British Microbiology Research Journal
4(12): 1299-1310, 2014

SCIENCEDOMAIN *international*
www.sciencedomain.org

Trans Sialidase Genes Allow Clustering of TcI *Trypanosoma cruzi* Mexican Isolates

V. M. Monteón[1*], R. López[1], A. Ramos-Ligonio[2] and K. Acosta-Viana[3]

[1]Center for Biomedical Research, University of Campeche, Mexico.
[2]Ladiser Immunology and Molecular Biology, Faculty of Science, Chemical, Orizaba, VER, Mexico.
[3]Academicians Biomedicine and Infectious Diseases Parasitic, CIR, Yucatan, Mexico.

Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Original Research Article

Received 25th April 2014
Accepted 8th July 2014
Published 20th July 2014

ABSTRACT

Aims: Polymorphism of the trans sialidase (TS) family of genes is common in *Trypanosoma cruzi*. Our goal was to cluster Mexican TcI DTU (Discrete Typing Unit) using a set of primers specific for TS genes.
Methodology: The DNA of 12 Mexican *T. cruzi* stocks (TcI) and reference strain were amplified using the CRP-1 primer, which anneals to the conserved 5' ends of CRP (Complement Regulatory Protein), TS, and FL-160 genes, and the CRP-2 primer, which anneals to conserved region within the GPI (Glycosil Phosphatidil Inositol) anchor sequence. Amplicons were analysed using PCR-SSCP (Single Strand Conformation Polymorphims) followed by construction of a nominal matrix data (presence/absence bands) to calculated the Jaccard and Dice similarity coefficient, and clustering with UPGMA.
Results: Mexican TcI stocks produced a common pattern of amplification products and cluster in a separate group to CL-Brener strain (TcVI). The PCR-SSCP revealed that within the TcI Mexican stocks there were a complex pattern, but *T. cruzi* from the Yucatan peninsula clustered in special and separate group.
Conclusion: The CRP-1 and CRP-2 primers were helpful for the analysis of genetic traits in *T. cruzi* DTU I and revealed the existence of special group in Yucatan Mexico.

**Corresponding author: Email: victormonteon@yahoo.com.mx;*

Keywords: Trypanosoma cruzi; discrete typing unit I; trans sialidase; PCR-SSCP.

1. INTRODUCTION

The surface of *Trypanosoma cruzi* is covered by a large number of GPI-anchored proteins, whose structure and chemical composition have been extensively studied [1] analysis of the *T. cruzi* genome indicated that 12% of the genes encode proteins anchored by GPI. These genes are encoded by thousands of polymorphic members of multigene families, such as trans sialidase (TcTS)/gp85 glycoprotein, with a diverse array of subfamilies, including the following: active trans sialidase, inactive trans sialidase, complement regulatory protein-CRP, Tc85 proteins, flagellum associated protein, the gp90 and gp82 proteins, several members of the Mucin (Mu) family (TcMUC I, TcMUC II, TcMUC III, TcSMUG S, and TcSMUG L), the Mucin- associated surface protein (MASP), and other families, such as Amastin, the gp63 proteins, and Mucin-like proteins [2]. The general structure of a GPI anchor includes a lipid tail containing either a phosphatidylinositol (PI) or an inositolphosphorylceramide (IPC) moiety attached to a glycan core consisting of a glucosamine (GlcN) residue followed by three mannose (Man) residues. In the third Man distal from the GlcN residue, there is usually an ethanol amine phosphate (EtNP) group attaches the GPI to the C-terminus of the protein [3].

Polypeptides targeted for attachment to GPI by transamidation within the endoplasmic reticulum following proteolytic cleavage of the C-terminal propeptide from the proprotein contain a short hydrophobic C-terminal consensus sequence of 14 to 25 amino acids. The site of attachment, which is called a GPI-modification site (ω-site), is a serine in 44% of cases, followed by a moderately polar spacer with a length of about 8–12 residues [4] . GPI-anchored proteins from *T. cruzi* contain a short hydrophobic C- terminal APTPGD sequence included within the last 20 amino-acids residues [2].

At the amino termini, proteins have the Signal Peptide Sequence (SPS), which allows for sorting to different organelles or secretion pathways. The deduced consensus SPS of MASP proteins, which are GPI- anchored and expressed in the cellular membrane, is MAMMMTGRVLLVCALCVLW [5]. In the CRP protein member of TcTS group III, the SPS is MSRNVFASAVLLLVVVMTSCSSEA [6]. In TCNA, a member of TcTS group I, the SPS is MVAIADARTETSSENSLLDTVA [7]. In GP82, a member of TcTS group II, the SPS is MSRRVFDSTILLLLVTTMCCDTCGAA [8].

Thus, members of the TcTS super family show a highly conserved SPS that targets nascent proteins to endoplasmic reticulum (MSRRVF/TSVLLLLF/LV). As consequence conserved sequences are useful to identify related proteins and genes.

