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A case of malignant melanoma patient complicating pneumonia of *Legionella pneumophila* Sg 1 infection and means to detect *Legionella* from the patient

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Legionella pneumonia is mainly caused by the inhalation of *Legionella* contaminated aerosol, aspiration or wound infection. Patients undergoing a severe immunosuppressive therapy such as organ transplant or cancer therapy are particularly at risk of infection. This study reported a case of *Legionella* pneumonia caused by *Legionella pneumophila* in a man with malignant melanoma. The method for diagnosing *Legionella* pneumonia by standard culture method was elaborate. A rapid diagnostic method derived from a two-step scheme assay with 99.42% accuracy for identification and differentiation of *L. pneumophila* and non- *L. pneumophila* in environmental *Legionella* isolates were also utilized in this study to evaluate whether this method was available for diagnosing *Legionella* infection. To confirm the diagnosis result of this rapid diagnostic method, sequencing of the bacteria in patient's sputum partial gene was also carried out. The diagnosis result of this rapid diagnostic method was consistent with the culture method which indicated that it was effective in diagnosing *L. pneumophila* infection.

Key words: *Legionella pneumophila*, malignant melanoma, pneumonia.

INTRODUCTION

Legionella pneumophila (*L. pneumophila*) is consistently reported among the most commonly identified pathogens in community-acquired and nosocomial pneumonias (Fields et al., 2002). Approximately, 24 different pathogenic species have been reported, with *L. pneumophila* accounting for more than 80% of human infections (Newton et al., 2010). The most commonly isolated *Legionella* spp. are *L. pneumophila* (85%), *Legionella longbeachae* (3.2%), *Legionella bozemanii* (2.4%), *Legionella micdadei*, *Legionella dumoffii* and *Legionella feeleii* (2.2% combined). *L. longbeachae* is more common in Australia (30.4%), where the infection has been associated with exposure to potting mixes (Kümpers et al., 2008). In addition to *L. pneumophila*, 20 *Legionella*

species have been documented as human pathogens on the basis of their isolation from clinical material (Burke et al., 2009), but they occur at very low frequencies (Siegel et al., 2010). The clinical features of pneumonia caused by *Legionella* spp. are diverse. Early symptoms include fever, malaise, myalgia, anorexia and headache. Cough is only slightly productive. During the course of *Legionella* pneumonia, fever is always up to 40°C, and stupor, respiratory or even multi-organ failure may develop (Stout and Yu, 1997). Elevation of creatine kinase and diarrhea have been reported as disease-specific symptoms for legionellosis in a comparative study (Sopena et al., 1998).

Legionellosis has been historically referred as an 'atypical' pneumonia, based on its clinical presentation and the

notion that chest radiographic findings are neither lobar nor consolidating, as seen in 'classic' pyogenic pneumonia (Winn, 1988). Many risk factors were associated with *Legionella* infection. These factors included immunosuppression especially those who suffered cancer and experienced chemotherapy and radiotherapy (Winn, 1988). Culture is gold standard for detection of *Legionella*. Culture isolation from BAL fluid or sputum is considered to be the gold standard for diagnosis of legionellosis (Muder and Yu, 2002), while the process of it is complex and time consuming. Therefore, many methods for detection of *Legionella* appear. These methods include urine antigen detection, direct fluorescent antibody (DFA) and sequence-based genotypic classification scheme such as PCR and real-time PCR (Armengol et al., 1992; Cloud et al., 2000; Massey et al., 2003; Thacker et al., 1992). Most commercially available assays based on PCR target species-specific regions within the 16S and 5S rRNA genes as well as in the mip gene. PCR techniques using ribosomal genes as a target have the potential to provide a rapid identification of several *Legionella* spp. with the use of readily obtainable respiratory tract specimens (Stølhaug and Bergh, 2006; Wilson et al., 2007). Urine antigen detection is most widely used but only be specific for *L. pneumophila* serogroup 1, will miss about 40% of legionellosis cases (Fields et al., 2002; Muder and Yu, 2002; Roig et al., 2002). DFA has a low sensitivity on diagnosis for respiratory samples. Moreover, these two methods cannot detect non-*L. pneumophila* spp.. Thus, rapid identification and differentiation of *L. pneumophila* and non-*L. pneumophila* is of critical importance for the diagnosis.

