Development of ELISAs for the Detection of Urogenital Chlamydia trachomatis Infection Targeting the pORF5 Protein[*]

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Abstract

Objective   To prepare antibodies against pORF5 plasmid protein of *Chlamydia trachomatis* and develop double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISAs) for the detection of genital *C. trachomatis* infections.

Methods   The pORF5 protein was expressed in *Escherichia coli* and used to immunize BALB/c mice and New Zealand rabbits to produce monoclonal antibodies (mAbs) and polyclonal antibody (pAb) for DAS-ELISAs. Clinical samples from 186 urogenital infection patients (groups I) and 62 healthy donors (groups II) were detected in parallel by the DAS-ELISAs developed in this study and by IDEIA PCE commercial ELISA.

Results   Two hybridoma cell lines, named 2H4 and 4E6, stably secreting specific mAbs against pORF5 were obtained. The mAb 2H4 was recognized by 32 (17.20%, positive recognition rate) and 25 (13.44%), mAb 2H4 by 0 (0%) and 2 (3.22%) samples from groups I and II, respectively. The sensitivities of mAbs 2H4 and 4E6 were 92.11% and 77.78% and the specificities were 100% and 96.88%, respectively in relation to the IDEIA PCE commercial ELISA. The sensitivities of detection for the DAS-ELISAs were 10 ng/mL (based on 2H4) and 18 ng/mL (based on 4E6).

Conclusion   Two DAS-ELISAs were developed in this study that provided a feasible and effective assay that could be considered alternative tools for the serodiagnosis of *C. trachomatis* infection.

Key words: *Chlamydia trachomatis*; Monoclonal antibody; Polyclonal antibody; pORF5; DAS-ELISA

INTRODUCTION

Chlamydia trachomatis is an obligate intracellular bacterial pathogen consisting of 19 serovars capable of causing different diseases in humans. Infection of human eyes with serovars A to C can lead to preventable blindness[1], whereas urogenital tract infection with serovars D to K often results in pelvic inflammatory disease, ectopic pregnancy, and tubal infertility[2-3].

Serovars L1 to L3 often invade lymphatic tissue, potentially resulting in systematic infection[4]. In addition, Chlamydial infections may also contribute to increased risk for Human Immunodeficiency Virus (HIV) infection and the development of invasive cervical cancer[5-7]. The majority of genital Chlamydial infections, however, are asymptomatic or produce mild symptoms, and infected individuals can also serve as important reservoirs for new infections. Thus, early detection using a rapid and sensitive assay that can direct treatment of *C. trachomatis* infections may reduce complications resulting from untreated infections.

Urogenital infections by *C. trachomatis* in humans can be confirmed through several approaches, such as isolation in cell culture, microimmunofluorescence (MIF), and nucleic acid detection. Isolation of *C. trachomatis* may provide a more accurate diagnosis, but culture is difficult, time-consuming, and relatively insensitive. MIF has long been considered the gold standard for the diagnosis of Chlamydial infections, but this method is not readily adaptable for routine use in diagnostic clinical laboratories because of the lack of standardization and cross-reactivity with other Chlamydial species[8-9]. Nucleic acid amplification tests provide promising sensitivity and rapid diagnosis, but these methods have some disadvantages, such as possible contamination, and the need for special equipment[10]. In this case, enzyme-linked immunosorbent assays (ELISAs) have been developed to facilitate the diagnosis of *C. trachomatis*; however, this method largely depends on the selection of target antigens[11-14]. The pORF5 protein, expressed by open reading frame 5 of the Chlamydial plasmid and secreted into the host cell cytosol, is a promising *C. trachomatis*-specific immunogen[15-18], suggesting that it may be a suitable candidate for Chlamydial detection. Indeed, ELISA using pORF5 protein has been previously reported for the serodiagnosis of *C. trachomatis* infections[19]; however, this assay was shown to be unreliable[20] because the titer of antibodies to *C. trachomatis* early in infection often does not increase significantly. IgG antibodies can last for a long period once produced, but antigen detection based on serological methods provides a rapid immunoassay that has shown its value for the diagnosis of Chlamydial infectious diseases.