On the epimastigote cell surface, the major GPI-anchored proteins expressed are members of the small mucin-like genes (TcSMUG S, whose mature polypeptide are short (56–85 amino acids) and highly glycosylated [2]. Other important GPI-anchored proteins are members of the TcTS family, which contains about 1439 proteins that range in size from 80 to >200 KDa and differ by about 5% in their primary sequence [9]. This data give evidence of molecular weight array of GPI-anchored proteins.

According to molecular weight, function, conserved motif (FRIP: xRxP located before Asp box and closer to N-terminal end; Asp box: SxDxGxTW, and TcS signature: VTVxNVxLYNR), chromosomal localisation, antigenic properties, and sequence identity, the members of TcTS superfamily were re-classified recently into eight groups [10]. Group I

contains active transialidases. Group II contains members without transialidase activity, such as gp82, gp85, gp90, implicated in host cell attachment. Group III consists of members, such as FL-160 and CRP. Group IV is composed of genes, such as TsTc13, with undefined biological function. Groups V and VI are related to group TcS II, and Groups VII and VIII to group TcS III.

Previous sequencing data revealed that there is great diversity in the TcTS family members. This diversity has been used as a classification tool. For example, the FL-160 and CRP genes have parologs in different *T. cruzi* strains, allowed the clustering of parasite strains into six groups that coincide with the previous Discrete Typing Unit (DTU) assignment: TcI, TcII, TcIII, TcIV, TcV, and TcVI [11-12]. It was recently recognised that the TcTS families encoded proteins with TS activity (group I) and TS inactive products (group II); The TS inactive products was observed exclusively in TcII, TcV and TcVI stocks, but was absent in TcI, TcIII, and TcIV stocks [13].

In Mexico, the most prevalent DTU reported is TcI [14-16]. Other DTUs have been found, but with lower frequency [17-19]. Analysis of the 350 bp intergenic region of the mini-exon revealed four subgroups within the TcI DTU, which are designated as genotype Ia-Id [20].

As a first approach to identify polymorphisms, the use of the simple PCR-Single Strand Conformational Polymorphism (PCR-SSCP) technique has been successfully used to detect polymorphisms of several genes, such as cruzipain, mini-exon, 24Salpha rRNA, and 18Sr RNA, in *T. cruzi* [21,22].

Taking advantage of the polymorphisms of TS family genes, in this study, we used the PCR-SSCP technique with a set of primers that annealed to the conserved 5' end of the CRP gene and a common region of TS family of trans sialidase genes to demonstrate diversity in twelve Mexican strains of *T. cruzi* that belong to TcI DTU and the reference CL-Brener strain (TcVI)

2. MATERIALS AND METHODS

2.1 Primers

In this work, we designed a set of primers from Y strain CRP-10 sequence (member of TcTS group III) (Gen Bank accession number U59297) described elsewhere [6]. The primer CRP-1 (5'ACTTACGGCAAGGAGGGCAATTCAAGGAAT-3') anneals to the conserved 5' end of the CRP gene sequence immediately after the SPS (MSRNVFASAVLLLVVVMTSCSSEA) and covers nucleotides 306 to 336, and is a common region in other TcTS members (Primer-blast tool http://www.ncbi.nlm.nih.gov/guide/dna-rna/), including TS/gp85 in the group I, and FL-160 and CRP in the Group III in the CL-Brener and Esmeraldo strains (Gen Bank accession numbers: XM810141.1, XM807757.1, XM802340.1, XM800042.1, X70948.1, U59297.1, XM805583.1, and XM811658.1)

The CRP-2 primer (5'AACACCCGAGACACACACCCATGCACGGTG 3') anneals to a conserved region within the GPI anchor sequence between nucleotides 3169 and 3199 at the 3`end of the CRP gene, and may detect any family member with a GPI anchor. The expected amplification fragment with this set of primers is approximately 2893bp.

2.2 Origin of *Trypanosoma cruzi* Isolates

Human Mexican isolates were donated by Reyes PA [15]. In brief blood sample (approximately 25mL) from seropositive subjects were cultured in LIT medium supplemented with 10% of Fetal Calf Serum for at least for 4 months. Every month the culture was examined for the presence of flagellates. Once positive culture was obtained, passage of parasite was carried out each week. The DNA of twelve *T. cruzi* isolated from diverse geographic regions in Mexico (Fig. 1) that belong to TcI [15,23] and the reference CL-Brener strain (TcVI), Y strain (II), and Sylvio strain (I) were used to amplify the trans sialidase genes.

