Vehicles of *Vibrio Cholerae* O1 Causing Cholera Outbreaks in Northern Vietnam

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**Abstract:** Strains of *V. cholerae* O1 were isolated from the sewage and a pond near the first patient’s house and also from domestic vegetables obtained at a neighboring market. From 24 October 2007 to 25 June 2009, 1,505 cases were confirmed positive for *V. cholerae* O1 (biotype El Tor, serotype Ogawa) in 22 cities and provinces in northern Vietnam. On May 8 and May 12, 2009, epidemic strains of *V. cholerae* O1 were isolated from dogs in slaughter houses in Hanoi and from dogs in cages in Thanh Hoa, respectively. Isolates of *V. cholerae* O1 in Laos and Thailand were found to be the same clone as those isolates from dogs, patients and environmental water samples in northern Vietnam. Although the transmission routes of cholera differed between the northern and southern provinces of Vietnam, the same clonality was observed among isolates from 2007 to 2010.

**Keywords:** Cholera, clonality, multiple drug resistance gene, tetA (class D).

**INTRODUCTION**

Cholera is a form of acute diarrhea caused by *Vibrio cholerae* O1 and O139. People living under poor sanitary conditions are exposed to the threat of cholera infection after consuming contaminated food and water. From 24 October 2007 to 25 June 2009, there were more than 7,000 cases suffering from acute severe, watery diarrhea in 22 cities and provinces in northern Vietnam. Of these cases, more than 1,500 were culture-positive for *V. cholerae* O1 (biotype El Tor, serotype Ogawa) in 22 cities and provinces in northern Vietnam. On May 8 and May 12, 2009, epidemic strains of *V. cholerae* O1 were isolated from dogs in slaughter houses in Hanoi and from dogs in cages in Thanh Hoa, respectively. Isolates of *V. cholerae* O1 in Laos and Thailand were found to be the same clone as those isolates from dogs, patients and environmental water samples in northern Vietnam. Although the transmission routes of cholera differed between the northern and southern provinces of Vietnam, the same clonality was observed among isolates from 2007 to 2010.

**Keywords:** Cholera, clonality, multiple drug resistance gene, tetA (class D).

**MATERIALS AND METHODS**

**Samples for Screening of *V. cholerae* O1**

**Sewage and the other Environmental Water**

Samples (500 ml) were collected in clean plastic bottles and immediately transported to the laboratory at ambient temperature.

**Vegetable**

Vegetables (herbs and raw vegetables) were collected at a market near the patient’s house and homogenized with Bag Mixer400 (Interscience, France).
**Dogs**

Rectal swabs were taken from dogs in slaughter houses in Hanoi and from dog-houses in Thanh Hoa (250 km south of Hanoi) where imported dogs were kept before delivery to slaughter houses in Hanoi. Each rectal swab was kept in a test tube containing 10 ml of alkaline peptone water (pH 8.6) and transported to the laboratory at ambient temperature.

**Knife, Floor, Table, Dog Meat in a Bucket in Dog Slaughter Houses in Hanoi**

Samples were collected from these materials by swabbing the surface with a sterile cotton swab and kept in 10 ml of alkaline peptone water and immediately transported to the laboratory at ambient temperature.

**Human Stools**

Rectal swabs were taken from individuals living with a cholera patient and processed in the same way as dog rectal swabs. In hospitals, stool specimens were collected in plastic containers, and then transported to National Institute of Health and Hygiene (NIHE) without delay.

**Bacteriological Examination**

**Water Samples**

The water sample (450 ml) was mixed with 50 ml of 10x alkaline peptone water in a 1 L flask and incubated overnight at 37 °C without shaking. For enrichment, two successive subcultures were done every 6-8 hr by shaking. One ml of original culture was transferred to 9 ml of fresh alkaline peptone water. A small aliquot of the culture was plated on thiosulfate-citrate-bile salts-sucrose agar (TCBS, Eiken, Tokyo, Japan) and alkaline agar plate (nutrient agar, pH 8.6) at each step (primary, secondary and tertiary plating).

