Trypanosoma cruzi in Mexican Neotropical vectors and mammals: wildlife, livestock, pets, and human population

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Abstract

Objective. To provide primary evidence of *Trypanosoma cruzi* landscape genetics in the Mexican Neotropics. **Materials and methods.** *Trypanosoma cruzi* and discrete typing units (DTU) prevalence were analyzed in landscape communities of vectors, wildlife, livestock, pets, and sympatric human populations using endpoint PCR and sequencing of all relevant amplicons from mitochondrial (kDNA) and nuclear (ME, 18S, 24Sα) gene markers. **Results.** Although 98% of the infected sample-set (N=2 963) contained single or mixed infections of DTUI (TcI, 96.2%) and TcVI (22.6%), TcIV and TcII were also identified. Sensitivity of individual markers varied and was dependent on host taxon; kDNA, ME and 18S combined identified 95% of infections. ME genotyped 90% of vector infections, but 60% of mammals (36% wildlife), while neither 18S nor 24Sα typed more than 20% of mammal infec-

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Resumen

Objetivo. Generar evidencia primaria sobre la genética del paisaje de *Trypanosoma cruzi* en el Neotrópico mexicano. **Material y métodos.** La prevalencia de *T. cruzi* y de las unidades taxonómicas discretas (DTU, en inglés) fueron analizadas en comunidades simpátricas de vectores, fauna silvestre, pecuarios, mascotas y población humana mediante PCR de punto final y secuenciación de marcadores genéticos mitocondriales (kDNA) y nucleares (ME, 18S, 24Sα). **Resultados.** A pesar de que 98% de las muestras infectadas (N=2 963) contenía infecciones únicas o mixtas de DTUI (TcI, 96.2%) y TcVI (22.6%), TcIV y TcII también fueron identificados. La sensibilidad de los marcadores individuales varió según el taxón del reservorio; la combinación de kDNA, ME y 18S identificó 95% de las infecciones. ME tipificó 90% de las infecciones por vectores, pero sólo 60% de los mamíferos (36%

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tions. **Conclusion.** Available gene fragments to identify or genotype *T. cruzi* are not universally sensitive for all landscape parasite populations, highlighting important *T. cruzi* heterogeneity among mammal reservoir taxa and triatomine species.

Keywords: *Trypanosoma cruzi*; landscape; lineages; reservoirs; Triatominae

en fauna silvestre), mientras que el 18S y el 24Sα tipificaron menos que 20% de las infecciones en mamíferos. **Conclusión.** Los fragmentos de genes disponibles para identificar o genotipificar *T. cruzi* no son universalmente sensibles para todas las poblaciones de parásitos del paisaje, y destaca la importante heterogeneidad entre los taxones de reservorios y las especies de triatominos.

Palabras clave: *Trypanosoma cruzi*; paisaje; linajes; reservorios; Triatominae

Trypanosoma cruzi is a parasite of new world mammals, geographically widespread in the American continent.[1,2] It is also the etiologic agent of Chagas Disease (CD), which is a major public health problem in all Latin American countries and increasingly in the United States, Europe, and Asia.[3,4] In Mexico, *T. cruzi* has been characterized and molecularly identified in bugs,[5-10] wildlife,[6,8,11] livestock and companion animals,[6,12] as well as in human populations.[6,13]

Molecular markers used to identify *T. cruzi* and genotype its discrete typing units (DTUs) differentiate six different discrete typing units (TcI, II, III, IV, V, VI) and one additional lineage for TcBat.[14] *Trypanosoma cruzi* and DTU identification in reservoirs and vectors requires the use of multiple gene fragments, since no individual marker is universally sensitive,[15,16] all have variable levels of methodological complexity and specificity affected by technical factors (volume and sample conservation, DNA isolation method, amplification conditions, gene sequences analyzed, and primer design).[16-20] The most commonly analyzed fragments and for which most reference sequences exist are the spliced leader mini-exon (ME), 18S rRNA (18S), 24Sα rRNA (24Sα) and minicircle kinetoplast DNA (kDNA), developed from *in vitro* selected South American (SA) isolates or clones.[20-25] There have been few analyses and evidence for marker sensitivity for all lineages/DTUs from native infections across North America (NA), or across ecotope-relevant host assemblages.[6,23,26-29] Natural infections are expected to be multi-clonal in all reservoirs[27,30,31] and hence methods to detect infections must overcome the difficulty of simultaneously amplifying multiple sequences and be specific for all parasite DTUs and haplotypes, ideally universal (conserved fragments) for all populations.[26,32,33]

The aim of the present study was to provide primary evidence of *T. cruzi* landscape genetics to detect and genotype *T. cruzi* in vectors and primary mammal reservoirs from seven neotropical Mexican landscapes using a multi-locus approach, and analyze the sensitivity and specificity of key primer sets for kDNA, ME, 18S, and 24Sα gene fragments. A better understanding of the reservoirs and diversity of *T. cruzi* populations circulating in sylvatic and human modified landscapes will allow us to design effective barriers for parasite dispersal to humans, develop more sensitive diagnostic tools for exposed human populations, and monitor the parasite landscape genetics.

