Identification of an IS6110 insertion site in plcD, the unique phospholipase C gene of Mycobacterium bovis

Cristina Viana-Niero,1 Cesar Alejandro Rosales Rodriguez,2 Fabiana Bigi,3 Marcos Santos Zanini,4 José Soares Ferreira-Neto,2 Angel Cataldi3 and Sylvia Cardoso Leão1

1Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, Rua Botucatu 862 3° andar, São Paulo, CEP 04023-062, Brazil
2Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Professor Dr Orlando Marques de Paiva 87, São Paulo, CEP 05508-900, Brazil
3Instituto de Microbiología y Zoología Agrícola del CICVyA – Instituto Nacional de Tecnología Agropecuaria (INTA), Castelar, Buenos Aires, Argentina
4Centro de Ciências Agrárias, Universidade Federal do Espírito Santo, PO Box 16, Alegre, Espírito Santo, Brazil

The IS6110 repetitive element is present in multiple copies in most Mycobacterium tuberculosis complex bacteria, except for Mycobacterium bovis strains, which usually contain a single copy of IS6110 located on a 1.9 kb PvuII fragment of the direct repeat region. IS6110 transposition can disrupt coding regions and is a major force of genomic variation. In a previous work it was demonstrated that phospholipase C genes are preferential loci for IS6110 transposition in M. tuberculosis clinical strains. Bacterial phospholipase C enzymes participate in pathogenic mechanisms used by different organisms, and have been implicated in intracellular survival, cytolysis and cell-to-cell spread. Four phospholipase C genes (plcA, plcB, plcC and plcD) were detected in the genomes of M. tuberculosis, Mycobacterium africanum, Mycobacterium microti and ‘Mycobacterium canetti’. M. bovis and the vaccine strain M. bovis Bacillus Calmette–Guérin contain only the plcD gene. In the present work, the existence of IS6110 insertions within plcD, the unique phospholipase C gene of M. bovis, has been investigated by PCR, Southern blot hybridization and sequencing analysis. In 18 (7.3 %) of 245 isolates analysed, the plcD gene was interrupted by the insertion of one copy of IS6110, which in all cases was transposed in the same orientation and at the same position, 1 972 894, relative to the genome of M. bovis AF2122/97. These 18 isolates were distributed in 6 different spoligotype patterns and contained 4 to 8 IS6110 copies. In contrast, strains showing an intact plcD gene contained one (87 %), two (9.4 %) or three (2.4 %) IS6110 copies, and only a single isolate (1.2 %) had four IS6110 copies. The implications of plcD gene disruption in M. bovis have not been fully investigated, but no differences in the organ distribution of the disease were detected when animals infected with strains from the same spoligotype patterns bearing plcD::IS6110 and intact plcD were compared.

INTRODUCTION

Genomes of Mycobacterium tuberculosis complex bacteria contain four highly related genes encoding phospholipase C enzymes (Cole et al., 1998). Three genes, plcA, plcB and plcC, are organized in tandem (plcABC locus). The fourth gene, plcD, is located in a different region. These four genes are present in most strains of M. tuberculosis, Mycobacterium africanum, ‘Mycobacterium canetti’ and Mycobacterium microti. Other complex members, the classical virulent M. bovis and the vaccine strain M. bovis Bacillus Calmette–Guérin, have only the plcD gene, because an 8.9 kb fragment containing the plcABC locus, the RD5 region, is consistently absent from strains of these species (Behr et al., 1999; Brosch et al., 2002; Gordon et al., 1999). In the laboratory strain M. tuberculosis H37Rv, plcD is truncated by insertion of a copy of IS6110 and by deletion of a 7.9 kb fragment, designated RvD2 (Brosch et al., 2002; Gordon et al., 1999).