**Vegetables**

After homogenization, 25 ml of the sample was mixed with 225 ml of alkaline peptone water and incubated overnight at 37°C. The enrichment method was applied as in the case of water sample.

**Swab Specimens**

Swab specimens were incubated overnight at 37°C and two successive subcultures for enrichment were performed as above.

Suspected colonies were tested for slide agglutination with *V. cholerae* O1-polyvalent antisera (BIO-RAD, CA, USA) and with monovalent antisera (Seiken, Niigata, Japan) and were also inoculated onto Kligler iron agar, semisolid sulfide indole motility medium, Falkow’s broth containing lysine, and Andrade’s peptone water containing mannitol for preliminary characterization. The organisms were differentiated by biotype on the basis of their ability to cause hemolysis of sheep red blood cells [5], sensitivity to polymyxin B [6], chicken blood cell agglutination [7] and sensitivity to group IV cholera phage [8]. Vibrio strains were stored at -80°C in Luria-Bertani (LB) broth supplemented with 30% glycerol.

**Preparation of Template DNA**

For screening of water sample for *V. cholerae* O1, DNA was extracted by the standard phenol-chloroform method from 10 ml of primary culture before enrichment.

**PCR Amplification**

The primers used in this study are listed in Table 1. PCR was carried out in 0.2 ml microcentrifuge tubes with 24 μl of the PCR mixture containing 1μl each of forward and reverse primers (20 pM/μl) and 1μl (ca. 0.1 μg) of template DNA

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5' to 3')</th>
<th>Amplicon Size (bp), Genes</th>
<th>Source or Reference; Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>101-F</td>
<td>CCT TCG ATC CCC TAA GCA ATA C</td>
<td>779 (txrR)</td>
<td>[9], M21249, AE004168</td>
</tr>
<tr>
<td>837-R</td>
<td>AGG GTT AGC AAC GAT GCG TAA G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTXII</td>
<td>CGG GCA GAT TCT AGA CCT CCT G</td>
<td>564 (ctxA)</td>
<td>[10]</td>
</tr>
<tr>
<td>CTXIII</td>
<td>CGA TGA TCT TGG AGC ATT CCC AC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VCO1F2-1,</td>
<td>GTT TCA CTG AAC AGA TGG G</td>
<td>192 (VCO1)</td>
<td>[11]</td>
</tr>
<tr>
<td>VCO1R2-1,</td>
<td>GGT CAT CTG TAA GTA CAA C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tetA (class D) -F</td>
<td>TCG CTG GTA GCT TCG CTA TT</td>
<td>1,327 (tetA class D)</td>
<td>In this study; AB450045.1</td>
</tr>
<tr>
<td>tetA (class D)- R</td>
<td>GCA ACT CTA ATG CCA CTG TG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>int1- F</td>
<td>ATG GCG TTA TCA GTT AGC TG</td>
<td>798 (int)</td>
<td>[12]; AF099172</td>
</tr>
<tr>
<td>int1- R</td>
<td>CTC TAT GGG CAC TGT CCA CAT TG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ia- F</td>
<td>CAA GGA AAG GTG AAC GAG TC</td>
<td>474 (dfrA1)</td>
<td>[13] ; X00926</td>
</tr>
<tr>
<td>Ia- R</td>
<td>CAC ATG CAG CCT TAT GCC AA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dfrA1-F</td>
<td>CAA GGA AAG GTG AAC GAG TC</td>
<td>1138 (dfrA1)</td>
<td>In this study;AB 485590.1</td>
</tr>
<tr>
<td>dfrA1-R</td>
<td>CAC ATG CAG CCT TAT GCC AA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>int9-F</td>
<td>AGC TGT TGT GAC CAG TAA CA</td>
<td>1,257 (int9)</td>
<td>In this study;AB 485590.1</td>
</tr>
<tr>
<td>int9-R</td>
<td>CTT CTC AGT AAT GTC TCC AC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
using Go-Taq Green Master Mix (Promega, Madison, Wis.). The solution was mixed, centrifuged briefly, and placed in an automated Eppendorf PCR Thermal Cycler (Hamburg, Germany). PCR amplification conditions were as follows: initial denaturation at 94°C for 2 min, and 30 cycles of 1 min-denaturation at 94°C, 1 min-annealing at 60°C, and 1 min-extension at 72°C with a final extension step at 72°C for 7 min at the end of 30 cycles, followed by maintenance at 4°C. PCR products were separated by 2% agarose gel electrophoresis in 1×TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), stained with ethidium bromide (1 μg/ml in water), and visualized under UV light. The primers used in this study are shown in Table 1.