Materials and methods

Ethics statement

Studies were conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the *Instituto Nacional de Salud Pública* (INSP), from Mexico (Ethics Committee Projects 1063, 1237, and 1369 to Janine M Ramsey). All subjects gave their informed consent for inclusion before they participated in the study, and all provided approval for secondary use of preserved blood sample aliquots. No human specimen is connected to any personal information. Wildlife were sampled according to international[34] and national (*Secretaría de Medio Ambiente y Recursos Naturales*, Sermarnat) regulations and collection permits (FAUT-023 to Janine M Ramsey), regional ejido community approval, and INSP Biosecurity and Ethics Committee approvals (#491, 1063, 1237, 1369), including informed consent by pet or livestock owners for blood sample extractions.

Sample origin

Samples from mammal wildlife, livestock, companion animals (pets), human population, and triatomines within the Mexican neotropical realm were analyzed from multiple integrated studies and sites between 2008 and 2017 in Campeche (19°50′55″N, 90°31′31″W), Chiapas (16°24′36″N, 92°24′31″W), Oaxaca (16°53′53″N, 96°24′51″W), and Morelos (18°44′51″N, 99°04′13″W) (figure S1, table S1 and S2[35]). Briefly, vectors were collected either by inhabitants in and around houses or technical personnel in landscapes which included domestic fragment (housing community), or using various collection methods (light traps, animal bait) in ecotone

(agricultural and livestock grazing areas) and sylvatic (conserved) habitats.[6,8,36,37] Specimens of *Triatoma dimidiata* haplogroup 1 (Hg1), *T. dimidiata* haplogroup 2 (Hg2), *T. dimidiata* haplogroup 3 (Hg3), *Triatoma pallidipennis*, *Triatoma phyllosoma*, and *Panstrongylus rufotuberculatus* were taxonomically identified and preserved in 70% EtOH, as previously described.[8] To determine which haplogroup of *T. dimidiata* was analyzed, ND4 fragments were amplified and sequenced, as previously reported.[36]

Wildlife specimens were collected as previously described across landscape habitats,[6,8] taxonomically identified, euthanized, and tissues (exclusively from heart tissue are reported herein) were preserved immediately in 95% molecular grade EtOH or DNazol (Invitrogen, San Diego, California, USA). Blood samples from livestock (5ml), pets (3ml) and one protected bat species (*Myotis velifer*; 0.3 ml) were drawn in guanidine buffer. In the framework of community-based Chagas surveillance and prevention programs (Santos Reyes Nopala and Salina Cruz counties in Oaxaca, Berriozabal county in Chiapas, and Zoh Laguna in Campeche), and based on self-recognition for bug exposure (correct bug recognition, having lived with bugs in homes, contact resulting in a "chinchoma" inflammation, or children of infected mothers), inhabitants requested diagnosis for *T. cruzi* infection. Following informed consent and assent, including permission for subsequent use of remaining aliquots, a blood sample (5ml) was drawn in EDTA for standard serology (2-5 tests only for humans) and an additional sample (5ml) preserved in guanidine buffer for molecular *T. cruzi* detection.[6]