Abbreviations: DR, direct repeat; DVR, direct variable repeat.
Genetic variations in plcABC and plcD loci caused by insertions of IS6110 mobile genetic elements, and deletions originating from homologous recombination of two IS6110 elements, have been characterized in M. tuberculosis clinical isolates (Nguyen et al., 2004; Talarico et al., 2005; Vera-Cabrera et al., 2001; Viana-Niero et al., 2004; Warren et al., 2000; Yesilkaya et al., 2005), indicating that these domains represent preferential regions for the integration of this insertion element (Warren et al., 2000; Yesilkaya et al., 2005). The IS6110 element is present at variable copy numbers (0–25) and at different insertion sites in the chromosome of different M. tuberculosis strains. In contrast, M. bovis isolates harbour few copies of this element, frequently only a single copy located in the direct repeat (DR) region (Hermans et al., 1991), suggesting that IS6110 transposition is a rare event in this species.

In this work, we investigated the existence of polymorphisms in the plcD gene, the unique phospholipase C gene of virulent M. bovis, and related these polymorphisms to IS6110 element insertions.

**METHODS**

**Mycobacterial strains.** A total of 245 M. bovis strains from 3 states in Brazil (São Paulo, Minas Gerais and Rio Grande do Sul) and 6 states in Argentina (Buenos Aires, Santa Fe, Entre Ríos, Formosa, Rosario and La Pampa) were used in this study (Table 1). M. bovis isolates from São Paulo were obtained during a surveillance study of bovine tuberculosis. Federal and State Inspection Services collected samples in slaughterhouses from different regions of São Paulo State. Bacteria from lesions were cultivated in Löwenstein–Jensen and Stonebrink–Leslie media (Centro Panamericano de Zoonosis, 1973). Two human isolates from São Paulo were obtained from a private clinical laboratory. M. bovis DNA of isolates from Minas Gerais, Rio Grande do Sul and Argentina were obtained from the collection of Centro de Investigaciones en Ciencias Veterinarias, Instituto Nacional de Tecnología Agropecuaria, Castelar, Argentina. All isolates were previously identified as M. bovis by bacteriological criteria (Vestal, 1975; Wayne, 1984) and/or spoligotyping (Kamerbeek et al., 1997). M. bovis AN5 was used as a control for PCR and hybridization experiments.

**PCR and sequencing.** The PCR of the plcD gene was set up using primers D1 5′-TTCGGGCGGAATTCTCTAG-3′ and D2 5′-TTCTGGGTGGGATAGTCCTGG-3′ for amplification of a 1478 bp fragment, as described previously (Viana-Niero et al., 2004) (Fig. 1a). Amplification of a 123 bp fragment from the IS6110 element was carried out using primers IS1 5′-GCGTGGAGCCGTAGGGCTGG-3′ and IS2 5′-GTCCTCCGGCCGCTTCG-3′, as described by Eisenach et al. (1990). Amplicons were sequenced using primers IS3 5′-CATCCGGCTTCATCCAGTAC-3′ directed outwards from the IS6110 sequence in an automated ABI PRISM 377 sequencer (Perkin-Elmer) using the BigDye Terminator Ready Reaction Cycle Sequencing kit v 3.0 (Applied Biosystems). Sequences were compared using BLAST – basic local alignment tool (http://www.ncbi.nlm.nih.gov/blast).

**Southern blotting and fingerprinting analysis.** Southern blotting was performed as described by van Embden et al. (1993). Briefly, 2 μg DNA were digested using PvuII and subjected to electrophoresis in 0.8% agarose in 0.5 × TBE pH 8.0, at 2 V cm⁻¹. A mixture of PvuII-digested supercoiled DNA ladder (Invitrogen) and HaeIII-digested λX174 DNA (Invitrogen) was used as an internal DNA size marker. DNA was blotted onto nylon membranes (Hybond-N+; Amershams Biosciences), and probed, in different experiments, with probes complementary to the plcD gene and IS6110, obtained by PCR using primers D1/D2 or IS1/IS2 as described previously. Blots were also hybridized with a probe complementary to the DR region (5′-GTCGTCAGACCCAAAACCCC-GAGAGGGGACGGAAAC-3′) (Hermans et al., 1991), and with an internal size marker. All probes were covalently labelled with peroxidase by the glutaraldehyde method, and detected using the ECL Direct system (Amersham) according to the manufacturer’s instructions. Membranes were exposed to X-ray films (X-OMAT; Kodak). Fingerprint autoradiograms were superimposed with internal marker autoradiograms for normalization, and were analysed using BioNumerics software version 4.0 (Applied Maths).