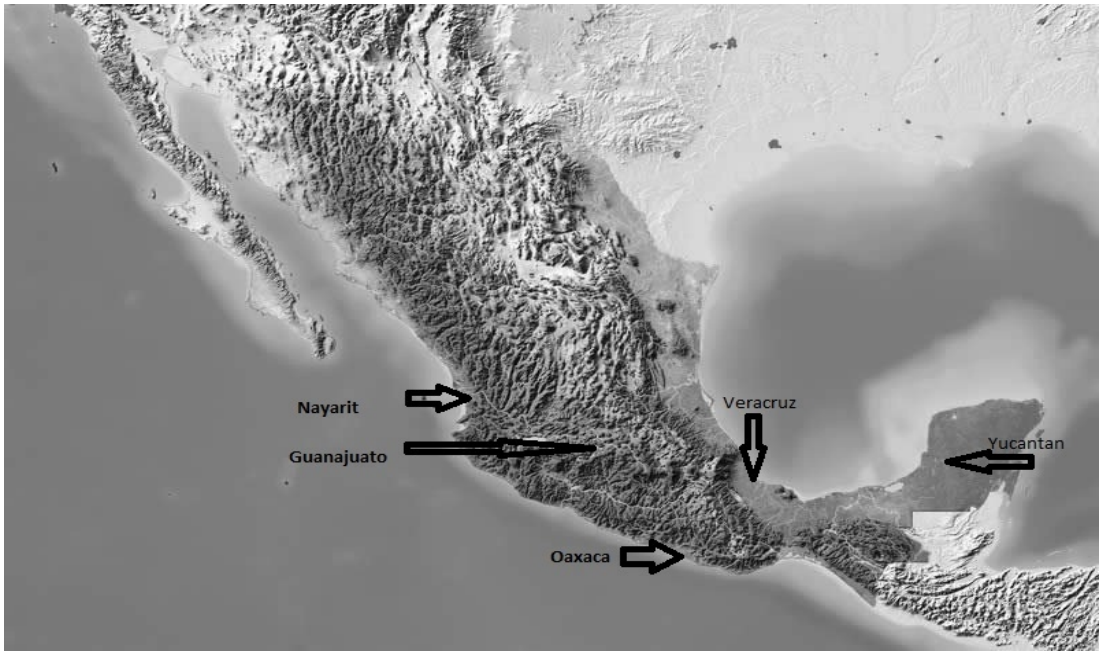

Fig. 1. Region of origin where *Trypanosoma cruzi* were isolated

2.3 PCR Amplification of TS Gene

The PCR reaction was performed in a final volume of 50µL, which contained 10ng of *T. cruzi* DNA, 10µM CRP-1 and CRP-2 primers, and 2.5 units of Taq pol, using the following cycling conditions: 94ºC for 5 min followed by 30 cycles of 95ºC for 1 min, 60ºC for 2 min, 72ºC for 3 min, and a final elongation step at 72ºC for 10min. The amplification products were analysed by electrophoresis on a 1% agarose gel before the Endonuclease Restriction (ER) assay and PCR-SSCP were performed in order to determine the existence of polymorphisms.

2.4 PCR-SSCP (Single Strand Conformational Polymorphism)

For the PCR-SSCP, 5µL PCR product of each *T. cruzi* isolate was mixed with 12µL SSCP loading buffer (95% v/v deionised formamide, 20mM EDTA pH 8.0, 10mM NaOH. 0.05% w/v xylene cyanol FF, and 0.05% w/v bromophenol blue). This mixture was incubated at 95ºC for 5 min and then immediately chilled in a bath of crushed ice to prevent re-hybridisation of the strands. Then, the entire sample was subjected to vertical slab polyacrylamide gel

electrophoresis in an 8% polyacrylamide gel containing 5% glycerol and using 0.5X TBE running buffer for 18 h at 500V, 15mA, and 6 watts at 18ºC. The gel was silver stained and photographed.

2.5 Enzyme Restriction (ER Assay of PCR Products)

The ER assay was incubated for 3h at 37ºC with Alu I, Sal I, and Hea III endonuclease restriction enzymes with the appropriate digestion buffer according to manufacturer's instructions. The reaction mixture consisted of 10µL of PCR product, 2µL of each enzyme, 2µL of 10X buffer, and 8µL of distilled water. The digestion pattern was visualised after agarose gel electrophoresis. The negative control had no restriction enzyme.

2.6 Phylogenetic Analyses

A nominal matrix data (presence/absence bands) using the results obtained with the PCR-SSCP was constructed. Then we calculated the Jaccard and Dice similarity coefficient, which is indicated for nominal data. After clustering with UPGMA, the dendrogram was obtained using the NTSYSpc software.

2.7 Ethical Considerations

The protocol was approved by the Research Committee of Universidad Autonoma de Campeche (2008/FI7FRP). This protocol did not include human beings.

3. RESULTS

All of the Mexican *T. cruzi* isolates belong to DTU Tc I as previously reported [15,23]. They were isolated from humans and vectors in towns located in the Pacific coast (INC- 1, Ninoa, and Nay), the Gulf of Mexico (INC-7-- INC-9), central Mexico (INC-5 and INC-10), and the Yucatan peninsula in the southeast (Camp-6, Camp-7, Calkini, and Calakmul) (Table 1, Fig. 1).

