Congenital Chagas disease involves *Trypanosoma cruzi* sub-lineage IId in the northwestern province of Salta, Argentina

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ABSTRACT

Trypanosoma cruzi is genetically classified into six discrete phylogenetic lineages on the basis of different genetic markers. Identifying lineages circulating among humans in different areas is essential to understand the molecular epidemiology of Chagas disease. In the present study, 18 *T. cruzi* isolates from congenitally infected newborns in the northwestern province of Salta-Argentina were studied by multilocus enzyme electrophoresis (MLEE) and random amplified polymorphic DNA (RAPD). All isolates were typed by MLEE and RAPD as belonging to *T. cruzi* IId. Analysis of minor variants of TcIId using probes hybridizing with hypervariable domains of kDNA minicircles, detected three variants with a similar distribution among the isolates. Our findings confirm the presence of *T. cruzi* IId among congenitally infected newborns in northwestern Argentina and support the assumption that human infection by *T. cruzi* in the Southern Cone countries of Latin America is due principally to *T. cruzi* II.

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1. Introduction

Congenital transmission is the principal mode of infection by *Trypanosoma cruzi*, the agent of Chagas disease, in areas where transmission by vectors and blood transfusions are controlled. In endemic regions, the transmission rates of congenital Chagas disease is extremely variable, ranging from 1% in Brazil to 4–12% in Argentina, Bolivia, Chile, and Paraguay (Gurtler et al., 2003; Carlier, 2005; Brutus et al., 2008). In addition, the morbidity and mortality of congenital infection also varies from being asymptomatic to severe and lethal clinical forms of disease (Torrico et al., 2004; Sanchez Negrette et al., 2005; Virreira et al., 2006). Host factors, such as the level of placental defense, and/or the maternal and fetal capacities to develop a specific immune response for the control of parasitic multiplication, can be involved in these differences (Hermann et al., 2002, 2004). An alternative hypothesis is that these differences are related to genetic polymorphism among parasite strains (Macedo and Pena, 1998).

Clonal populations of *T. cruzi* harbor considerable genetic polymorphism and different parasite genotypes might play an important role in creating variation in the pathology they cause in different regions (Macedo and Pena, 1998). Multilocus enzyme electrophoresis (MLEE) is currently the gold standard for strain typing which has lead to sub-division of the taxon into two major lineages, *T. cruzi* I and *T. cruzi* II (Barnabe et al., 2000; Brisse et al., 2000). Each lineage is genetically heterogeneous and despite a failure to identify sub-divisions within *T. cruzi* I, five sub-groups of *T. cruzi* II have been proposed on the basis of biochemical and molecular markers (Brisse et al., 2000). These six discrete genetic subdivisions, or lineages, have been proposed as a reference framework for genetic variability and biological characterization of *T. cruzi* stocks. Therefore, identifying lineages circulating among humans in different areas is important to evaluate the possible influence of infective genotypes on the broad spectrum of observed clinical disease.

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In previous works we have reported a congenital transmission rate of 9% and a family clustering of vertical transmission of *T. cruzi* in the province of Salta, Argentina (Mora et al., 2005; Sanchez Negrette et al., 2005). Since knowledge about the genetic diversity of *T. cruzi* isolates circulating within a country is essential to understand the molecular epidemiology of Chagas disease, we aimed to characterize *T. cruzi* lineages in 18 isolates from congenitally infected newborns in the province of Salta by using three different molecular markers. MLEE and RAPD analyses were used as standard markers for lineage identification. Additionally, new sensitive probes designed to distinguish variants within the *T. cruzi* IId sub-lineage were used to asses genetic variability of *T. cruzi* isolates.

2. Materials and methods

2.1. Study area

This study was conducted in the city of Salta, in northwestern Argentina, with approximately 500,000 inhabitants. Triatomine vectors were eradicated from the city in 1970s, and no re-infestations have been registered in the area where this study was performed.