Molecular detection of *T. cruzi*

Genomic DNA was extracted from mammal heart tissues (approximately 50% of total tissue if small specimens and 10% from medium-sized specimens) and bug midgut contents using DNAzol (Invitrogen, San Diego, California, USA) following manufacturer's instructions and from venous blood (exposed human population, livestock, pets, and one protected bat species) preserved in guanidine buffer using a phenol-chloroform protocol.[38] Extracted DNA was re-suspended in 80 μL of nuclease-free water and maintained at -20 °C prior to amplification. Primers from one mitochondrial and three nuclear genes (four of the five genes having most *T. cruzi* sequences registered in GenBank) were used to amplify samples. The conserved region of the kDNA (S34/S67primers) was chosen as the primary multiple copy mitochondrial fragment for *T. cruzi* infection in all taxa as previously described.[25] Satellite (SAT) DNA was not used due to lower specificity of amplicons from wildlife taxa, which would have created a bias due to non-specific amplification of mammal DNA from most taxa.[39] All samples were analyzed herein using the kDNA and the conserved repeat of the spliced leader mini-exon (ME), and all samples amplified using either marker, 100% of kDNA negative vector, bat and human samples, and a random selection of 25% of all other kDNA negative samples were also amplified using primers for the small subunit rDNA 18S gene using the primers SSU561F/SSU561R (N=2103).[22] A random selection of 70% of kDNA positive samples and all equine and livestock samples were also analyzed using the 24Sα gene using the D71/ D72 primers.[40] All amplicons of expected band size of the kDNA (120bp), those between 300 and 350 bp of the ME, all amplification products between 520-720 bp of the 18S, and all 24S amplicons within the range of 110 to 125 bp were sequenced. *Trypanosoma cruzi* DNA from Mexican TcI parasite strain CARI06 and TcVI (CL-Brener), and negative amplification controls were run with samples, and all samples not amplifying parasite controls were analyzed using the cyt *b*;[39] samples of questionable DNA quality represented <1% of all samples and were eliminated from analyses. Electrophoresis bands of expected size from PCR products (kDNA, ME, 18S, 24Sα) were purified using QIAquick Gel Extraction kits (QIAGEN, Valencia, CA) and to determine the amplification specificity of gene marker tools (primers), capillary sequencing was carried out on an Applied Biosystems 3730XLs (Macrogen, Korea). To avoid cross-contamination of amplicons among specimens and positive controls, internal plate controls for sequencing quality were included.

Forward and reverse sequences from all samples were used to generate consensus using MEGA v.10.[41] A specimen was considered infected if the sequence of at least one gene fragment had identity (GenBank) to *T. cruzi*. Representative haplotypes were deposited in GenBank: accession numbers for the 18S fragments: MW326771-MW326773; accession numbers for the ME fragments: MW520200-MW520203; accession numbers for the kDNA fragments: OQ236562-OQ236565.

Trypanosoma cruzi infection prevalence was calculated as the number of *T. cruzi* positive samples using the combined results of the four gene markers/total samples analyzed.

Statistical analyses

Type I error for parasite detection has been reduced by classifying presence of *T. cruzi* based only on confirmed sequence identity (not only amplification of expected size bands), while type II error was reduced by using a multi-locus approach. Specificity of gene markers and sensitivity of each as well as the multiple loci approach

were analyzed initially from vectors, since we assumed no quantitative bias of parasite load in bugs. The success to genotype *T. cruzi* samples was calculated individually for each 18S, ME, and 24Sα genes, as well as for their combined result, using sequence identity (number of samples amplifying/sequence with all or individual gene/total *T. cruzi* samples). Sensitivity of any gene to detect infection was calculated from the number of sequences with identity/total *T. cruzi* samples. Specificity of individual markers for *T. cruzi* in each host taxon was calculated as the ratio of *T. cruzi* sequences/all samples amplifying expected-size band for the specific marker. *Trypanosoma cruzi* infection rates, success to genotype, DTU prevalence, and sensitivity or specificity of genes were analyzed for independence at species and taxa (order) levels, assuming all host taxa have similar average rates, using a Bonferroni-corrected chi-square, and 95% confidence intervals.

Results

Trypanosoma cruzi infection overall was 19.6%, 36.0% from six triatomine species (N= 814; table S1[35]) and 13.4% from 58 mammal species (N= 2 149; table S2[35]). TcI was the dominant DTU (96.2%) followed by TcVI (22.6%) for all reservoirs, while DTUII was identified in only one rodent species, and DTUIV only identified in two vector species. Parasite populations were genotyped from 78.8% of infected triatomines but only 48.8% from infected mammal reservoirs. Sensitivity of the ME gene was 61.2% overall (45.0% mammals, 77.1% triatomines), while for the 18S it was 21.0% (18.3% mammals, 23.5% triatomines), and 14.3% for the 24Sα (18.7% mammals, 9.9% triatomines).