**PFGE**

PFGE was performed as previously described by Albert et al. [14], with some modifications. In brief, several colonies of a test strain were collected by a sterile cotton swab and resuspended in 1 ml of cell suspension buffer (CLS, 100 mM Tris, 100 mM EDTA, pH 8.0). The concentration of the cell suspension was adjusted to an optical density of 0.8-1.0 at 610 nm. An equal volume of bacterial suspension and 2% SeaKem Gold agarose (Bio-Rad, Hercules, Calif) was prepared and dispensed into a plug mold. Bacterial cells were lysed with 25 μl of proteinase K (20 mg/ml) in 5 ml of cell lysis buffer (CLB, 50 mM Tris, 50 mM EDTA, pH 8.0, 1% Sarcosyl) at 54°C for 1 h and washed in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Restriction digestion was performed with 20 U NotI at 37°C for 4 h. The agarose plug was stored in TE buffer at 4°C. The restriction fragments were separated using the contour-clamped homogeneous electric field method on a CHEF-DRIII system (Bio-Rad) and a 1% pulsed-field-certified agarose in 0.5× TBE buffer. Electrophoresis was performed at 6 V/cm at a temperature of 14°C with the following pulse times: 2 to 10 s (13 h) and 20 to 25 s (6 h) at an angle of 120°. The gel was stained with ethidium bromide for 30 min, destained, and photographed on a UV transilluminator. The DNA size standards used were a bacteriophage lambda ladder consisting of concatemers starting at 48.5 kbp and increasing to 1,000 kbp (Bio-Rad) and a salmonella braenderup strain.

**Analysis of Drug-Resistance Gene Clusters**

The genetic organization of the gene cluster in SXT conjugative mobile element was determined essentially by primer walking referring to the sequence of SXT \(^{\text{3MO10}}\) [15]. Amplified DNA was purified before sequencing using a QIAGEN Gel Extraction kit (Qiagen, Hilden, Germany) and the nucleotide sequence was determined using a cycle sequencer with an AmplitaqFS Dye Terminator kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, PerkinElmer, Foster, CA, USA).

**RESULTS**

**Recovery of V. cholerae O1 from Raw Vegetables and Dog Meat in a Market**

Strains of V. cholerae O1 were isolated from raw vegetable and dog meat samples collected on 6 January 2008 at Khuong Trung market in Hanoi. Two water samples from Nhue River (Duong Noi Commune) and one sample from a pond near a patient’s house collected on 4 April 2008 were found to be positive for V. cholerae O1. One water sample collected from a pond in Phu Tho province on 7 April 2008 was found positive for V. cholerae O1.

**Isolation of V. cholerae O1 from Dogs in Slaughter Houses in Duongnoi and Dog Houses in Thanhhoa**

The delivery route of dogs imported from north-eastern provinces of Thailand is shown in Fig. 1. In Duong Noi Commune (Hanoi), 37 samples were collected from 4 slaughter houses on 8 May 2009 and 11 samples were found positive for V. cholerae O1. The positive samples were as follows: 1 rectal swab from a live dog, 1 rectal swab from a slaughtered dog, 2 from knives, 4 from dog-meat, 2 from water samples in the buckets to wash dog-meat, 1 from the floor surface in a slaughter house. On 13 May 2009, 54 samples were collected in dog houses in Thanh Hoa province and 3 samples were found to be positive for V. cholerae O1 (two rectal swabs from dogs and one bowl containing drinking water for dogs). After one week on 20 May 2009, more than 300 rectal swabs from imported dogs were examined at the Centre for Preventational Medicine, Thanhhoa. All samples were negative for V. cholerae O1.