**RESULTS**

**Analysis of plcD integrity by plcD PCR**

All 245 isolates were tested by plcD PCR. A total of 41 isolates from Argentina and 186 isolates from Brazil (13 from Rio Grande do Sul, 22 from Minas Gerais and 151 from São Paulo) produced amplicons of 1478 bp. In contrast, 18 (7.3%) isolates from São Paulo produced PCR amplicons of approximately 3 kb, which were larger than expected. In all cases, reamplification of these large amplicons using primers IS1 and IS2, specific for the IS6110 sequence, generated 123 bp products, confirming the insertion of this element into plcD gene (plcD::IS6110).

**Corroboration of IS6110 insertion in plcD by fingerprinting**

DNA from 42 isolates from São Paulo was available for fingerprinting with the plcD-complementary probe. For 12 isolates with plcD::IS6110, hybridization with this probe revealed a four-banded pattern, with bands of approximately 900, 1150, 3900 and 4200 bp (Fig. 2a). For 30 isolates...
that generated 1478 bp plcD amplicons, hybridization patterns produced by the plcD-derived probe revealed 3 bands of 538, 3900 and 4200 bp, also observed with M. bovis AN5 control strain, confirming the presence of an intact plcD gene (Fig. 2a).

Hybridization experiments with the IS6110 complementary probe (IS6110 RFLP) were performed on the 42 isolates from São Paulo. For an additional 55 isolates from Minas Gerais, Rio Grande do Sul and Argentina, the IS6110 copy number was estimated from previous experiments and publications (Zanini et al., 2001, 2005). The 12 isolates with plcD::IS6110 contained four or more IS6110 copies (Fig. 2b). In contrast, 74 (87 %) from 85 isolates without insertion in the plcD had a single IS6110 copy, which in 73 isolates was detected as a 1-9 kb band and in 1 case was detected as a 1-1 kb band (Fig. 2b). Eight (9-4 %) isolates had two IS6110 copies, two (2-4 %) isolates had three copies and only one (1-2 %) isolate presented four copies (Fig. 2b, data not shown).

The superimposition of plcD and IS6110 RFLP films showed that both probes hybridized with fragments of the same length, about 1150 bp, in strains with plcD::IS6110, further substantiating the presence of an IS6110 copy in the plcD gene (Fig. 2a, b). Hybridization with DR-derived probe and

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**Fig. 1.** (a) Schematic representation of the plcD locus from M. bovis AF2122/97 (Garnier et al., 2003). (b) Schematic representation of isolates with plcD::IS6110. Gene names are shown in (a). Small white arrows (not to scale) above the line represent primers D1 and D2. PvuII restriction sites are represented as small vertical arrows. The small black horizontal arrow in (b) represents the direction of transcription of IS6110 transposase. a, b, c, d and e are fragments of 4200, 538, 3900, 1150 and 900 bp, respectively, detected by hybridization of Southern blots with a plcD-derived probe.