The CRP-1 and CRP-2 primers produce a doublet band of 2750 and 2700, and a third band near to 900bp in Mexican *T. cruzi* isolates, and in the reference CL-Brener strain, the doublet was 2850 and 2500bp (Fig. 2). All Mexican TcI isolates have an identical pattern, which is different from the South-American reference CL-Brener strain Tc VI. However, the Y (Tc II) and Sylvio (Tc I) strains also had a third band of 900bp (data not shown).

Restriction digestion with Sal I (Fig. 3) generates two different restriction patterns in Mexican *T. cruzi* isolates. One pattern includes the INC8 isolate from Veracruz in the Gulf of Mexico, Camp6, Camp7 from the Yucatan peninsula, and Nay from Pacific coast in the north west of Mexico. The rest of isolates make up the second group. It was striking that the reference CL-Brener strain showed a different pattern than the Mexican isolates, in which bands of approximately 900bp and 1700bp are absent. Restriction digestion with Alu in the CL-Brener and Y strains showed a similar pattern, but different than the Mexican and Sylvio strains (data not shown). Digestion with the HeaIII enzyme showed no differences in the banding patterns.

Table 1. General background of the Mexican *Trypanosoma cruzi* isolates

Isolates	From	Location (State/Country)	Tc DTU
MHM/MX/1994/INC-1	Human	Oaxaca/México	I
MHM/MX/1994/INC-5	Human	Guanajuato/México	I
MHM/MX/2001/INC-7	Human	Veracruz/Mexico	I
MHM/MX/2001/INC-8	Human	Veracruz/Mexico	I
MHM/MX/2001/INC-9	Human	Veracruz/Mexico	I
MHM/MX/2000/INC-10	Human	Guanajuato/Mexico	I
TDIM/MX/2007/Cam-6	*Triatoma dimidiata*	Campeche/Mexico	I
TDIM/MX/2007/Cam-7	*Triatoma dimidiata*	Campeche/Mexico	I
TDIM/MX/2007/Calakmul	*Triatoma dimidiata*	Campeche/Mexico	I
TDIM/MX/2007/Calkini	*Triatoma dimidiata*	Campeche/Mexico	I
TPIC/MX/1990/Nay	*Triatoma picturata*	Nayarit/Mexico	I
MHOM/MX/1988/Ninoa	Human	Oaxaca/Mexico	I
CL-B	*Triatoma infestans*	Brazil	VI

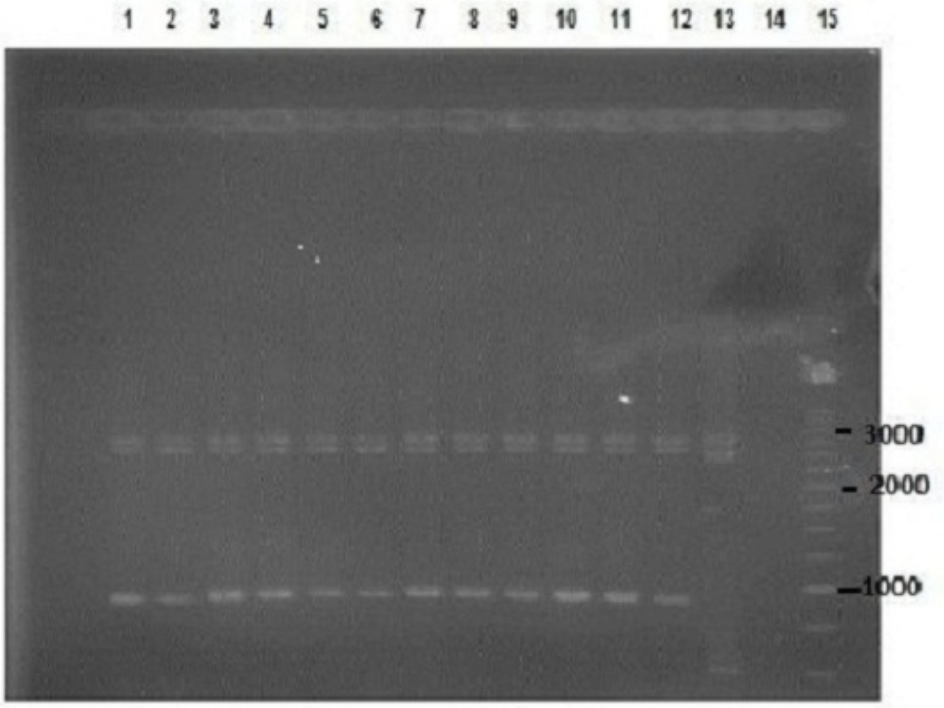

Fig. 2. PCR products of Mexican *Trypanosoma cruzi* isolates with CRP1 and CRP2 primers for CRP and Trans sialidase genes

Lane 1 INC1; lane 2 INC5; lane 3 INC6; lane 4 INC8; lane 5 INC9; lane 6 INC10; lane 7 Camp6; lane 8 Camp7; lane 9 Calak; lane 10 Calki; lane 11 Nay; lane 12 Ninoa; lane 13 CL-Brener