We have previously reported a two-step scheme for rapid identification and differentiation of *L. pneumophila* and non-*L. pneumophila* species. The scheme was based on our finding that the "ACNGT" base sequence appeared in 178 to 182 bp site of a 226 bp fragment of 16S rRNA gene of *L. pneumophila* while non-*L. pneumophila* strains had a variable sequence in this site. We designed PCR assay to detect genus *Legionella* and HpyCH4III endonucleases restriction analysis which identify the "ACNGT" base sequence to differentiated *L. pneumophila* and non-*L. pneumophila*. Among 242 *Legionella* strains including 42 ATCC strains and 200 environmental isolates, 99.59% *L. pneumophila* and non-*L. pneumophila* strains were correctly identified and differentiated by this scheme which proved favourable accuracy. The study shown that the first-step of the *Legionella* genus specific PCR assay could detect all *Legionella* strains and amplified 226 bp products. If the 226 bp PCR products from the PCR assay could be digested to be 180 and 46 bp, it was *L. pneumophila* and if not it was non-*L. pneumophila*. Total time needed for this scheme was about 4 h. However, there has been no clinical isolate or clinical specimen to test this scheme and there also has been no scheme for rapid identification and differentiation of *L. pneumophila* and non-*L. pneumophila* in clinical specimen.

In this study, we will report a case of malignant melanoma patient complicating pneumonia of *L. pneumophila* infection. We will utilize the traditional culture method and other methods to identify and differentiate *L. pneumophila* and non-*L. pneumophila*. To be compared, the method derived from this two-step scheme will also be used.

Case report

A 51-year-old man was admitted to the emergency room of Nanfang Hospital of Southern Medical University on November 6, 2009 to start his second treatment cycle of Docetaxel and Carboplatin for malignant melanoma. He was initially diagnosed with malignant melanoma in February 2009 at the first affiliated hospital of Kunming Medical College. However, the patient had only been on chemotherapy for 9 months prior to this admission when he began to experience fatigue, anorexia, progressive lymphadenectasis and left pleural effusion. Two months later, he experienced frequent hemoptysis, chest stuffiness, anhelation and weight loss of approximately 5 kg. On admission, his temperature was 36.7°C, heart rate was 88 bpm, blood pressure was 122/62 mmHg and respiratory rate was 18. Physical examination revealed that the patient maintained mild yellow skin and mucosa with lymphadenectasis of left groin. The largest lymph node was about 5 x 6 cm, of which is slightly hard. The patient had no superficial lymph node enlargement. Other physical findings included an 8 cm visible post-operative cicatrix on the left heel, hepatomegaly and legs edema. After hospitalization, the inspection revealed remarkable change in routine blood tests. The patient had mild decrease of leukopenia white blood cell (WBC), count of which was $3.38 \times 10^9/L$ with 82% neutrophils, 13% lymphocytes and 5% monocytes. The patient had mild anemia with 85.0 g/L hemoglobin red blood cell (RBC) count was $3.30 \times 10^{12}/L$, platelet (PLT) was $83 \times 10^9/L$, and elevated C-reactive protein (CRP) 209 mg/L.

Artery blood gas (ABG) analysis confirmed moderate hypoxia and respiratory alkalosis due to hyperventilation (PO_2 9.29 kPa, PCO_2 5.53 kPa, pH 7.090, HCO_3^- 12.00 mM, PO_2 (A) 13.82 kPa, serum K^+ 4.44 mM, Na^+ 130.6 mM, Cl^- 96.4 mM, Ca^{2+} 2.18 mM, Mg^{2+} mM). Liver function of the patient was injured due to moderately elevated liver enzymes (aspartate aminotransferase 95 IU/L, alanine aminotransferase 196 IU/L). Blood biochemical test were performed as follows: total protein 60.0 g/L, albumin 30.9 g/L, globulin 29.1 g/L, glucose 5.8 mM, total bilirubin 88.9 μ M, direct bilirubin 54.6 μ M, indirect bilirubin 34.3 μ M, total bile acid 163.9 μ M, prealbumin 105 mg/L, blood Urea nitrogen 7.7 mM, creatinine 68 μ M and uric acid 617 μ M. The myocardial enzyme Spectrum showed abnormal changes as LDH 4403 IU/L, HBDH 2569 IU/L, CK 70 IU/L, CK-MB 42 IU/L and AST 95 IU/L. Other serum enzyme changes included the following: ALP 860 IU/L, γ -GT 1242 IU/L, CHE 3.3 U/mL and AFU 31.2 IU/L. Chest radiograph revealed multiple nodule photocopying with blur edge and