**Screening of the Samples from Dogs and Environmental Water Samples for V. cholerae O1 with Cholera Toxin (ctxA) Gene**

Before the isolation of V. cholerae O1, DNA was extracted from the primary culture of water samples and rectal swabs from dogs and used as template DNA for multiplex PCR using primers for toxR, ctxA and VCO1 genes (Fig. 2). Three water samples and 4 dog rectal swab samples showed positive for V. cholerae O1 with ctxA gene. One environmental water sample (lane 4 in Fig. 2) and a rectal swab from a dog showed the presence of non-O1 vibrios.

**Clonality of Epidemic Strains Isolated between 2007 and 2009 in Vietnam**

The gene tetA (class D) encoding resistance to tetracycline was first identified in V. cholerae O1 strain HN1 isolated in 2007 in Hanoi (accession no: AB450045.1). This new gene may work as a marker for the epidemiological study on cholera. When characterized by multiplex PCR using primers for toxR, ctxA and VCO1 genes (Fig. 2), three water samples and 4 dog rectal swab samples showed positive for V. cholerae O1 with ctxA gene. One environmental water sample (lane 4 in Fig. 2) and a rectal swab from a dog showed the presence of non-O1 vibrios.

**Clonality of Epidemic Strains in Laos, Thailand and Vietnam During 2007 and 2009**

Four strains of V. cholerae O1 (2 isolates from 2007 and 2 isolates from 2008) and 12 DNA extracts of V. cholerae O1 (4 each year from 2007 through 2009) were provided by the Center for Laboratory and Epidemiology, Laos and by National Institute of Health, Thailand. When characterized by multiplex PCR by using primers for dfrA1, tetA (class D) and int9 genes, the same clonality was observed among isolates isolated from different sources between 2007 and 2009 in northern Vietnam (Fig. 3).

**Clonality of Epidemic Strains in Laos, Thailand and Vietnam During 2007 and 2009**

Four strains of V. cholerae O1 (2 isolates from 2007 and 2 isolates from 2008) and 12 DNA extracts of V. cholerae O1 (4 each year from 2007 through 2009) were provided by the Center for Laboratory and Epidemiology, Laos and by National Institute of Health, Thailand. When characterized by multiplex PCR by using primers for dfrA1, tetA (class D) and int9 genes, the same clonality was observed among isolates isolated in Laos and Vietnam (Fig. 4). Furthermore, when characterized by single PCR by using primers for tetA (class D) and dfrA1 genes, the same clonality was observed among isolates in Thailand and Vietnam (Fig. 5). These data suggest that the epidemic clones in Laos, Vietnam and Thailand between 2007 and 2009 are the same.
Fig. (1). The delivery route of dogs imported from north-eastern Thailand.

Fig. (2). Multiplex PCR to detect toxR, O1, and ctxA genes in water, and dog samples in Hanoi and Thanhhoa in May, 2009. Lanes M: 100 bp ladder PC: positive control (strain HN1 isolated in Hanoi in 2007); lanes 1-5: environmental water samples collected from water sources near slaughter houses in Duongnoi (Hanoi); 6, 7: Dog-rectal swabs from Duongnoi; lanes 8-10: dog-rectal swabs from Thanhhoa. Template DNAs were extracted from primary cultures before isolation of V. cholerae O1.
Fig. (3). Detection of \textit{tetA} (class D), \textit{int}, and \textit{dfrA1} genes in \textit{V. cholerae} O1 strains from dogs, slaughterhouses (SH) and its environment in Hanoi in May, 2009. Lane 1: floor in a slaughter house; lane 2: dog (killed), 3: dog (live with diarrhea), lane 4: knife, lane 5: dog (killed), lanes 6, 7: water to wash dog meat; lane 8: dog meat; lane 9: dog (live) lane 10: equipment in a slaughter house lane 11: dog meat, 12: Nhue River (1 km away from SH), PC: positive control (HN1), NC: negative control, 13: cholera patient’s stool (Thanhhoa), M: 1k bp ladder.