The aim of this study was to establish double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISAs) based on specific antibodies for the pORF5 protein for antigen detection. The assay developed in this study will facilitate the development of an effective serological test for diagnosis of *C. trachomatis* infections.

MATERIALS AND METHODS

Patients and Clinical Specimens

Clinical samples used in this study were collected from patients at the hospital in Hengyang, China, during August 2008 - June 2009. The study subjects were categorized into two groups: Group I included 186 patients (80 men, 106 women, between the ages of 15-60 years; median, 22 years) with urogenital infections, diagnosed on the basis of an infectious syndrome accompanied by pelvic inflammatory disease, urethritis or prostatitis; Group II consisted of 62 healthy donors (age range, 22-65 years; median, 30 years) determined to not have *C. trachomatis* infection, based on negative *C. trachomatis* MIF analysis. All urethral or endocervical specimens were collected according to standard procedures and stored at -80 °C.

Preparation of pORF5 Fusion Protein

The open reading frame encoding pORF5 plasmid protein (GenBank accession no. NC_001372.1) was amplified from *C. trachomatis* serovar D and cloned into the pGEX-6p-1 prokaryotic expression vector (GE Healthcare Biosciences, Pittsburgh, PA, USA). The recombinant prokaryotic expression vector, designated pGEX-6p/pORF5, was used to transform *E. coli* XL1 Blue cell and was induced to express pORF5 fusion protein induced with isopropyl-β-D-thiogalactoside (IPTG, Invitrogen, Carlsbad, CA, USA). The fusion protein was purified using glutathione-conjugated agarose beads (GE Healthcare Biosciences) as previously described[15,21-22].

Production of Monoclonal Antibodies (mAbs) Against pORF5 Protein

Pathogen-free female BALB/c mice (6-8 weeks old) were first immunized subcutaneously with 100 μg of purified pORF5 protein emulsified with Freund's complete adjuvant (CFA; Sigma, St. Louis, MO, USA) and boosted twice with 50 μg of pORF5 protein in Freund's incomplete adjuvant (IFA; Sigma) at 2-week intervals. Three days before sacrifice, each mouse was administered with 50 μg of immunogen in sterile phosphate-buffered saline (PBS), the whole

spleen was aseptically isolated, and splenocytes were fused with SP2/0 myeloma cells in 50% polyethylene glycol 4000 (PEG 4000; Merck, Darmstadt, Germany). Hybridoma cell lines secreting antibodies were screened and subcloned by limiting dilution at least three times. The culture supernatants mAbs were collected and purified using an affinity chromatography column (GE Healthcare Biosciences).

Generation and Purification of Polyclonal Antibody (pAb) Against pORF5 Protein

For pORF5 polyclonal antibody production, three New Zealand rabbits were subcutaneously administered 1 mg purified pORF5 protein in 0.5 mL of PBS emulsified with an equal volume of CFA or IFA for a total of three immunizations. Ten days after the final boost, all rabbits were exsanguinated by heart puncture under general anesthesia. Sera were separated from blood cells by centrifugation at 500 ×g for 15 min at 4 °C. This crude serum was purified using saturated ammonium sulfate precipitation and stored at −80 °C.

Western Blot Analysis

Western blot was carried out as described elsewhere[23-24]. Briefly, pORF5 fusion was subjected to 12% SDS-PAGE and the same amount of CPAF and MOMP fusion protein were also loaded in the same gel as a loading control. Proteins were then transferred to nitrocellulose membranes and incubated with different mAbs and pAbs, and bound antibodies were probed with a horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and visualized using an enhanced chemiluminescence (ECL) substrate solutions (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immunofluorescence Assay (IFA)

HeLa cells infected with *C. trachomatis* serovar D were fixed with 2% paraformaldehyde (Sigma) for 30 min at room temperature, followed by permeabilization with 1% saponin (Sigma) for an additional 30 min. After washing and blocking, cell samples were subjected to different anti-pORF5 antibodies and chemical staining for 1 h at 37 °C. Bound antibodies were visualized using fluorescent-conjugated secondary antibodies (Jackson ImmunoResearch) under an AX-70 fluorescence microscope (Olympus, Melville, NY, USA).