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**Fig. 2.** Fingerprint analysis of M. bovis isolates using probes complementary to plcD (a) and IS6110 (b), analysed using BioNumerics v. 4.0. Specific bands hybridizing with each probe are shown on the left and hybridization patterns on the right. *Bands that co-hybridized with IS6110- and DR-derived probes. #Bands that co-hybridized with IS6110 and plcD-derived probes. A–E, IS6110-RFLP patterns of isolates with intact plcD; F–J, IS6110-RFLP patterns of isolates with plcD::IS6110. A, M. bovis AN5; B, 1 isolate representative of 73 isolates with a single IS6110 copy located in the 1-9 kb DR region; C, D, E, single isolates with 1, 2 and 4 IS6110 copies, respectively, and without insertion in plcD; F, representative of 3 isolates from spoligotype sb0881 and 1 each from spoligotypes sb1033, sb0121 and ST1853; G, single isolate from spoligotype sb0134; H, representative of 3 isolates from spoligotype sb0881; I, single isolate from spoligotype sb0881; J, single isolate from spoligotype sb0295.
superimposition with IS6110 RFLP films showed that both probes always hybridized with fragments of the same length, confirming the presence of one IS6110 copy in the DR region in all isolates tested (Fig. 2b).

**Distribution of spoligotype patterns in isolates with plcD::IS6110**

Considering all 245 isolates, a total of 42 different spoligotypes were observed (data not shown). The 18 isolates with plcD::IS6110 were distributed in 6 previously nominated spoligotypes: sb0881, sb0121, sb1033, sb0134, sb0295 (from the *M. bovis* spoligotype database at http://www.mbovis.org/) and ST1853 (from the SpolDB4 database) (Table 2). A total of 111 additional isolates with intact plcD genes, as shown by plcD PCR, were allocated in these same 6 spoligotype patterns (Table 2).

**Localization of IS6110 insertion site by sequencing**

Sequence analysis of the 3 kb amplicons corroborated the presence, in all 18 strains, of the IS6110 element at the same position, 1972894, relative to the genome of *M. bovis* AF2122/97 (Garnier et al., 2003), in the same orientation, and disclosed a DR of three nucleotides CTC:CTC at the ends of the insertion sequence (Fig. 1b).

**DISCUSSION**

Insertion of a copy of IS6110 in the plcD gene was detected in 7-3% of the isolates studied here. All isolates bearing plcD::IS6110 presented an indistinguishable four-banded hybridization pattern with the plcD-derived probe and presented the same insertion site in the plcD gene by DNA sequencing. Isolates were distributed in six spoligotype patterns and five IS6110 RFLP profiles. Eight different strains could be recognized by a combination of both methods (Table 2).

The observation that all plcD::IS6110 isolates originated from the same region (São Paulo, Brazil) and shared two IS6110 hybridizing bands carrying particular IS6110 insertions (plcD::IS6110 and an IS6110 copy in the DR region) suggests that they represent a unique evolutionary event.

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**Table 2. Distribution of spoligotype and IS6110 RFLP patterns in isolates with plcD::IS6110 and intact plcD**