The PCR-SSCP results revealed a more complex banding pattern for the Mexican *T. cruzi* isolates (Fig. 4). With nominal matrix of presence/absence bands and the Jaccard and Dice similarity coefficient allowed to clustering with UPGMA and the dendrogram revealed three main clusters. Cluster 1 includes only isolates from the Yucatan peninsula (Camp6, Camp7,

Calkini, and Calakmul), which are all geographically related and isolated exclusively from Triatoma dimidiata vector. In cluster 2, there were three subclusters: 2a (INC-8, INC-9, and INC-10 isolates), 2b (Nay, Ninoa, and INC-7), and 2c (INC-5 isolates). While they are not geographically related, all of them were isolated from human cases, with the exception of the Nay isolate. Cluster 3 had only INC-1, which was isolated from a chronic human case in the Pacific coast in Oaxaca. It was the same as the Ninoa isolates, but clustered in group 2b. An expected finding was that the CL-Brener strain clustered in a separate group from Mexican strains (Fig. 5).

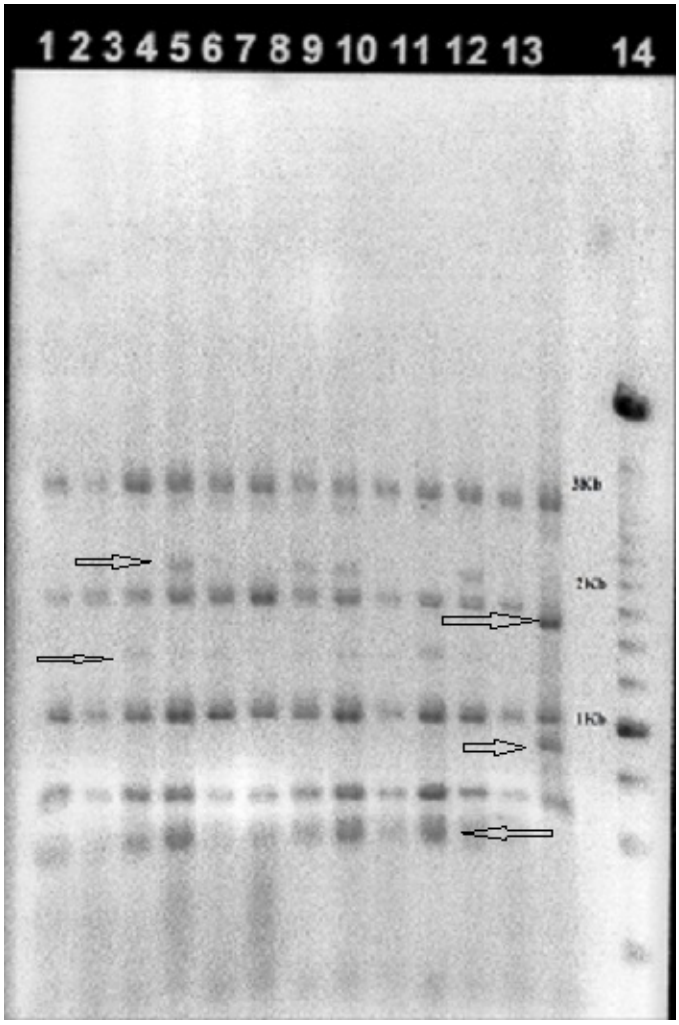

Fig. 3. Restriction digestion with Sal I of PCR products of Mexican *Trypanosoma cruzi* isolates and CL-brener strain

Lane 1 INC1; lane 2 INC5; lane 3 INC6; lane 4 INC8; lane 5 INC9; lane 6 INC10; lane 7 Camp6; lane 8 Camp7; lane 9 Calak; lane 10 Calki; lane 11 Nay; lane 12 Ninoa; lane 13 CL-Brener

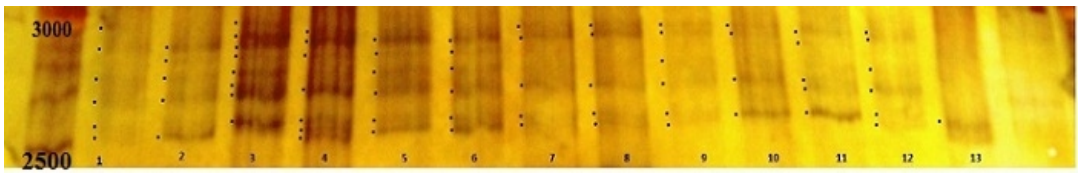

Fig. 4. PCR-SSCP patterns of the CRP and trans sialidase genes in the Mexican *Trypanosoma cruzi* isolates

Lane 1 INC1; lane 2 INC5; lane 3 INC6; lane 4 INC8; lane 5 INC9; lane 6 INC10; lane 7 Camp6; lane 8 Camp7; lane 9 Calak; lane 10 Calki; lane 11 Nay; lane 12 Ninoa; lane 13 CL-Brener. In the left lane it is indicated the molecular marker