Trypanosoma cruzi populations in triatomine vectors

Trypanosoma cruzi infection rates for five vector species collected from all sites (figure S1, table S1 and S2[35]) are reported in table I; a combined "other triatomine" group, is included having three specimens of *P. rufotuberculatus* and 105 specimens of *T. dimidiata* which had not been individually genotyped, the latter from communities where haplogroups Hg1 (30%) and Hg2 (70%) were sympatric. Infection prevalence was significantly high in *T. dimidiata* Hg1 (χ^2=14.64; df= 5; p<0.0001) and *T. pallidipennis* (χ^2=7.21; df= 5; p<0.01), but significantly low in *T. dimidiata* Hg2 (χ^2=19.15; df = 5; p<1.2E-5). Only 74.1% overall of amplified kDNA sequences had identity to *T. cruzi*, the remaining infections were confirmed using one or more of the three nuclear genes (ME, 18S, or 24Sα). By combining results from the kDNA and ME, all *T. cruzi* infections were detected in three species (*T. pallidipennis*, *T. phyllosoma*, *T. dimidiata* Hg3), while remaining infected samples (15%) of *T. dimidiata* Hg1 and *T. dimidiata* Hg2 were detected using the 18S gene (figure S2[35]). Comparatively, kDNA specificity was significantly lower for *T. cruzi* populations from *T. dimidiata* Hg2 (figure S2; χ^2=24.88; df=5; p<6.1E-7[35]).

Genotype success rates (calculated from combined results of nuclear genes) and TcI prevalence were similar in parasite populations from all triatomine species, with 78.8% of all infected triatomines genotyped (table I, figure 1A, table S3[35]). The TcIA subtype was identified in 60.4% of all TcI populations, significantly high in *T. pallidipennis* (81.2%; χ^2=29.65; df=5; p<0.0001), but significantly low in *T. dimidiata* Hg3 (0.0%; χ^2=5.89; df=5; p<0.01) (table I). None of the remaining non-TcIA populations had identity to other registered GenBank subtypes. Neither TcIV nor TcVI were amplified from either *T. dimidiata* Hg3 or *T. phyllosoma*, while TcIV was not amplified from *T. dimidiata* Hg1. Both TcIV (2.2% overall) and TcVI (17.3% overall) were similarly prevalent in *T. dimidiata* Hg2 and *T. pallidipennis* (figure 1A).

Individual nuclear gene sensitivity to amplify parasite populations among vector species was not uniform (figure S2, table S3[35]). ME sensitivity was moderately high overall (77.1%) and similar among parasite populations from all triatomine species, although that for the 18S gene was significantly low (23.5% overall). The 18S sensitivity was significantly high in *T. dimidiata* Hg1 (48.0%, χ^2=8.30; df=5; p<0.01), and it was significantly low in *T. pallidipennis* (8.8%, χ^2=13.83; df=5; p<0.001). The 24Sα sensitivity was significantly high in *T. dimidiata* Hg2 (100.0%, χ^2=8.93; df=5; p<0.01) and significantly negative for parasite populations in *T. pallidipennis* (χ^2=12.52; df=5; p<0.001), also did not amplify in either *T. dimidiata* Hg3 (N=10) or *T. phyllosoma* (N=23). Specificity of *T. cruzi* amplicons was on average high using the ME (85.9%), 18S (86.3%) and 24Sα (85.3%) (table S3[35]).

It is worthwhile mentioning that the three *P. rufotuberculatus* specimens included in "other triatomines" were all *T. cruzi* TcIA infected, and parasite populations from the three amplified using kDNA, ME, 18S and 24Sα (2 of 3 amplified using the latter). *Panstrongylus rufotuberculatus* specimens were co-collected (same nest) with 20 infected specimens of *T. dimidiata* Hg1, of which 66.7% were identified infected using kDNA, 83.3% using the ME, and 75% using the 18S (none of 20 amplified using the 24Sα).

Table I

TRYPANOSOMA CRUZI INFECTION PREVALENCE, TOTAL POPULATION (N), AND CONFIDENCE INTERVAL > 95%CI FOR INDIVIDUAL *DIMIDIATA* AND *PHYLLOSOMA* COMPLEX SPECIES AND *PANSTRONGYLUS RUFOTUBERCULATUS*,* SENSITIVITY OF KDNA (SEQUENCE IDENTITY GENBANK/TOTAL INFECTED), OVERALL GENOTYPE SUCCESS OF INFECTED SPECIMENS,‡ PREVALENCE OF *T. CRUZI* LINEAGES TCI, SUBTYPE TCIA, TCIV AND TCVI IN GENOTYPED POPULATIONS