Double-antibody Sandwich (DAS)-ELISA Assay for Use with Clinical Specimens

Microtiter plates were coated with 1 μg purified 2H4 or 1 μg purified 4E6 mAb in 100 μL coating buffer (0.06 mol/L carbonate-bicarbonate buffer; pH 9.6) per well and incubated overnight at 4 °C. The plates were blocked with 1% BSA in PBS with 0.05% Tween 20 at room temperature for 1 h. Clinical specimens were added to the plates at 37 °C for 1 h, then incubated with rabbit anti-pORF5 antibody, then HRP-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch) was added to each well and re-incubated at 37 °C for 1 h. Finally, each well was incubated with 100 μL of soluble substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma) at room temperature for 10 min. The color reaction was stopped by adding 50 μL of 2.0 mol/L sulfuric acid. The optical density (OD) was measured at 490 nm in a microplate reader (Multiskan MK3, Thermo Labsystems, Vantaa, Finland).

Commercially Available ELISA

ELISA kits (IDEIA PCE Chlamydia) used to detect Chlamydia lipopolysaccharide (LPS) antigen were provided by Dako (Ely, UK). Monoclonal anti-Chlamydia LPS and alkaline phosphatase-conjugated anti-Chlamydia antibodies were used in this study as directed by the manufacturer.

Statistical Analysis

The data were analyzed with SPSS 13.0 for Windows version (SPSS Inc., Chicago, IL, USA). Statistical significance of the differences between groups was assessed by the chi-square test. Significance was established as $P<0.05$.

RESULTS

Expression and Purification of the Recombinant pORF5 Protein

The pORF5 gene was successfully amplified and cloned into the pGEX-6p prokaryotic expression vector. Recombinant plasmids were used to transform *E. coli* XL1 Blue cells for protein expression. After affinity purification, the fusion protein with molecular weight of approximately 54 kD was observed on SDS-PAGE but absent in non-transformed *E. coli* XL1 Blue (Figure 1). The pORF5 fusion protein was mainly presented in a soluble form, while inclusion bodies could also be
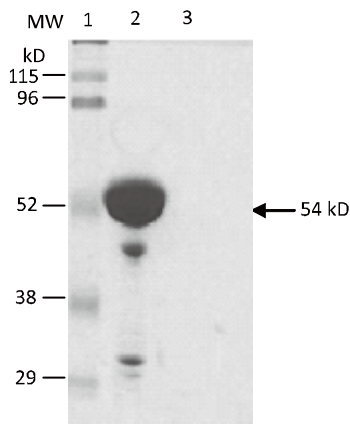
detected (data not shown).

Figure 1. Expression and purification of the pORF5 recombinant protein. The bacteria harboring the pGEX-6p/pORF5 were induced with IPTG, and after purification with glutathione agarose beads, the protein was resolved on an SDS gel and protein bands visualized using Coomassie brilliant blue staining. Lane 1, molecular weight markers; lane 2, purified product from XL1Blue *E. coli* transformed with pGEX-6p/pORF5 recombinant plasmid; lane 3, lysate of XL1Blue *E. coli*. The molecular weight of the pORF5 fusion protein was approximately 54 kD.

Production and Characterization of Antibodies Against pORF5 Protein

Through the procedures of immunization, fusion and clonal selection, two hybridoma strains, named 2H4 and 4E6, were obtained and both were found to stably produce anti-pORF5 mAbs. The 2H4 mAb isotyped as an IgG2a (Figure 2A), and the 4E6 mAb was as an IgG1 isotype (Figure 2B).

To verify the specificity of the mAbs and pAb against pORF5 protein, western blot assay was carried out and the two mAbs and pAb were shown to recognize the pORF5 fusion proteins without obvious cross-reactivity with GST-CPAF and GST-MOMP heterologous fusion proteins (Figure 3). Despite all fusion proteins containing a common GST fusion tag, the anti-fusion protein antibodies failed to recognize GST in unrelated fusion proteins.