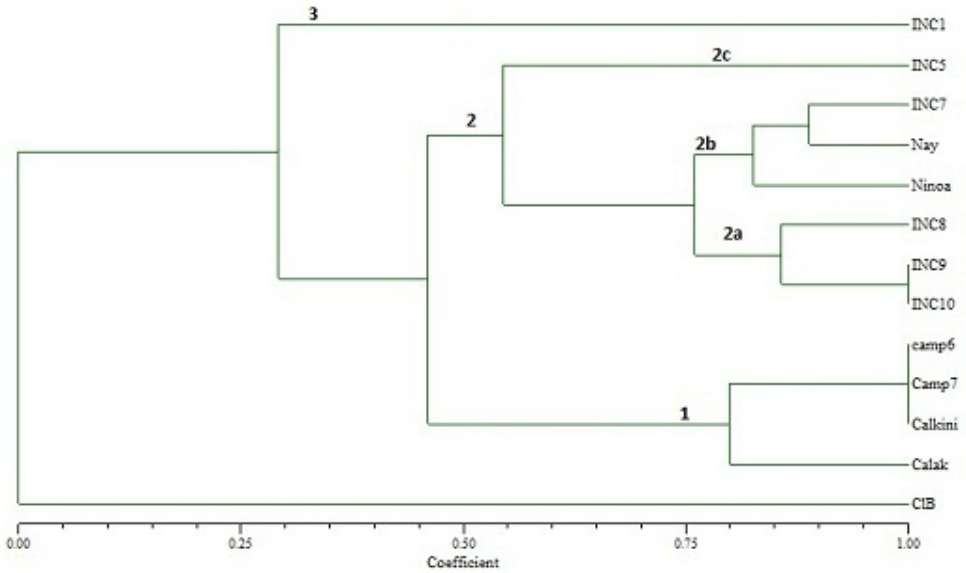

Fig. 5. PGMA tree of the CRP and Trans sialidase genes in Mexican and CL-Brener *Trypanosoma cruzi*

4. DISCUSSION

According to Primer-blast tool (http://www.ncbi.nlm.nih.gov/guide/dna-rna/), the CRP1 and CRP2 primers can anneal TS in group I, gp85 in group II, and FL-160 and CRP in group III. The PCR product from the CRP gene is about 2893bp, and 2870 for the Trans-sialidase genes. In theory, three kinds of TS members can be detected. The Mexican *T. cruzi* isolates produced three products that were 2700, 2750, and 900bp, while the CL-Brener strain had two products, one larger than 2750 bp and a second band close to 2500bp (Fig. 2). This finding is reasonable since the primers could amplify members of three different families of TcTS. In theory TS, CRP, and FL-160 gene products are expected with similar size, and only the gp85 product would be smaller. The most interesting finding is that all Mexican stocks belonging to TcI DTU, including the Sylvio strains (Tc I), showed the same pattern, which was clearly different to the reference CL-Brener strain, which belonged to TcVI DTU (Fig. 2). This simple PCR method can be useful for distinguishing TcI from TcVI, but not Y strain, which belongs to TcII (data not shown). It is known that in Mexico, the most prevalent DTU reported is TcI [14-16].

The restriction patterns after digestion of the PCR products with the Sal I enzyme generated three different patterns. Two of which included the Mexican strains and one that more clearly differentiated the CL-Brener strain (Fig. 3). The first group consists of INC-8, INC-9, Camp7, Calak, and Nay *T. cruzi* isolates. With the exception of the Nay *T. cruzi* isolates, all came from the Gulf of Mexico in Veracruz and Campeche states, where the main vector was *Triatoma dimidiata*. The other group contains the rest of the Mexican isolates, including the Y and Sylvio reference strains (data not shown). The restriction pattern resulting from Alu I digestion produced a similar pattern in the Mexican and Sylvio strains, but it was different in the Y strain (TcI; data not shown). These findings were in agreement with previous reports that the TS family of genes are heterogeneous, but that *T. cruzi* stocks clusters coincide with previous DTU assignments [11-13].

It is accepted that *T. cruzi* is a monophyletic, but genetically heterogeneous group and that this genetic diversity seems to be correlated with geographical distribution, evolution in the insect vector, and human infection [24]. In this context, our findings seems to be consistent with this point of view since Mexican *T. cruzi* stocks share geographic region, reservoirs, and insect vectors, whereas CL-Brener is distributed in south America and clusters in separate group.