2.2. Sample collection

Samples were collected from July 1997 to December 2001 in the framework of a collaborative project to improve diagnostic methods for Congenital Chagas disease in Salta city (Mora et al., 2005). Mothers were identified as bearing *T. cruzi* infection by using standard parasite-specific serological tests (ELISA and indirect hemagglutination IHA) in duplicate samples as previously described (Mora et al., 2005). Samples from newborns were mainly obtained from umbilical cords and occasionally from venous punctures performed during the first 15 days of life. Umbilical cords were collected in the Hospital Materno Infantil of Salta, as previously described (Mora et al., 2005). All congenital infections were subjected to treatment. Informed consent was obtained from mothers of babies participating in this study, under a protocol approved by the Ministry of Public Health of the Province of Salta.

2.3. Parasite isolation and reference strains

Eighteen isolates of *T. cruzi* were obtained from umbilical cords and venous puncture following hemoculture in liver infusion tryptose (LIT) medium. The isolated parasites were maintained by passage in LIT medium at 28 °C. Table 1 summarizes the geographical origin and clinical data of the 18 stocks. Four strains were used for reference: X10cl1 (*T. cruzi* I), CANIIIcl1 (*T. cruzi* IIa), Mncl2 (*T. cruzi* IId) and CL-Brener (*T. cruzi* IIe). X10cl1 and CANIIIcl1 correspond to the formerly described zymodemes I and III, respectively (Miles et al., 1978).

2.4. Sample preparation

Stocks were harvested by centrifugation ($2800 \times g$, 20 min, 4 °C) and washed in PBS (Na_2HPO_4 10 mM, NaCl 150 mM, pH 7.2). Cells were lysed on ice for 20 min in an equal volume of hypotonic enzyme stabilizer (EDTA 2 mM, dithiothreitol 2 mM, 1-aminocaproic acid 2 mM). The soluble fraction was stored at −70 °C until used in MLEE analyses, whereas the pellet of lysed cells was used for DNA extraction, as described elsewhere (Brisse et al., 2000). DNA concentration was estimated by spectrophotometry at 260 nm.

Table 1
Origin of 18 *Trypanosoma cruzi* stocks examined and clinical features of respective congenital chagasic patients.

Stocks	Geographical origin of the mother	Clinical form
CONG 1	Salta, Santa Victoria	Asymptomatic
CONG 2	Salta, Colonia Sta Rosa	Anemia
CONG 3	Salta, Cerrillos	Asymptomatic
CONG 4	Salta, Cerrillos	Asymptomatic
CONG 5	Salta, Metan	Asymptomatic
CONG 6	Salta, Gral San Martin	Anemia
CONG 7	Salta, Campo Quijano	Asymptomatic
CONG 8	Salta, Oran	Anemia
CONG 9	Salta, Gral San Martin	Asymptomatic
CONG 10	Salta	Asymptomatic
CONG 11	Salta	Asymptomatic
CONG 12	Salta	Asymptomatic
CONG 13	Salta	Anemia
CONG 14	Salta	Asymptomatic
CONG 15	Salta, Gral San Martin	Jaundice
CONG 16	Salta, Santa Victoria	Asymptomatic
CONG 17	Salta, Lesser	Asymptomatic
CONG 18	Salta, Santa Victoria	Asymptomatic

2.5. Protocol for isoenzyme analysis

Multilocus enzyme electrophoresis analysis was performed as described elsewhere, with slight modifications (Ben Abderrazak et al., 1993). The following 9 enzyme systems were used: diaphorase (EC 1.6.99.2, DIA), glutamate dehydrogenase, NAD^+ (EC 1.4.1.2, GDH-NAD^+), glutamate dehydrogenase $NADP^+$ (EC 1.4.1.4, GDH-$NADP^+$), glutamate oxaloacetate transaminase (EC 2.6.1.1, GOT), glucose-6 phosphate dehydrogenase (EC 1.1.1.49, G6PD), glucose-6-phosphate isomerase (EC 5.3.1.9, GPI), malate dehydrogenase (EC 1.1.1.37, MDH), malic enzyme (EC 1.1.1.40, ME) and phosphoglucomutase (EC 5.4.2.2, PGM). These 9 enzyme systems correspond to 11 different genetic loci, since diaphorase and malic enzymes both exhibit activity of two distinct loci.