	Infection T. cruzi % (N)	*Sensitivity kDNA % (N)*	*Genotype success‡ % (N)*	*Prevalence*			
				TcI % (N)	*TcIA % (N)*	*TcIV % (N)*	*TcVI % (N)*
T. dimidiata Hg1	**65.8§ (38)**	84.0 (25)	88.0 (25)	100.0 (22)	50.0 (22)	0.0 (22)	18.2 (22)
T. dimidiata Hg2	**24.0# (308)**	**48.6# (74)**	89.2 (74)	100.0 (66)	50.0 (66)	1.5 (66)	24.2 (66)
T. dimidiata Hg3	24.4 (41)	100.0 (10)	50.0≠ (10)	100.0 (5)	**0.0& (5)**	0.0 (5)	0.0 (5)
T. pallidipennis	**43.8≠ (260)**	81.6 (114)	72.8 (114)	96.4 (83)	**81.2§ (80)**	2.4 (83)	12.0 (83)
T. phyllosoma	39.0 (59)	73.9 (23)	60.9& (23)	100.0 (14)	42.9 (14)	0.0 (14)	0.0 (14)
Other triatomines*	43.5 (108)	85.1 (47)	87.2 (47)	97.6 (41)	55.0 (40)	4.9 (41)	24.4 (41)
All triatomines [95%CI]	36.0 (814) [32.7,39.3]	74.1 (293) [69.0,79.1]	78.8 (293) [77.7,96.8]	98.3 (231) [96.6,99.9]	60.4 (227) [54.0,66.7]	2.2 (231) [0.3,4.1]	17.3 (231) [12.4,22.2]

* Other triatomines includes 3 *P. rufotuberculatus* and 105 non-genotyped *T. dimidiata* (30% Hg1 and 70% Hg2).
‡ Combined ME, 18S, 24Sα genotype success
Bonferroni adjusted X^2 significance in bold: § $p<0.0001$ (greater); # $p<1.0E-5$ (lower); & $p<0.01$ (lower); ≠ $p<0.01$ (greater)

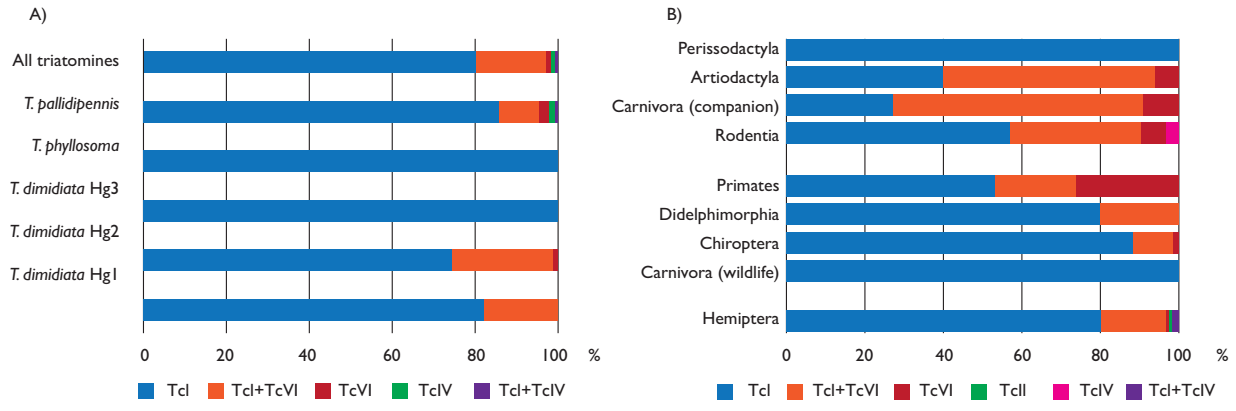

FIGURE 1. COMPARATIVE PROPORTIONS OF *T. CRUZI* DTUS IN GENOTYPED PARASITE POPULATIONS FROM WHOLE LANDSCAPE NEOTROPICAL MEXICAN TRIATOMINE SPECIES (A) AND MAMMAL RESERVOIRS (B)

Trypanosoma cruzi populations in mammal reservoirs

Following validation of the four molecular markers for native Mexican *T. cruzi* populations in vectors, we compared parasite prevalence, genotype success and DTU prevalence from the most abundant small and medium-sized mammal reservoir assemblages from the same sites (table S2[35]). Since vector and mammal populations were co-collected (same transects, locations and sylvatic, ecotone and human habitats), we expected that most of the parasite populations found in vectors, would be identified with similar sensitivity and specificity in mammal taxa. However, this was not the case as summarized in table II and figure 2A. Infection prevalence was significantly low in all synanthropic mammals: pets (4.7%; χ^2=16.88, df=7, p<0.0001), Artiodactyla livestock (6.9%; χ^2=7.96, df=7, p<0.01), and rodents (9.9%; χ^2=6.17, df=7, p<0.01). In contrast, infection prevalence was significantly high in wildlife: Chiroptera (18.0%; χ^2=15.64, df=7, p<0.0001) and wildlife carnivores (44.4%; χ^2=7.43, df=7, p<0.01).