<table>
<thead>
<tr>
<th>No. of isolates</th>
<th>Origin</th>
<th>plcD PCR</th>
<th>IS6110 RFLP</th>
<th>Spoligotype</th>
<th>Pattern</th>
</tr>
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<tr>
<td>3</td>
<td>SP</td>
<td>plcD::IS6110</td>
<td>F</td>
<td>11011111011111011111111110000011111100000 sb0881</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>SP</td>
<td>plcD::IS6110</td>
<td>H</td>
<td>11011111011111011111111110000011111100000 sb0881</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>SP</td>
<td>plcD::IS6110</td>
<td>I</td>
<td>11011111011111011111111110000011111100000 sb0881</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>SP</td>
<td>plcD::IS6110</td>
<td>NE</td>
<td>11011111011111011111111110000011111100000 sb0881</td>
<td></td>
</tr>
<tr>
<td>2</td>
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<td>plcD</td>
<td>1*</td>
<td>11011111011111011111111110000011111100000 sb0881</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>SP</td>
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</tr>
<tr>
<td>5</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>49</td>
<td>SP</td>
<td>plcD</td>
<td>1‡ or 2§</td>
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<td></td>
</tr>
<tr>
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<td>F</td>
<td>11011111011111011111111110000011111100000 sb0881</td>
<td></td>
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<tr>
<td>5</td>
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<td>plcD</td>
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<tr>
<td>1</td>
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<tr>
<td>3</td>
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</tr>
<tr>
<td>1</td>
<td>SP</td>
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</tr>
<tr>
<td>3</td>
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<td>plcD</td>
<td>1*</td>
<td>11011111011111011111111110000011111100000 sb0881</td>
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*One isolate evaluated.
†Thirteen isolates evaluated.
‡Four isolates evaluated.
§Two isolates evaluated.
followed by clonal expansion and divergence of spoligotype and IS6110 RFLP patterns. This hypothesis is reinforced by
the similarities of spoligotype and IS6110 RFLP patterns, but is somewhat contradicted by the recognition of 11 additional
isolates belonging to the same spoligotype patterns but showing intact plcD genes (Table 2). Forty-two of these
isolates were also evaluated by IS6110 RFLP. All had a copy of IS6110 inserted in the 1.9 kb fragment of the DR region
but not in the 1.4 kb fragment (data not shown). Therefore, an independent evolution of the different genetic events
cannot be excluded.

The DR region is a polymorphic locus in the genome of
M. tuberculosis complex members comprising a cluster of
directly repeating sequences of 36 bp, separated by unique spacer sequences of 36–41 bp. One repeat sequence and the
following spacer sequence have been named DVR (direct
repeat variable). Polymorphisms in this region can originate
from transposition of the IS6110 element inside the DR
region or successive deletions from a primordial DR locus,
probably mediated by homologous recombination between
IS6110 elements, neighbouring or distant directly repeating
sequences and/or by slippage during DNA replication (Fang
et al., 1998; Groenen et al., 1993; van Embden et al., 2000;
Warren et al., 2002). IS6110-dependent mechanisms of
evolution would not be frequent in M. bovis isolates due to
the low-copy number of IS6110 elements. In this context,
spoligotype pattern sb0121 showed the most intact structure,
and patterns sb0295, sb0881 and ST1853 could have
originated from a common ancestor with the same DR
structure as sb0121 by successive loss of DVRs in dependent
or independent events. Spoligotype patterns sb1033 and
sb0134 retain DVR21, which is missing in the other spoligo-
type patterns, and therefore were probably generated by
independent events. Except for spoligotype pattern ST1853,
which was found only in isolates from Brazil, the other five
spoligotype patterns were previously observed in M. bovis
isolates from France, the Netherlands, Belgium and Australia,
according to data from M. bovis spoligotype database at
http://www.mbovis.org/. They were not found in 600 M.
bovis isolates from Argentina (data not shown). Spoligotype
patterns sb0121 and sb0134 were predominant in a study on
M. bovis isolates from Europe (Haddad et al., 2001). Due
to the wide geographic distribution of these spoligotype
patterns it is also possible that they have evolved conver-
gently through preferential deletion of specific DVRs (Fang
et al., 1998; Warren et al., 2002). This would justify the
existence of isolates with and without insertion in the plcD
gene belonging to the same spoligotype pattern.

The majority of M. bovis isolates have a single copy of IS6110
stably inserted in the 1.9 kb fragment of the DR region
(van Embden et al., 2000). On rare occasions, this element
replicates and inserts into different regions of the genome.
Transposition can be assisted by activation of adjacent
promoters, and transposition frequency is higher if the
insertion sequence is inserted into transcriptionally active
sites (Wall et al., 1999). plcD is a transcriptionally active site
(Raynaud et al., 2002; Viana-Niero et al., 2004) and, there-
fore, insertion into this gene can potentially activate IS6110
transposition events. In addition to the two IS6110 copies
common to all plcD::IS6110 isolates, seven isolates had a
further two common IS6110 copies on 4.3 kb and 2.5 kb
PvuII fragments and one of these isolates subsequently
acquired another IS6110 copy (patterns F and G). The other
five plcD::IS6110 isolates belong to three IS6110 RFLP
patterns that also had additional common IS6110 copies on
6.3, 4 and 2.2 kb PvuII fragments (patterns H and I), and on
2.2 and 1.7 kb PvuII fragments (patterns I and J). Isolates of
spoligotype pattern sb0881 showed higher IS6110 trans-
position activity, comprising three different IS6110 RFLP
patterns, from which at least one (F) could have evolved
independently from the other two (H and I), suggesting
parallel evolution of the IS6110 banding pattern in these
strains.