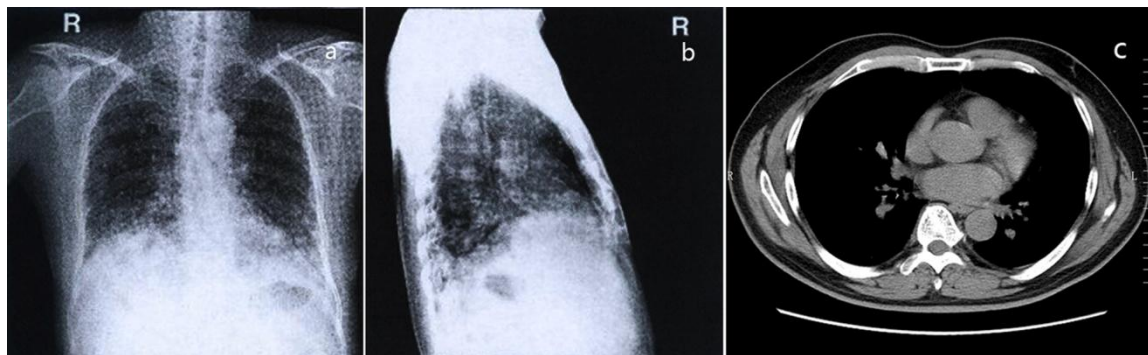


Figure 1. Chest radiograph on admission showing multiple nodules photocopying, edge blur and uniform density of the double lung lower lobe (a, b). Chest-CT additionally revealed multiple millet grain nodules in the double lower lung, which was regarded as the metastatic carcinoma focus (c).

uniform density of the double lung lower lobe, which were regarded as the metastasis of carcinoma. In addition, chest-CT revealed multiple millet grain nodules beneath the double lung as the metastatic carcinoma focus (Figure 1).

MATERIALS AND METHODS

Bacterial culture and bacterial biochemistry tests

Culturing *Legionella* species on BCYE α agar is the gold standard for the diagnosis of legionellosis. Sputum specimen collected from the patient was plated to buffered charcoal yeast extract (BCYE α -DGVPC) agar medium supplemented with AKCF supplement agents which were composed of ACES 10.0 g buffered (*N*-(2-acetamido)-2-aminoethanesulfonic acid, 1.0 g α -ketoglutarate, 0.25 g L-Cysteine-HCl, H₂O 0.4g, Fe₄(P₂O₇).9H₂O in 1000 ml H₂O. Selective antimicrobial agents (DGVPC) included dye mixture, glycin, vancomycin, polymyxin B, cycloheximide were added to the media. Plates were incubated at 37°C in 5% CO₂ with 80% relative humidity. The culture was examined daily and held for 5 days before final reporting of results. Visible colonies were subcultured onto a BCYE α agar and a blood agar plate with 5% sheep blood. Bacteria that grew on the BCYE α agar, but not on the blood agar plate were considered to be *Legionella* spp..

To confirm the species of the bacteria, the isolate was done by biochemistry tests. These tests included nitrate reduction test, urease test, glucose fermentation test, catalase test, hippurate hydrolysis and gelatinase test.

PCR-enzymatic digestion analysis

A *Legionella*-genus specific seminested PCR-enzymatic digestion assay which could detect *L. pneumophila* and non-*L. pneumophila* in the sputum specimen and km 1108 isolate from the patient were used in this study. PCR assay was previously described in our laboratory as a conventional method (Zhan et al., 2010). Genus-specific PCR primers amplified a 386-bp portion of the 16S rRNA gene (base 451 to base 837) of *Legionella*. The sequence of the forward primer (Leg386F) was 5'-AGGGTTGATAGGTTAAGAGC-3'. The reverse primer (Leg386R) was 5'-CCAACAGCTAGTTGACATCG-3'. Another primer was designed to mutate site 195 of the 226-bp fragment which was the upstream sequence in the 386 bp fragment from T to G. The forward primer sequence of Leg226F was the same as Leg386F. The reverse primer Leg226R was 5'-ATTCCACTACCCTCTCCCACTCGAGTCAACC-3'.