Parasite populations from individual taxa amplified differentially to each gene marker and no single marker was capable of universally identifying all *T. cruzi* populations (figure 2A). Although all *T. cruzi* populations did amplify using kDNA, the specificity of amplicons was moderate (61.6%). Overall, *T. cruzi* specificity of ME amplicons from mammals was high (97.7%), while it was moderate for 18S (60.9%) and 24Sα (72.0%) (table S4[35]). ME specificity was significantly low only in human Primates (81.8%; χ^2=12.65, df=7, p<0.001), while there was no significant difference of 18S or 24Sα amplicon specificity among taxa. The kDNA and ME together identified 83.4% of infections (100% of didelphids and wildlife carnivores), which was 98.6% if additionally considering the 18S (figure 1B).

Although similar among most mammal taxa, kDNA sensitivity was significantly high only in Didelphimorphia (100.0%; χ^2=8.83, df=7, p<0.01) (table II). Sensitivity of the ME was average overall in mammals (45.0%), although significantly high in didelphids (88.9%; χ^2=7.01, df=7, p<0.01), pets (83.3%; χ^2=7.13, df=7, p<0.01), and livestock (80.0%; χ^2=7.43, df=7, p<0.01), while significantly low in bats (34.6%; χ^2=6.94, df=7, p<0.01). Overall, 18S sensitivity was lower than that for the ME (18.3%), but in contrast to the pattern for ME, it was significantly high in bats (26.4%; χ^2=6.92, df=7, p<0.01) while low in rodents (5.3%; χ^2=6.51, df=7, p<0.01), indicating particularly distinct genetic populations in this latter taxon. The 24Sα had similar overall sensitivity to that for 18S (18.7%), but it did not amplify in wildlife carnivores, and as was the case for the ME, also had significantly low sensitivity in bats (10.1%; χ^2=7.78, df=7, p<0.01). The 24Sα was particularly important as the only gene to amplify most *T. cruzi* populations from an equine and livestock (80.0%; χ^2=37.12, df=7, p<1.1E-9) (figure 2A).

Only 48.8% of mammal *T. cruzi* populations were genotyped using primers for any of the three nuclear

Table II

TRYPANOSOMA CRUZI INFECTION PREVALENCE, TOTAL POPULATION (N), AND CONFIDENCE INTERVAL > 95%CI, IN MAMMAL TAXA, SENSITIVITY OF KDNA (SEQUENCE IDENTITY GENBANK/TOTAL INFECTED), OVERALL GENOTYPE SUCCESS OF INFECTED SPECIMENS,* AND INDIVIDUAL SENSITIVITY OF NUCLEAR MARKERS (ME, 18S, 24Sα)

	Infection T. cruzi % *(N)*	*Sensitivity kDNA* % *(N)*	*Genotype success** % *(N)*	*Sensitivity ME* % *(N)*	*Sensitivity 18S* % *(N)*	*Sensitivity 24Sα* % *(N)*
Primates	**23.7‡ (135)**	65.6 (32)	46.9 (32)	28.1 (32)	15.6 (32)	18.8 (32)
Chiroptera	**18.0§ (884)**	52.8 (159)	**39.0# (159)**	**34.6# (159)**	**26.4& (159)**	**10.1# (159)**
Didelphimorphia	19.1 (47)	**100.0 (9)**	55.6 (9)	**88.9& (9)**	0.0 (9)	22.2 (9)
Rodentia	**9.9# (575)**	73.7 (57)	52.6 (57)	59.6 (57)	**5.3# (57)**	21.1 (57)
Carnivora (wildlife)	**44.4& (9)**	75.0 (4)	50.0 (4)	50.0 (4)	25.0 (4)	0.0 (4)
Carnivora (companion)	**4.7≠ (256)**	83.3 (12)	**91.7& (12)**	**83.3& (12)**	8.3 (12)	41.7 (12)
Artiodactyla	**6.9# (217)**	60.0 (15)	**100.0§ (15)**	**80.0& (15)**	6.7 (15)	**80.0§ (15)**
Perissodactyla	3.8 (26)	0.0 (1)	100.0 (1)	0.0 (1)	0.0 (1)	100.0 (1)
All mammals [95%CI]	13.4 (2 149) [12.0-14.9]	61.6 (289) [56.0-67.2]	48.8 (289) [43.0-54.6]	45.0 (289) [39.2-50.7]	18.3 (289) [13.9-22.8]	18.7 (289) [14.2-23.2]