The plcD locus has been identified as a preferential site for
IS6110 insertion in M. tuberculosis (Gordon et al., 1999; Ho
et al., 2000; Sampson et al., 1999). Yesilkaya et al. (2005)
identified 9 distinct IS6110 insertion sites within the plcD
gene in 161 clinical M. tuberculosis isolates, and Kong et al.
(2005) reported the existence of 27 different insertion sites
within the plcD gene in 144 clinical M. tuberculosis isolates.
However, insertions at the same position of the plcD gene in
different M. tuberculosis isolates were also reported (Kong
et al., 2005; Viana-Niero et al., 2004; Warren et al., 2000).
The insertion position identified here, which is different
from insertion positions reported in M. tuberculosis isolates,
was the same in all M. bovis isolates, regardless of differences
in spoligotype and IS6110 RFLP patterns. Together these
findings suggest that IS6110 insertions at the same site in the
plcD gene could potentially originate from independent
events. Independent occurrences of IS6110 insertion at the
ipl loci (iplA and iplB) in different M. tuberculosis isolates
were also reported (Fang et al., 2001). Further research, using
additional markers or sequencing will disclose if M. bovis
with insertion in plcD has arisen from an ancestral clone or
resulted from independent events of transposition.

Deletions in the plcD locus have been reported in M. tuber-
culosus clinical isolates (Talarico et al., 2005; Viana-Niero et al.,
2004). Lari et al. (2001) showed that 15 out of 45 isolates of
M. tuberculosis studied presented the RvD2 deletion. We
have shown that deletions of plc genes in M. tuberculosis
isolates originate from homologous recombination between
two copies of IS6110 (Viana-Niero et al., 2004). Deletions in
plcD gene were not found in any of 245 M. bovis isolates
studied here, as the primer sites at each end of the plcD gene
were always present, irrespective of the presence of the
IS6110 element. The low-copy number of IS6110 elements in
M. bovis could explain the absence of this occurrence, as
proximity of two copies of the insertion element in the same
orientation is necessary for homologous recombination, and
this proximity is not expected to occur in M. bovis.

No difference in the organ distribution of lesions was observed
when animals infected with strains with plcD::IS6110 were
comparing to animals infected with strains with intact plcD of the same spoligotype pattern (data not shown). Therefore, it seems that the plcD product is not involved in the organ dissemination of M. bovis. Despite the well-known role of phospholipases C in the virulence of several intracellular bacterial pathogens (Berka et al., 1981; Logan et al., 1991; Vazquez-Boland et al., 1992), the role of these enzymes in the pathogenicity of mycobacteria has not yet been completely understood. In a previous study (Viana-Niero et al., 2004) we demonstrated that lack of transcription of all four plc genes did not impair the ability of M. tuberculosis isolates to cause disease in their hosts. Raynaud et al. (2002) concluded that the plcD gene did not contribute to the virulence of M. tuberculosis but could act in association with other plc genes. Yang et al. (2005) demonstrated that M. tuberculosis bearing plcD gene mutations generated more extra-pulmonary tuberculosis than strains with intact plcD. As yet unresolved questions are whether insertions in plcD represent a biological advantage for the bacterium, and what the precise role of this gene in bacterial survival and pathogenicity is.

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