Nucleic acids extracted from an equivalent of 0.5 ml digestive sputum (QIAamp DNA Mini kit; Qiagen) were used in PCR assay as

template. After the first step of the PCR assay, the 226 bp product was purified and digested with HpyCH4III for 30 min, results were shown as electrophoresis.

PCR assay

Each PCR reaction included 25 μ L 2 \times Taq PCR MasterMix including polymerase, reaction buffer, deoxynucleoside, 3 mM MgCl₂, 20 mM Tris-HCl (pH 8.3) and 100 mM KCl in a ready-to-use formulation (TIANGEN BIOTECH CO., LTD). Primers, 5 μ L of template and ddH₂O were added for a total volume of 50 μ L. A GeneAmp PCR system (Applied Biosystems) was used for the reaction. The thermal cycling profile consisted of an initial incubation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 30 s and 72°C for 5 min. The PCR product was used as the template for the second round reaction shown as follows:

Seminested PCR

A seminested PCR was performed to enhance the sensitivity for detection of *Legionella*. 5 μ L of PCR product from the first reaction mixture was used as a template for the second PCR reaction, with ampler enclosing a 226-bp fragment of 16S rRNA gene for the genus *Legionella*. These 226-bp fragments form the upstream fragment of the 386-bp fragments. Seminested PCR has the same amplification parameters as the first-step PCR. Each reaction product was electrophoresed on a 2.0% agarose gel with ethidium bromide.

HpyCH4III enzymatic digestion analysis assay

PCR products were purified by TIANquick Midi Purification Kit (TIANGEN BIOTECH CO., LTD, Beijing, China), according to the manufacturer's instructions. The endonuclease of the HpyCH4III utilized in the enzymatic digestion analysis of purified PCR products was from New England Biolabs (NEB-China, Beijing, China). Each enzymatic digestion system included a 10 \times NEB buffer 4, ddH₂O, HpyCH4III endonuclease and 8 μ L of purified PCR product for a total volume of 12 μ L. Each digestion reaction was carried out at 37°C for 45 min. After enzymatic digestion, 6 μ L of each reaction product was used for 2% agarose electrophoresis analysis.

Legionella gene sequencing detection

Genome DNA of km 1108 and bacteria in patient's sputum specimen were extracted. They were assumed as *Legionella* spp or contain *Legionella* spp. Partial 16S rRNA gene and (microphage infec-