2.6. Random amplified polymorphic DNA analysis

We selected six primers that yielded the most discriminating patterns as previously described (Brisse et al., 2000). The primers correspond to the A, B, F and U kits from Operon Technologies: A10 (GTGATCGCAT), B11 (GTAGACCCGT), B19 (ACCCCCGAAG), F5 (CCGAATTCCC), U11 (AGACCCAGAG) and U14 (TGGGTCCCTC). The amplification reactions were performed in a final volume of 60 μl containing 0.9 units Taq Polymerase (Boehringer), 100 μM each dNTP, 200 nM primer, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris ± HCl, pH 8.3, and 20 ng template DNA. Forty-five cycles (denaturation: 1 min at 94 °C; annealing: 1 min at 36 °C; elongation: 2 min at 72 °C) were followed by a final elongation step of 7 min at 72 °C. Random amplified polymorphic DNA products were analyzed by electrophoresis in 1.6% agarose gels in TAE buffer (Tris-acetate 40 mM, EDTA 1 mM), stained with ethidium bromide and visualized by ultra-violet light.

2.7. Hybridizations with synthetic oligodeoxynucleotide probes for variants of lineage IId

Identification of variants of the sub-lineage TcIId was performed by hybridization of DNA amplicons resulting from the Tc121/Tc122 PCR amplification, with oligonucleotide probes as previously described (Virreira et al., 2006). The TcIId variants were grouped according to their relative hybridization pattern with probes Oli-IId-1 and Oli-IId-2, as described by Virreira et al., 2006.
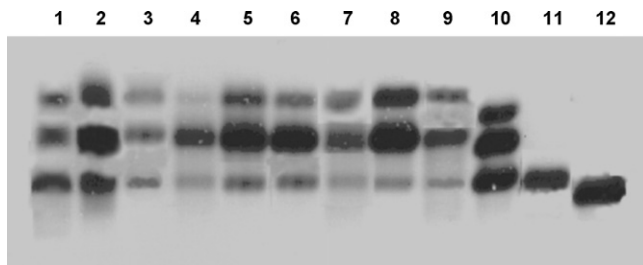
Fig. 1. Patterns obtained for the glucose phosphate isomerase (GPI) locus for 8 of the 18 *T. cruzi* stocks under study. Lines 1–8 correspond to stocks listed in order in Table 1. Line 9: Mncl2 (reference strain for *T. cruzi* IId), Line 10: CL-Brener (reference strain for *T. cruzi* IIe), Line 11: CANIIIcl1 (reference strain for *T. cruzi* IIa), Line 12: X10cl1 (reference strain for *T. cruzi* I).

2.8. Data analysis

MLEE patterns were compared with those described for the major lineages and for additional subdivisions of *T. cruzi* II lineage (Barnabe et al., 2000; Brisse et al., 2000) using the four reference strains. Every band was counted, excepting central bands in heterozygous patterns that showed three bands. These patterns in *T. cruzi* have been attributed to heterozygous genotypes for dimeric enzymes, in which central bands do not correspond to an allele (Tibayrenc et al., 1985).

3. Results

3.1. Multilocus enzyme electrophoresis analysis

Multilocus enzyme electrophoresis analysis was performed at 11 isoenzyme loci on all 18 isolates. According to the patterns observed, an allelic interpretation of MLEE variability was performed based on the hypothesis that *T. cruzi* is a diploid organism (Tibayrenc et al., 1986). Each enzyme extract was analyzed at least twice in parallel with the reference strains. Among all the isolates studied (*n* = 18) only one multilocus genotype was detected from the analysis of zymograms. All stocks presented identical electromorphic patterns at the 11 loci analyzed, corresponding to patterns for the Mncl2 reference. Fig. 1 shows the electrophoresis of glucose-6-phospate isomerase (Gpi). Since all isolates exhibited the specific character Gpi 2/4 genotype of the *T. cruzi* IId lineage all stocks were assigned to this Discrete Typing Unit.