* Combined ME, 18S, 24Sα genotype success.
Bonferroni adjusted X^2 significance in bold greater: ‡ p<0.001 (greater); § p<0.0001 (greater); # p<0.01 (lower); & p<0.01 (greater); ≠ p<0.0001 (lower)

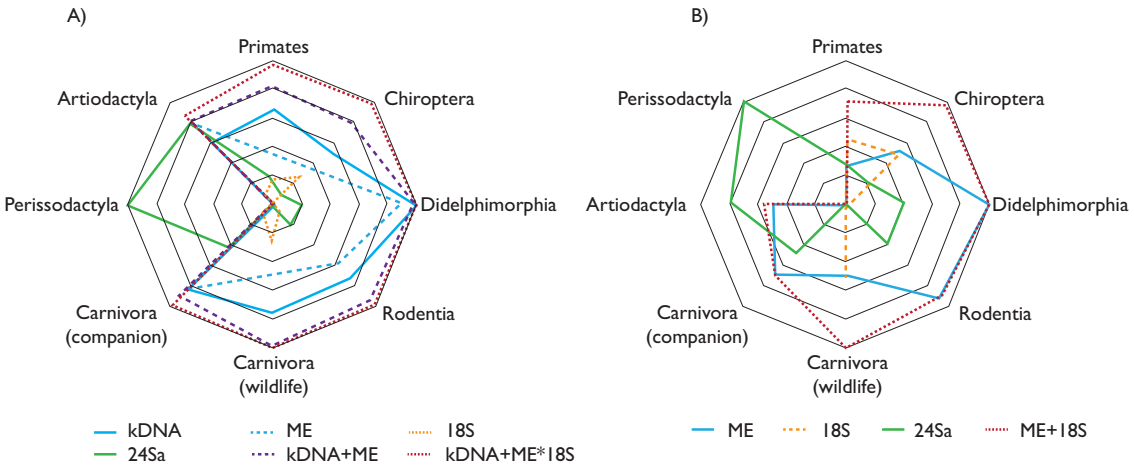

Figure 2. Comparative ratios (20% radial lines) of infections identified by single (kDNA, ME, 18S, 24Sα) or combinations of gene markers (kDNA + ME; kDNA + ME + 18S) in mammal taxa for (A) all *T. cruzi* populations and (B) only for TcI populations

genes (ME, 18S, or 24Sα), being significantly high in livestock (100.0%; χ^2=15.74, df=7, p<0.0001) and pets (91.7%; χ^2=8.83, df=7, p<0.01), but low in bats (39.0%; χ^2=6.11, df=7, p<0.01) (table II). Genotype success of TcI was highest by combining the ME and 18S, particularly for human Primates, bats and wildlife carnivores, while the 24Sα identified 43% of TcI in livestock and an equine (figure 2B). Most TcVI were typed using the ME, the exception being 1.2% (N=83) using the 24Sα. The single TcII identified in a rodent specimen amplified using the ME.

Proportional DTU profiles for all taxa and specifically for mammal taxa are summarised in figure 1B and table S3,[35] respectively. Generally, 93% of genotyped populations had TcI and 31% had TcVI, while 25% had co-infections of TcI and TcVI. TcII was identified in only one rodent species (*Heteromys desmarestianus;* 0.7% infection rate). Overall, 23.7% (95%CI 16.4,31.0) of TcI had identity to subtype sequences registered in GenBank for TcIA and no identity for any other subtype; there was no significant difference of DTUIA prevalence among taxa. Human population had significantly high single TcVI infection (26.7%; χ^2=10.33, df=7, p<0.001) as compared to all mammals and significantly low overall TcI prevalence (73.3%; χ^2=8.72, df=7, p<0.01). A similar trend was seen in pets, with significantly high TcVI (72.7%; χ^2=8.83, df=7, p<0.01) and combined TcI-TcVI infections (63.6%; χ^2=8.88, df=7, p<0.01), but significantly low single TcI prevalence (27.3%; χ^2=15.64, df=7, p<0.0001). Livestock also had significantly high combined TcI-TcVI (53.3%; χ^2=6.53, df=7, p<0.01) and overall TcVI infections (60.0%; χ^2=5.79, df=7, p=0.016). In contrast to the three former synanthropic taxa, bats had significantly high single TcI infections (88.7%; χ^2=12.14, df=7, p<0.001), but low TcVI (11.3%; χ^2=11.45, df=7, p<0.001) or combined TcI-TcVI prevalence (9.8%; χ^2=7.62, df=7, p<0.01).