Very interesting findings were observed in the UPGMA tree based on nominal data (presence/absence bands). First, all of the *T. cruzi* isolated in Yucatan Mexico clustered together in a separate group from the rest of the Mexican strains. It is known that the main and exclusive vector in the Yucatan Peninsula of Mexico is *Triatoma dimidiata.* In traits and behaviours [25]. Taking into account that the transmission cycle is mainly sylvatic and peridomestic, and that only one vector is implicated in this region, it is likely that circulating parasites in Yucatan, Mexico are composed of very few *T. cruzi* clones and, as a consequence, form a more homogeneous population and cluster together. However, in order to confirm this hypothesis, it is necessary to carry out sequencing of clones from different *T. cruzi* stocks from this region.

The remaining eight *T. cruzi* Mexican isolates were not geographically related, but clustered in two main branches (2 and 3). They were more dissimilar or heterogenous, and probably consist of many clones spread throughout the country. All of them were isolated from humans, with exception of Nay strain, which was isolated from the *T. picturata* vector. In group 2a (stocks INC8, INC9, and INC10), all stocks were isolated from asymptomatic human cases and clustered together. Group 2b contains the parasites isolated from an acute and asymptomatic human case, and from a vector.

In spite of the pattern in PCR-SSCP in our work represent the sum of polymorphisms of the different gene TS family, it resulted helpful to gather *T. cruzi* stocks depending on genetic traits. A striking finding is that the CL-Brener strain (TcVI DTU) clustered in a separate group (Figs. 4,5). Our findings are in agreement with a recently published paper [13], in which the authors reported the existence of differential distribution of the genes encoding the virulence factor trans sialidase along the *T. cruzi* DTU.

In our work, we demonstrated that the use of CRP-1 and CRP-2 primers, in combination with PCR-SSCP, can be used to analyse the genetic traits within *T. cruzi* Mexican DTU I, which allowed us to identified a very special group in Yucatan Mexico.

This work received financial support from Conacyt grant 153764.

5. CONCLUSION

The CRP-1 and CRP-2 primers were helpful for the analysis of genetic traits in *T. cruzi* DTU I and revealed the existence of special group in Yucatan Mexico.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Previato JO, Jones C, Xavier MT, Wait R, Travassos LR, Parodi J, *Mendonça-previato L.* Structural characterization of the major glycosylphosphatidylinositol membrane-anchored glycoprotein from epimastigote forms of *Trypanosoma cruzi* Y-strain. Journ Biol Chem. 1995;270(13):7241–7250.
2. Nakayasu ES, Yashunsky DV, Nohara LL, Torrecilhas AC, Nikolaev AV, GPIomics: Global analysis of glycosylphosphatidylinositol-anchored molecules of *Trypanosoma cruzi*. Mol System Biol. 2009;261:1-17 DOI: 10.1038/msb.2009.13. Epub 2009 Apr 7.
3. Ferguson MA. The structure, biosynthesis and functions of glycosylphosphatidyl inositol anchors, and the contributions of trypanosome research. Journ Cell Scient. 1999;112(pt17):2799–2809.
4. Eisenhaber G, Bork P, Eisenhaber F. Sequence properties of GPI-anchored proteins near to ω-site: Constraints for the polypeptide binding site of the putative transamidase. Protein Engineering. 1998;11(12):1155–1161.
5. De Pablos L, Osuna A. Conserved regions as markers of different patterns of expression and distribution of the Mucin-Associated Surface Proteins *Trypanosoma cruzi*. Infect Immun. 2012;80(1):169-174.
6. Norris K, Schrimpf J, Szabo M. Identification of the gene family encoding the 160-Kilodalton *Trypanosoma cruzi* complement regulatory protein. Infect Immun. 1997;65(2):349–357.
7. Pereira, M, Mejia S, Ortega-Barria E, Matzilevich D, Prioli R. The *Trypanosoma cruzi* neuraminidase contains sequence similar to bacterial neurminidase YWTD repeats of the low density lipoprotein receptor and type III modules of fibronectin. Journ Exp Med. 1991;174(July):179-191.
8. Correa P, Cordero E, Gentil L, Bayer-Santos E, Da Silveira J. Genetic structure and expression of the surface glycoprotein GP82, the main adhesion of *Trypanosoma cruzi* metacyclic trypomastigotes. The Scientific World Journal; 2013. DOI: 10.1155/2013/156734.
9. Cremona M, Campetella O, Sanchez D, Frasch A. Enzymatically inactive members of trans-sialidase family from *Trypanosoma cruzi* display beta-galactose binding activity. Glycobiology. 1999:9(6):581–587.
10. Freitas L, Dos Santos S, Rodrigues-Luiz G, Mendes T, Rodrigues T, Gazinelli R, Teixeira S, Fujiwara R, Bartholomeu D. Genomic analyses, gene expression and antigenic profile of the Trans-sialidase superfamily of *Trypanosoma cruzi* reveal and undetected level of complexity. Plos one. 2011;6. DOI: 10.1371/journal.pone.0025914.
11. Beucher M, Norris K. Sequence diversity of the *Trypanosoma cruzi* complement regulatory protein family. Infect Immun 2008;76(Feb): 750–758.