3.2. Random amplified polymorphic DNA polymorphism

As all isolates studied were typed within the same lineage *T. cruzi* IId, we performed RAPD with 6 decameric primers in order to assess genetic polymorphism of *T. cruzi* isolates. All amplifications were performed at least twice confirming the reproducibility of the
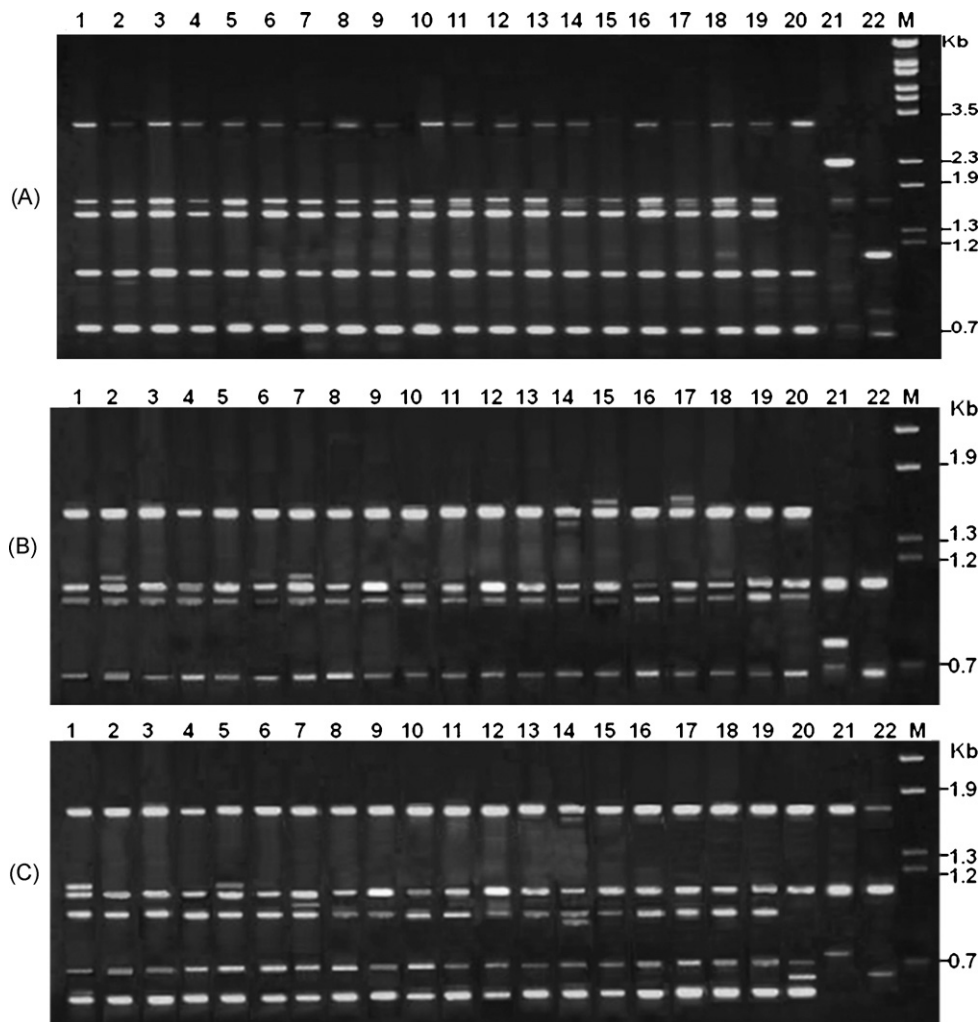

Fig. 2. Agarose gels (1.6%) stained with ethidium bromide showing random amplified polymorphic DNA (RAPD) patterns generated by primers F5 (A), B19 (B) and U14 (C) for 18 *T. cruzi* stocks under study. The numbers above the lanes 1–18 correspond to the stock numbers listed in order in Table 1. Line 19: Mncl2 (reference strain for *T. cruzi* IId), Line 20: CL-Brener (reference strain for *T. cruzi* IIe), Line 21: CANIIIcl1 (reference strain for *T. cruzi* IIa), Line 22: X10cl1 (reference strain for *T. cruzi* I). M: Molecular marker: phage lambda DNA digested with BstEII.
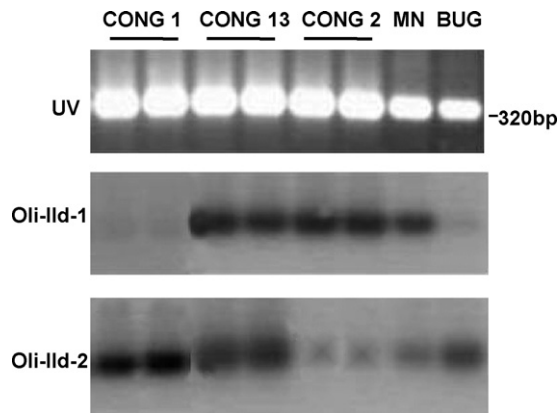
Fig. 3. Relative hybridization of kDNA amplicons with synthetic oligonucleotides probes. The hypervariable regions of *T. cruzi* kDNA were amplified (first line) and the intensity of amplicon fluorescence is compared with autoradiography after hybridization with indicated oligodeoxynucleotides probe Oli-IId-1 and Oli-IId-2 (second and third lines). Amplicons were analyzed in duplicate. MN: Reference strain MNcl2. BUG: Reference strain Bug2148.