Detection of Endogenous pORF5 Protein by Immunofluorescence Assay

We also used the different anti-pORF5 antibodies to detect the distribution of the endogenous pORF5 protein in Chlamydia-infected cells. *C. trachomatis*-infected HeLa cells were stained with anti-pORF5 antibodies and analyzed by IFA. Both mAbs and pAb produced strong signals in the Chlamydia-infected cell cytosol, and a small amount of the fluorescence signal could be detected within the inclusion bodies (Figure 4).
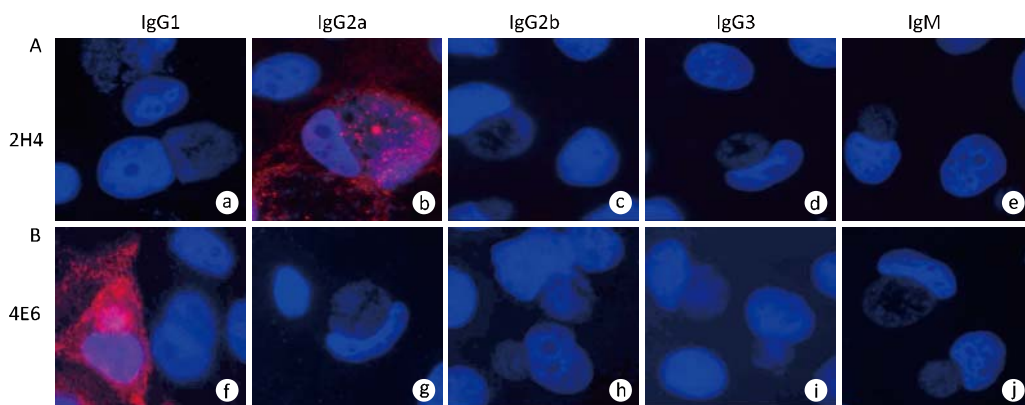

Figure 2. Isotype determination of mAbs against pORF5 protein. (A) Isotyping of 2H4 mAb; (B) Isotyping of 4E6 mAb. *C. trachomatis*-infected HeLa cells were probed with different anti-pORF5 mAbs, the mAb bindings were visualized with Cy3-conjugated goat anti-mouse isotype-specific secondary antibodies (red), and the nuclear DNA was visualized with Hoechst DNA dye (blue). The 2H4 mAb specifically bound to goat anti-mouse IgG2a secondary antibody but not to goat anti-mouse IgG1, IgG2b, IgG3, and IgM secondary antibodies, while the 4E6 mAb specifically bound to goat anti-mouse IgG1 antibody, but not to goat anti-mouse IgG2a, IgG2b, IgG3, and IgM secondary antibodies. Specific fluorescent signals in the cytoplasm of Chlamydia-infected cells were observed in panels b and f, but no significant specific staining signals were observed in panels a, c-e and g-j.
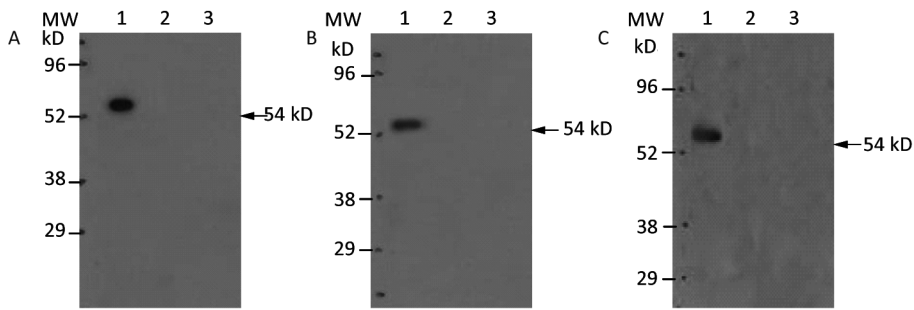
Figure 3. Specificity analyses of mAbs and pAb against pORF5 protein by western blot. GST-pORF5, GST-MOMP, and GST-CPAF fusion proteins were resolved in 12% SDS gels and transferred to nitrocellulose membranes for reaction with mAbs and pAb against pORF5 protein (fusion proteins reacted with 2H4(A), 4E6(B), and pAb(C)). Protein bands recognized by primary antibodies were visualized using HRP-conjugated secondary antibody and enhanced chemiluminescence. Lane 1, GST-pORF5; lane 2, GST-MOMP; lane 3, GST-CPAF. Both mAbs and pAb recognized the pORF5 fusion protein without cross-reactivity with heterologous fusion proteins.
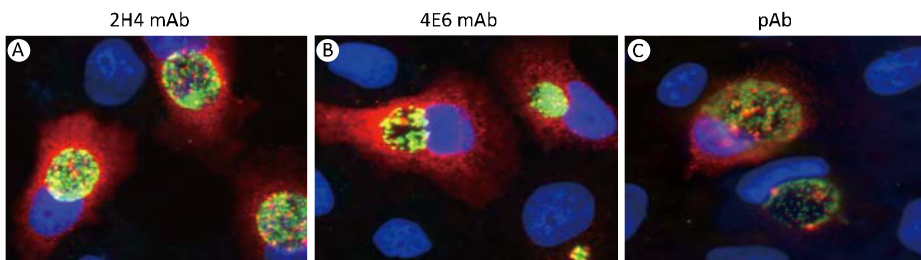