12. Mathieu-Daudé F, Lafay B, Touzet O, Lelièvre J, Parrado F, Bosseno M, Rojas M, Fatha S, Ouaissi A, Brenière S. Exploring the FL-160-CRP gene family trough sequence variability of the complement regulatory protein (CRP) expressed by the trypomastigote stage of *Trypanosoma cruzi*. Infect Gen Evol. 2008;8:258–266. DOI10-1016/j.meegid2007.12.010.
13. Burgos JM, Risso MG, Brenière SF, Barnabé C, Campetella O, Leguizamón MS. Differential distribution of genes encoding the virulence factor trans-sialidase along *Trypanosoma cruzi* Discrete typing units. PLoS One. 2013;8:e58967. DOI:10.1371/journal.pone.0058967.
14. Bosseno M, Barnabé C, Magallón-Gastellum E, Lozano- Kasten F, Ramsey J, Espinoza B, Brenière F. Predominance of *Trypanosoma cruzi* I lineage in Mexico. Journ Clin Microbiol. 2002;40(2):627–632.
15. Ruíz-Sánchez R, De León P, Matta V, Reyes PA, López R, Jay D, Monteón V. *Trypanosoma cruzi* isolates from Mexican and Guatemalan acute and chronic chagasic cardiopathy patients belong to *Trypanosoma cruzi* I. Mem Inst Oswaldo Cruz. 2005;100(3):231–234.
16. Martinez I, Nogueda B, Martinez-Hernandez F, Espinoza B. Microsatellite and Mini-Exon analysis of Mexican human DTU I *Trypanosoma cruzi* strains and their susceptibility to nifurtimox and benznidazole. Vector Borne Zoonotic Disease. 2013;13(3):181–187.
17. Risso G, Sartor P, Burgos J, Briceño L, Rodríguez EM, Guhl F, Chavez OT, Espinoza B, Monteón VM, Russomando G,Schijman AG, Bottasso OA, Leguizamón MS. Immunological identification of *Trypanosoma cruzi* lineages in human infection along the endemic area. Am J Trop Med Hyg. 2011;84(1):78–84.
18. Ibañez-Cervantes G, Martínez-Ibarra A, Nogueda-Torres B, López-Orduña E, Alonso AL, Perea C, Maldonado T, Hernández JM, León-Avila G. Identification by Q-PCR of *Trypanosma cruzi* lineage and determination of blood meal sources in triatomine gut samples in Mexico. Parasitol International. 2013;62(1):36–43.
19. Ramos-Ligonio A, Torres-Montero J, López-Monteon A, Dumonteil E. Extensive diversity of *Trypanosoma cruzi* discrete typing units circulating in Triatoma dimidiata from central Veracruz, Mexico. Infect Genet Evol. 2012;12(7):1341–1343.
20. Falla A, Herrera C, Fajardo A, Montilla M, Vallejo GA, Guhl F. Haplotype identification within *Trypanosoma cruzi* I in Colombian isolates from several reservoirs, vectors, and humans. Acta Trop. 2009;110:15-21. DOI:101016/j-actatropica. 2008.12.003.
21. De Leon MP, Yanagi T, Kikuchi M, Mu J, Ayau O, Matta V, Paz M, Juarez S, Kanbara H, Tada I, Hirayama K, Characterisation of *Trypanosoma cruzi* populations by DNA polymorphism of the cruzipain gene detected by single-stranded DNA conformation polymorphism (SSCP) and direct sequencing. Int Journ Parasitol. 1998;28(12):1867–1874.
22. Higo H, Miura S, Agatsuma T, Mimori T, Yanagi T, Iwagami M, De Arias AR, Matta V, Hirayama K, Takeuchi T, Tada I Identification of *Trypanosoma cruzi* sublineages by the simple method of single-stranded conformation DNA polymorphism (SSCP). Parasitol Res. 2007;100:1023–1031.
23. Monteon V, Alducin C, Hernandez J. Ramos-Ligonio A, Lopez R. High frequency of human blood in *Triatoma dimidiata* captured inside dwellings in a rural community in the Yucatan Peninsula, Mexico, but low antibody seroprevalence and electrocardiographic findings compatible with Chagas disease in humans. Am Journ Trop Med Hyg. 2013;88:566–571.

24. Zingales B, Miles M, Campbell D, Tibayrenc M, Macedo AM, Teixeira MM, Schijman AG, Llewellyn MS, Lages-Silva E, Machado CR, Andrade SG, Sturm NR. The revised *Trypanosoma cruzi* subspecific nomenclature: Rationale, epidemiological relevance and research applications. Infection Genetic Evolution. 2012;12:240–253.
25. Dorn P, Calderon M, Melgar S, Moguel B, Solorzano E, Dumonteil E, Rodas A, De la Rua N, Garnica R, Monroy C. Two distinct *Triatoma dimidiata* (Latrulle, 1811) Taxa are found in sympatry in Guatemala and Mexico. Plos Neglected Tropical Disease. 2009;3:393–408.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=605&id=8&aid=5412