patterns. This analysis generally found limited genetic variability among all isolates, since patterns generated with all six primers were very similar. Patterns generated using DNA from Mncl2 reference strains were very similar to those generated for all congenital *T. cruzi* stocks analyzed in the present study. Moreover, the *T. cruzi* IId lineage was specifically identified for all isolates by the presence of an identical pattern obtained with F5 primer that detects specific diagnostic RAPD fragments as previously described (Fig. 2A). Therefore, these results confirm the identity of all stocks as belonging to the *T. cruzi* IId lineage, as previously demonstrated by MLEE analyses. Nevertheless, we detected intraspecific genetic polymorphism among *T. cruzi* isolates using the B19 and U14 primers. Fig. 2B and C shows the patterns obtained with these primers. Pattern differences obtained with these primers were characterized by the absence of some bands in a few isolates, or the presence of an extra band for others.

3.3. T. cruzi typing by hybridization of synthetic oligodeoxynucleotide probes identifying variants of sub-lineage IId

Further characterization with synthetic oligodeoxynucleotide probes was performed for all isolates in order to identify variants of sub-lineage IId, as previously described (Virreira et al., 2006). These probes allow sensitive hybridization of kDNA minicircle derived-amplicons obtained from PCR with primers Tc121/Tc122. The hybridization patterns of the reference strains with Oli-IId-1 and Oli-IId-2 probes allow samples to be classified as the following *T. cruzi* IId variants: MN-like, Bug-like, or TPK-like. Isolates displaying the pattern MN-like and Bug-like hybridized more strongly with Oli-IId-1 and Oli-IId-2, respectively, whereas the TPK-like hybridized equally with both probes. The amplification of the 320-bp was performed in duplicate for all isolates in order to obtain the hybridization pattern. This method allowed *T. cruzi* IId typing of all isolates with the following variant distribution: 6 MN-like, 5 Bug-like, and 7 TPK-like. Hybridization patterns of some stocks are shown in Fig. 3.

The pattern of variation detected in the stocks by RAPD analysis was not correlated with that detected by the hybridization approach (data not shown).

4. Discussion

In the present work we report the genotypic analysis of 18 *T. cruzi* stocks isolated from congenitally infected newborns in the province of Salta, Argentina by using three molecular markers. All isolates were unequivocally typed by MLEE and RAPD as belonging to the *T. cruzi* IId sub-lineage, showing the clear predominance of this sub-lineage among the studied congenitally infected neonates.

Despite the limited number of isolates investigated and the lack of statistical data analysis (both sample size and genetic variability are too small for this purpose) our RAPD analysis showed low genetic polymorphism among isolates with 2 out of 6 decameric primers used in our study. Further analysis by using a new comparative hybridization approach (Virreira et al., 2006) allowed us to detect three variants of the IId with a similar distribution among the isolates studied. This result confirms the previous heterogeneity of this sub-lineage, suggesting the existence of families of lineage-specific sequences (Telleria et al., 2006). Surprisingly, the lack of concordance between the polymorphism revealed by RAPD and the hybridization approach suggests no genetic linkage among these markers, as would be expected for a clonal propagation of this parasite. Further studies are needed to understand the relationship among these molecular markers.

Genetic variability studies in human cultured isolates have been found to belong to a single lineage in other studies (Barnabe et al., 2001; Bosseno et al., 2002; Montilla et al., 2002). Nevertheless identification of *T. cruzi* lineages from cultured stocks may underestimate the parasite diversity originally present in the host. Therefore, we could not discard the possibility that some newborns in our study could be infected by parasites from more than one *T. cruzi* lineage. Indeed, mixed infections with *T. cruzi* I and *T. cruzi* II lineages have been reported in Bolivia and Chile (Breniere et al., 1998; Torres et al., 2004).