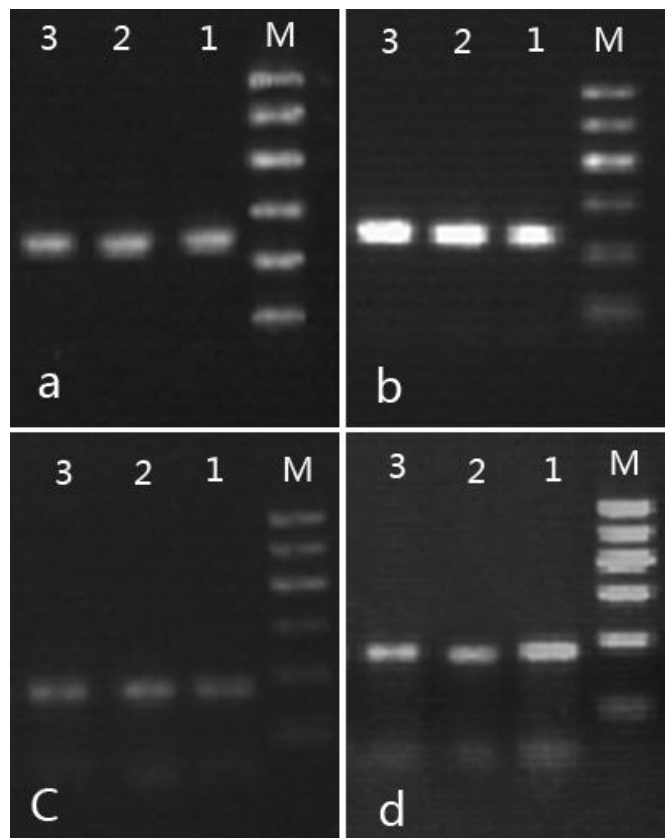


Figure 2. Seminested PCR amplification and enzymatic digestion of 226-bp fragments of 16S rRNA genes. PCR amplification electrophoregram of (a) sputum samples and (b) km 1108 isolate. Enzymatic digestion electrophoregram of (c) sputum samples and (d) km 1108 isolate. ladder 1, *L. pneumophila* Sg1 ATCC-33152; 2, *L. pneumophila* Sg 6 ATCC 33215, 3 (a and c), sputum samples; 3 (b and d), km 1108 isolate; M, 100-bp DNA ladder.

infectivity potentiator) *mip* gene fragment were amplified by PCR using 16S rRNA gene specific primers and *mip* gene specific primers which were reported elsewhere. The PCR products were sent to Invitrogen Trading (Shanghai) Co. Ltd. for sequencing analysis. PCR products were purified with the QIAquick PCR purification kit according to the manufacturer's instructions and were held at -20°C until sequencing. Sequencing was performed using Big Dye chemistry with the ABI Prism 377 Sequencer. Sequence analysis was performed by a BLAST search of the GenBank database (Cloud et al., 2000).

RESULTS

Bacterial culture and bacterial biochemistry tests

For three days culture, the clone of bacteria appeared on the BCYE DGVPC agar and it could not grow on blood plate. This result made us consider it as *Legionella* spp. The results of the biochemistry test of the isolated bacteria were negative for nitrate reduction, urease, glucose fermentation and catalase; positive for hippurate hydrolysis and gelatinase. The results of biochemical tests indi-

cated that this isolate belonged to *L. pneumophila*. Serum agglutination test revealed that it was serogroup 1 of *L. pneumophila*. We named the strain isolate from patient's sputum specimen as km 1108.

PCR assay

The results of seminested PCR detection for the sputum samples and the km 1108 isolate of the patient all showed a 226 bp fragment product, the same as the positive control strains which were *L. pneumophila* Sg1 ATCC-33152 strain and *L. pneumophila* Sg 6 ATCC 33215 strain. This result strongly indicated that the km 1108 isolates and bacteria in patient's sputum samples belonged to *Legionella* spp. (Figure 2a and 2b).

HpyCH4III enzymatic digestion analysis assay

PCR product from the seminested PCR assay were purified by kits and then digested by HpyCH4III endonuclease. Based on the analysis of genus *Legionella* 16S rRNA gene and our previous study, the target 226 bp of PCR products from *L. pneumophila* would be digested to two fragments, one is 46 bp and another is 180 bp. However, the non-*L. pneumophila* would have none of such appearance as *L. pneumophila*. The products of digestion were estimated by agarose electrophoresis. The electrophoresis result of HpyCH4III enzymatic digestion shown that both 180 and 46 bp zones appeared, the digestion analysis results of the km 1108 isolate and bacteria in patient's sputum samples were also the same as the positive control strains which were *L. pneumophila* Sg1 ATCC-33152 strain and *L. pneumophila* Sg 6 ATCC 33215 strain and it proved the bacteria from the patient's sputum specimen and km 1108 isolate were *L. pneumophila* (Figure 2c and 2d).

Legionella gene sequencing

The PCR product of partial 16S rRNA gene and *mip* gene fragment from km 1108 or *Legionella* spp in patient's sputum specimen were sequenced and gene sequence of which were obtained. BLAST search of the obtained gene sequences shown that the 16S rRNA gene sequences of km 1108 and *Legionella* spp in patient's sputum specimen were 100% with the corresponding *L. pneumophila* isolate 16S rRNA gene fragment. Although, the *mip* gene fragment of km 1108 was not 100% with the corresponding *L. pneumophila* isolate *mip* gene fragment, the 99.34% sequence identity shown it was *L. pneumophila* (Table 1). The sequencing results mentioned here indicated the km 1108 isolate and *Legionella* spp in patient's sputum specimen were *L. pneumophila*.

DISCUSSION

In this case report, the patient presented with mild

Table 1. Results of partial 16S rRNA and mip gene sequencing for patient sputum samples and km 1108 isolate.