Fig. (4). Single PCR to detect antibiotic resistance genes of \textit{V. cholerae} O1 isolates in Laos in 2007-2008. Lanes: M; 100 bp ladder, 1,2, 5,6, 9, 10; 2007- isolates, 3,4,7, 8, 11, 12; 2008-isolates, PC ; positive control (HN1)

Fig. (5). Detection of \textit{tetA} (class D) and \textit{dfrA1} genes in Thailand isolates of \textit{V. cholerae} O1 between 2007 and 2009. Lanes: M; 100 bp marker DNA: PC: positive control (strain HN1); lanes 1-10:Thailand isolates:VC49/50, VC54/50, VC170/50, VC440/50, VC26/51, VC52/51, VC/59/51, VC126/51, VC1/52, VC2/52, VC3/52, VC9/52, respectively.
One V. cholerae O1 isolate from 1995, 2 isolates from 2002 and 2003, 4 isolates from 2004, 3 isolates from 2007 and 2 isolates from 2008 were analyzed by PFGE after NotI restriction enzyme digestion. Of the 14 tested, 9 isolates from 1995 to 2004 produced similar patterns that differed from the isolates from 2007 to 2008 (Fig. 6a). Of the 13 isolates from 2010 in the southern provinces of Vietnam, two patterns, designated A and B were seen (Fig. 6b). Three samples from Angiang province (lanes: 1, 2 and 5 in Fig. 6b) were obtained from Cambodian citizens who came to Angiang for treatment of acute diarrhea. One strain isolated in Nam Dinh (a northern province) in 2009 produced pattern B.

Nucleotide Sequence Accession Number

The DNA sequence described in this article has been deposited in the DDBJ database under accession numbers AB450045.1 and AB485590.1.

DISCUSSION

As hypothesized earlier by Sack (3), dogs from cholera endemic areas caused the transmission of the infection in northern Vietnam. Between 1 January and 31 October 2007, there were 600 cases of cholera spread across 36 provinces in Thailand [16]. In Laos, in December 2007, cholera cases started to be reported in Sekong province with the index case being reported on 19 December. As of 19 January 2008, a total of 362 cases were registered including 3 deaths in 32 villages located in 2 districts of the province: Thateng and Lamam [17]. Acute diarrhea spread to 14 localities in Vietnam’s northern and central regions, affecting 1,907 people from 23 October 2007 to 31 December 2007 [18]. The 14 affected provinces included the capital city, Hanoi, and surrounding provinces. Hanoi had seen the largest number of acute diarrhea cases... At the beginning of the outbreak, no one suspected dogs as the vehicle of cholera transmission. The common food taken by patients was the shrimp paste,
because shrimp paste is always served as a sauce for dog meat.

At the end of December 2007, we collected samples from dog meat, table, floor, knife and water at slaughter houses in Duc Giang Commune, Hoai Duc District, Hanoi, but were unable to detect *V. cholerae* O1. Rectal swabs and the other swabs to collect samples were carried in test tubes with screw cap containing alkaline peptone water, it seemed there was no marked difference between rectal swab and conventional stool culture. Ponds near patients’ houses were easily contaminated with the epidemic strain from human stools. Data from interviews with cholera patients conducted during the first 2 weeks of the epidemics shows that 64-83% of the patients ate dog meat. An investigation of slaughter houses was conducted in Hanoi in May 2009 to collect rectal swabs from dogs, and water samples in buckets used for washing dog meat. We found several samples positive for *V. cholerae* O1. This is the first report of cholera transmission possibly caused by dogs imported from cholera endemic areas. Washing dog meat. We found several samples positive for epidemic strains of *V. cholerae* and *Photobacterium damsalae subsp. piscicida*. Osorio CR, et al. [21], this gene was never reported previously in isolates of *V. cholerae* O1. Therefore we used the tetA (class D) gene as a marker to discriminate strains isolated in Laos, Vietnam and Thailand together with the other genes (dfra K, int, and intI9). In northern Vietnam including Hanoi, fresh dog meat is prepared in Hanoi from dogs imported across the border with Laos. These imported dogs are gathered in dog houses in Thanh Hoa province and then transferred to Hanoi. Since we isolated *V. cholerae* O1 from the rectal swabs of 2 dogs from slaughter houses in Hanoi, we went to Thanh Hoa province to collect samples from dogs and from the environment around dog houses. Two rectal swabs from dogs and one drinking water sample were found to be positive for *V. cholerae* O1. The isolates of *V. cholerae* O1 from patients, imported dogs and environmental water sources collected from 2007 to 2009 were found to be the same clone as those isolates from Laos and Thailand, when judged by PCR and PFGE.