Despite the identification of *T. cruzi* lineages from cultured stocks, our results agree with previous works indicating *T. cruzi* IId as the main lineage infecting newborns in Argentina and Bolivia (Virreira et al., 2006; Burgos et al., 2007). These authors found a high prevalence of *T. cruzi* IId (94% and 95%, respectively) in congenital infections by using PCR-based technology that avoids the possible selection of a particular parasite lineage.

In addition, these studies reveal that *T. cruzi* IId was the main lineage in adults of the same area and of mothers who gave birth to uninfected children (Virreira et al., 2006; Burgos et al., 2007). Therefore, the predominance of the IId lineage detected in our study cannot be attributed to a preferential association of this lineage with congenital infection, but rather as indicative of the general distribution of *T. cruzi* lineages in the province of Salta, Argentina.

In this regard, our results agree with previous findings suggesting that, in the Southern Cone countries of Latin America, human infection by *T. cruzi* is due principally to the *T. cruzi* II lineage. In these countries, molecular and immunological markers corroborated that human chagasic infection is due principally to *T. cruzi* II (Breniere et al., 1998; Zingales et al., 1998; Di Noia et al., 2002; Buscaglia and Di Noia, 2003). This contrasts with the genetic characterization of human isolates in Mexico, Colombia and Venezuela where a high predominance of the *T. cruzi* I lineage has been reported (Bosseno et al., 2002; Montilla et al., 2002; Anez et al., 2004).

It is important to point out that *T. cruzi* typing studies are performed mainly from blood samples and consequently the presence of other lineages in infected organs cannot be ruled out. Recent data have shown the presence of different lineages of *T. cruzi* in blood and brain tissue from an Argentinean patient with chagasic reactivation due to AIDS. Interestingly, *T. cruzi* I was only found in the cerebrospinal fluid from this patient and not in peripheral blood, suggesting a tropism of this lineage for target organs (Burgos et al., 2008). These authors suggest that the *T. cruzi* I lineage circulates at higher frequencies in the Southern countries of America and that the low rate of detection of this lineage was

due to the low bloodstream parasitic load as a consequence of higher tropism for target organs (Burgos et al., 2008).

Several host as well parasitic factors might be involved in vertical transmission of *T. cruzi*. Regarding the genetic variability of parasite strains, previous studies do not support a direct association between the *T. cruzi* lineage or minicircle signature with the occurrence of congenital infection (Virreira et al., 2006; Burgos et al., 2007). In cases of transmission, both studies reveal that whole maternal bloodstream populations were transmitted to newborns since sub-lineages of *T. cruzi* were found to be identical in mothers and their neonates.

These findings support the hypothesis that *T. cruzi* infection in human fetus/neonates, or adults, depends more on host resistance/susceptibility governed by its genetic background, as well as environmental and social factors (Hermann et al., 2002; Campbell et al., 2004; Cruz-Robles et al., 2004). This assumption is also reinforced by previous data showing that congenital transmission of *T. cruzi* is associated with high parasitic loads and peripheral deficient immunological responses in mothers (Hermann et al., 2004). In addition, data from our group suggest the association of the HLA-DRB1*08 haplotype from the major histocompatibility complex (MHC) with the congenital transmission of *T. cruzi* in the province of Salta (Sanchez Negrette, unpublished data). Taken together, these data indicate that several other variable features from both parasites and humans have an important influence on the clinical outcome of Chagas disease, in addition to the genetic diversity of *T. cruzi* clonal populations.

In conclusion, data reported in this study confirms for the first time the presence of the *T. cruzi* IId lineage among congenitally infected newborns in the province of Salta, Argentina. These data support the assumption that in the Southern Cone countries of Latin America, human infection by *T. cruzi* is due principally to *T. cruzi* II. Nevertheless, further studies are needed to ascertain the role of both parasite and human factors in the development of the clinical outcome of Chagas disease in addition to the genetic diversity of *T. cruzi* as defined by their lineages.

Acknowledgements

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