Figure 4. Anti-pORF5 antibodies were used to detect the distribution of the endogenous pORF5 protein in *C. trachomatis*-infected cells. HeLa cells infected with *C. trachomatis* were fixed and probed with anti-pORF5 mAbs and pAb, bound antibodies were visualized with Cy3-conjugated secondary antibodies (red), the Chlamydial organisms were visualized with Cy2-conjugate antibodies (green), and the nuclear DNA was visualized with Hoechst DNA dye (blue). All antibodies [2H4(A), 4E6 (B)], and pAb (C) localized the endogenous pORF5 antigen inside the cytosol of the *C. trachomatis*-infected cells, with a small amount present inside the inclusions.

Intra- and Inter-assay Precision of the DAS-ELISAs

Intra-assay precision was assessed using 10 replicates from the same donor in the same run; inter-assay precision was assessed by performing 10 assays on different days. The coefficients of variation (CV) for intra-assay runs were 5.52% (2H4 coated-plate) and 6.03% (4E6 coated-plate) and the inter-assay CVs were 6.69% (2H4 coated-plate) and 7.58% (4E6 coated-plate), respectively (Table 1).

Detection Sensitivity of the DAS-ELISA

The pORF5 protein was serially diluted two-fold and then analyzed using DAS-ELISA. The DAS-ELISAs were able to detect pORF5 at as low as 10 ng/mL (2H4

coated-plate) and 18 ng/mL (4E6 coated-plate), respectively (Table 1).

Clinical Application of the DAS-ELISAs

To assess the use of the DAS-ELISAs for pORF5 detection in clinical sampled, a total of 248 samples were collected and tested. Each sample was detected by the two DAS-ELISAs and a commercial IDEIA PCE at the same time. The 2H4 mAb recognized 32 (17.20%, positive recognition rate) and 25 (13.44%) samples, the 2H4 mAb by 0 (0%) and 2 (3.22%) samples from groups I and II, respectively (Table 2). The sensitivities of 2H4 and 4E6 mAbs were 92.11% and 77.78%, and the specificities

Table 1. Intra-assay Precision, Inter-assay Precision, and Sensitivity of the DAS-ELISA

Plates Coated with	Number	Intra-assay CV	Inter-assay CV	Detection Sensitivity
2H4	10	5.52%	6.69%	10 ng/mL
4E6	10	6.03%	7.58%	18 ng/mL

Table 2. Comparison of the DAS-ELISAs with IDEIA PCE

ELISA Results	LPS mAb (IDEIA PCE)		2H4 mAb		4E6 mAb	
	Positive	Negative	Positive	Negative	Positive	Negative
Group I (186)	35	151	32	154	25	161
Group II (62)	5[*]	56	0[*]	62	2[*]	60
Total (248)	46	202	32	216	27	221

Note. [*]denotes *P*<0.05.

were 100 and 96.88%, respectively, in relation to IDEIA PCE (Table 3). The 2H4-DAS-ELISA was significantly more sensitive than the 4E6-DAS-ELISA (*P*<0.05), but only slightly more specific than the 4E6-DAS-ELISA (*P*>0.05). Of the 248 samples tested, the positive rates in group I were 18.82%, 17.20%, and 13.44% using IDEIA PCE, 2H4-DAS-ELISA, respectively; the positive rates in group II were 8.92%, 0, and 3.23%, respectively. The Chlamydial positive rate in urogenital infection patients was much higher than that in healthy controls (*P*<0.05). Interestingly, there were three samples from group I that were negative when tested with the prepared DAS-ELISAs based on pORF5 mAbs, but positive when tested IDEIA PCE based on the LPS mAb.