The first isolate of *V. cholerae* O1 in Hanoi (HN1) in 2007 was analyzed genetically to characterize its properties. We found tetA (class D) in the conjugative mobile element (SXT element) which carries multiple drug resistance genes. The gene, tetA (class D) was first reported in a Spanish isolate of *Photobacterium damsalae subsp. piscicida* by Osorio CR, et al. [21], this gene was never reported previously in isolates of *V. cholerae* O1. We therefore used the tetA (class D) gene as a marker to discriminate strains isolated in Laos, Vietnam and Thailand together with the other genes (dfra K, int, and intI9). In northern Vietnam including Hanoi, fresh dog meat is prepared in Hanoi from dogs imported across the border with Laos. These imported dogs are gathered in dog houses in Thanh Hoa province and then transferred to Hanoi. Since we isolated *V. cholerae* O1 from the rectal swabs of 2 dogs from slaughter houses in Hanoi, we went to Thanh Hoa province to collect samples from dogs and from the environment around dog houses. Two rectal swabs from dogs and one drinking water sample were found to be positive for *V. cholerae* O1. The isolates of *V. cholerae* O1 from patients, imported dogs and environmental water sources collected from 2007 to 2009 were found to be the same clone as those isolates from Laos and Thailand, when judged by PCR and PFGE.

The results of PCR for Thailand isolates showed discrepancies because of the template DNA. The template DNA was prepared by the supernatant of heat-treated whole cells. Current epidemic strains in Vietnam seem to be different from those isolated before 2007. Therefore, they were probably introduced into Vietnam in 2007. Although the route of cholera transmission seemed to differ between North and South Vietnam, the same clone was seen among the isolates from 2007 to 2010. A minor difference observed in PFGE profile between isolates in 2010 from the southern provinces may be derived from some mobile elements such as SXT element and integrated filamentous phage genes. Furthermore, currently epidemic El Tor strains in Thailand [19], Laos [22], and Vietnam [23] carry *ctx B^c* (El Tor variant). The results of multiplex PCR and PFGE [19, 22] suggested that the same epidemic clone is spread widely in Southeast Asian countries. The PFGE profile suggested that three Cambodian strains seemed to be the same clone with those isolated in Vietnam. Multilocus sequencing typing (MLST) was reported to have better discriminatory ability for typing *V. cholerae* than does pulse-field gel electrophoresis [24], and multi-virulence locus sequencing typing (MLVST) was reported to be more discriminatory than MLST [25]. Although, we used a new gene (tetA class D) as a specific marker for the epidemiological study on cholera, new methods such as LAMP in rectal swab could improve detection of *V. cholerae* O1.

In Vietnam, people never eat dog meat for 2 or 3 days at the beginning of every lunar month, because they believe that eating dog meat at the beginning of the month is auspicious. This custom reflects the low incidence of acute diarrhea in the first two weeks of the lunar calendar (data not shown). Not all the dogs imported were necessarily infected with *V. cholerae* O1; some dogs in some batches were infected. A review of importation practices of animals from cholera-endemic regions may be required to prevent future transmission.

**CONFLICT OF INTEREST**

The authors have no conflicts of interest to report.

**ACKNOWLEDGEMENT**

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