Gene	Species or sample tested	Gene sequence
16S rRNA	<i>L.pneumophila</i> gb CP001828.1	AGGGTTGATA TAGCTGTTGG (bases 451 to 837)
	Sputum	AGGGTTGATA TAGCTGTTGG (bases 451 to 837)
	km 1108	AGGGTTGATA TAGCTGTTGG (bases 451 to 837)
mip	<i>L.pneumophila</i> gb AF022332.1 AF022332	CTGCAGCTGT.....G ^aG ^bT ^cC ^dGGCCCACGTA (base 4 to 609)
	km 1108	CTGCAGCTGT..... A ^aA ^bC ^cA ^dGGCCCACGTA (base4 to 609)

^abase 212, ^bbase 260, ^cbase 437 and ^dbase 554.

leukopenia, mildly anemic hemoglobin and markedly elevated CRP. Chest radiograph revealed multiple nodules photocopying with blur edge, and uniform density of the double lung low lobe. Chest-CT additionally revealed multiple millet grain nodules beneath the double lung as the metastatic carcinoma focus. By contrast, legionellosis has been historically referred to as an 'atypical' pneumonia based on its clinical presentation and the long-held belief that chest radiographic findings are neither lobar nor consolidating. Three-quarters of patients with *L. pneumophila* pneumonia present with an abnormal chest X-ray. Patchy pneumonic infiltrates, predominantly of the lower lobes and circumscribed or even lobar consolidations are observed, while cavitations are rather uncommon (Tan et al., 2000). Numerous case reports suggest a higher rate of non-*L. pneumophila* among immunocompromised patients than in immunocompetent hosts (McClelland et al., 2004). In an immunocompetent patient, community-acquired pneumonia is caused by *L. longbeachae* (Diederer et al., 2005).

Numerous cases have been reported related to *L. pneumophila* and non-*L. pneumophila* infection around the world, such as perimyocarditis (Burke et al., 2009), community-acquired lung abscess (Girard and Gregson, 2007), legionella endocarditis (Massey et al., 2003), rheumatoid arthritis (Chang et al., 2001), chronic lymphocytic leukemia (Hendrick, 2001; Siegel et al., 2010; Thacker et al., 1992), Myocarditis (Armengol et al., 1992), cancer (Jacobson et al., 2008) and invasive aspergillosis (Saijo et al., 2008). However, the case of malignant melanoma patients complicating pneumonia of *L. pneumophila* Sg 1 infection had not been reported before. In this study, we isolated *Legionella* from patient's sputum specimen using culture method. Take into consideration of patient's clinical symptoms and chest X-ray feature and the patient's clinical background which was long-term chemotherapy for malignant melanoma, the diagnosis of legionella pneumonia is out of question. To be noted, it is the first report of this disease complicating *L. pneumophila* infection. We also utilized a two-step assay derived method which we have previously report as a rapid and accurate means to detect and differentiate *L. pneumophila* and non-*L. pneumophila* to detect the *Legionella* from patient's sputum specimen. Zhan et al. (2010) has reported that this two-step assay utilize 5 base sequence differen-

ces in the 16s rRNA gene between *L. pneumophila* and non-*L. pneumophila* to differentiate *Legionella* genus to different species (Zhan et al., 2010). This two-step assay include a genus specific PCR assay and a species specific enzymatic digestion analysis assay (Zhan et al., 2010).

In this study, to enhance the sensitivity and specificity of PCR assay, we carried out a seminested PCR assay to detect *Legionella* genus from patient. The two-step assay has proved the bacteria from sputum specimen and the isolate belonged to *L. pneumophila*. Time needed for the detection and differentiation of *Legionella* in sputum specimen is about 4 h, while culture method costs 7 days. Although, culture and the after biochemistry tests showed that the bacteria is *L. pneumophila* Sg 1 and the two-step assay could not identify serotype, we still recommend this two-step assay utilized in the suspected patients sputum specimen. It is also the first study that utilizes this rapid two-step assay to identify and differentiate *L. pneumophila* and non-*L. pneumophila* in clinical isolates and clinical specimen. Results of culture method, 16S rRNA gene and mip gene sequencing method all proved the validity of this assay.

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