Table 3. Sensitivity and Specificity of DAS-ELISA Compared with IDEIA PCE

DAS-ELISA Based on	Sensitivity (%)	% Specificity
2H4	92.11	100.00
4E6	77.78[*]	96.88[**]

Note. [*] denotes *P*<0.05; [**] denotes *P*>0.05.

DISCUSSION

Detection of Chlamydial antigens and/or genomes can provided early and rapid diagnosis of urogenital infections can be followed by appropriate treatment. As described in the background, nucleic acid detection is not readily adaptable for routine use in clinical diagnosis[10], therefore antigen detection based on serologic methods has shown its value for the diagnosis of Chlamydial infectious diseases. Many studies have been performed to use mAbs for antigen detection in ELISA, such as LPS mAb for LPS detection, and promising results were obtained with these tests[25]. However, cross-reactivity persisted because of the presence of other genus-specific epitopes. On the basis of the high immunogenicity and specificity[16-18], the pORF5 protein appeared to be a suitable antigenic marker for the diagnosis of *C. trachomatis* infection. In the

present study, we described the production and characterization of several specific antibodies against the pORF5 protein of *C. trachomatis* and the subsequent development of DAS-ELISAs that allowed its detection.

Since isolation of pORF5 protein from crude *C. trachomatis* preparations is very limited and artificial synthesis of the pORF5 protein may have some concerns on production cost, we first attempted to produce pORF5 protein in a recombinant *E. coli* system to produce mAbs and pAb. In this study, we used the prokaryotic expression vector pGEX-6p with *E. coli* XL1Blue to express pORF5 recombinant protein. The recombinant pORF5 protein was then used as an antigen to produce specific mAbs and pAb. The anti-pORF5 antibodies strongly reacted with the recombinant pORF5 protein but not with other Chlamydial proteins, and specifically localized the endogenous pORF5 antigen inside the host cell cytosol of the *C. trachomatis*-infected cells. These observations demonstrated that the anti-pORF5 antibodies are specific to pORF5 protein.

After characterization of the antibodies, DAS-ELISAs were developed for antigen detection. The mAbs 2H4 and 4E6 were selected as the capture antibodies, and pAb was used as the detecting antibodies to pORF5 protein. Validation of the DAS-ELISAs showed that the developed tests were easy to handle and very sensitive tools for the detection of pORF5 protein. Moreover, these assays displayed a low limit of detection at 10 ng/mL (based on 2H4) and 18 ng/mL (based on 4E6), suggesting that a more widely applicable enzyme immunoassay based on 2H4 mAb may be helpful for the serodiagnosis of *C. trachomatis* infection.

To evaluate the DAS-ELISAs prepared in this study, 248 samples from different groups were selected and tested. The highest sensitivity was observed in IDEIA PCE test, but this determination had a low specificity possibly because of LPS as a genus-specific antigen. Meanwhile, our results indicated that there was a much higher rate of Chlamydial infections in urogenital tract infection patients than in healthy controls when analyzed by

ELISA. Interestingly, significant variation was observed when samples were tested with different mAbs; one sample reacted with 2H4, 4E6, but not with LPS mAb; two samples reacted with 2H4 and LPS mAbs, but not with 4E6; another three samples reacted only with LPS mAb. The different reactivities observed here are likely the result of the surface exposure of the antigen with different immunoaccessibility. Further evaluation, however, will be performed with more samples to determine the specificity of the assay in a more quantitative way.

CONCLUSION

DAS-ELISAs based on 2H4 and 4E6 mAbs were developed in this study, where 2H4 was found to bind pORF5 protein with good specificity and sensitivity. The newly developed DAS-ELISAs provide feasible, convenient, and effective assays that could be considered as alternative tools for aiding the serodiagnosis of *C. trachomatis* infections. However, further validation in large field trials is needed.

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