Comparative parameters of *T. cruzi* populations from vectors and the two major mammal groups

Mammal taxa clustered into two subgroups based primarily on *T. cruzi* infection rates (low vs. high) and secondarily on genotype success. Mammal group 1 (LI-HG) had low infection prevalence and high genotype success (Rodentia, companion Carnivora, Artiodactyla, and Perissodactyla), while mammal group 2 (HI-MG) had high infection prevalence and average genotype success (wildlife Carnivora, Chiroptera, human Primates, and Didelphimorphia) (table III).

The mean *T. cruzi* infection rate in the synanthropic LI-HG mammal subgroup 1 (LI-HG) was significantly low (7.9%; χ^2=93.59, df=2, p<3.9E-22), while that in triatomines high (36.0%; χ^2=137.91, df=2, p<7.6E-32). While the 24Sα sensitivity was significantly high in LI-HG (35.3%; χ^2=30.75, df=2, p<2.9E-8), 18S sensitivity (5.9%; χ^2=11.67, df=2, p<0.001) and 18S specificity (41.7%; χ^2=6.01, df=2, p<0.01) were significantly low. Single TcI infections (47.4%; χ^2=25.13, df=2, p<5.3E-7) and the proportion of TcIA (23.1%; χ^2=11.38, df=2, p<0.001) were also significantly low in the LI-HG, although all single TcVI (50.9%; χ^2=26.11, df=2, p<3.2E-7) and mixed TcI-TcVI infections were significantly high (43.9%; χ^2=21.23, df=2, p<4.1E-6).

Table III

Comparative *T. cruzi* infection parameters for mammal subgroup (1) LI-HG (low infection – high genotype success: Rodentia, companion Carnivora, Artiodactyla, Perissodactyla; N), subgroup (2) HI-MG (high infection – medium genotype success: Primates, Chiroptera, wildlife Carnivora, Didelphimorphia; N), and all Triatominae (N): infection rate (%) and confidence interval > 95%CI, amplification specificity of kDNA, ME, 18S and 24Sα (%), % genotype success (combined ME, 18S, 24Sα), and sensitivity of individual nuclear markers ME, 18S, 24Sα (% of all *T. cruzi* infections), prevalence of total infections for lineages TcI, TcIV, TcVI, TcII and subtype TcIA and single or mixed TcI and TcVI infections

	Mammal 1 LI-HG % (N)	*Mammal 2 HI-MG* % (N)	*Triatominae* % (N)	*All Hosts* % (N) [CI]
T. cruzi infection	**7.9* (1 074)**	19.0 (1075)	**36.0‡ (814)**	19.6 (2 963) [18.2-21.1]
kDNA specificity	71.8 (85)	**57.4§ (204)**	**74.1# (293)**	67.9 (582) [64.1-71.7]
Genotype success	67.1 (85)	**41.2* (204)**	**78.8‡ (293)**	63.9 (582) [60.0-67.8]
ME sensitivity	65.9 (85)	**36.3* (204)**	**77.1‡ (293)**	61.2 (582) [57.2-65.1]
ME specificity	98.2 (57)	97.4 (76)	98.3 (230)	98.1 (363) [96.7-99.5]
18S sensitivity	**5.9§ (85)**	23.5 (204)	23.5 (293)	21.0 (582) [17.7-24.3]
18S specificity	**41.7& (12)**	64.0 (75)	**86.3ᵠ (80)**	73.1 (167) [66.3-79.8]
24Sα sensitivity	**35.3‡ (85)**	11.8 (204)	**9.9∞ (293)**	14.3 (582) [11.4-17.1]
24Sα specificity	69.8 (43)	75.0 (32)	85.3 (34)	76.1 (109) [68.1-84.2]
Single TcI	**47.4§ (57)**	82.1 (84)	80.5 (231)	75.8 (372) [71.5-80.2]
Total TcI	91.2 (57)	94.0 (84)	97.8 (231)	96.2 (372) [94.3-98.2]
TcIA	**23.1§ (52)**	**24.1ø (79)**	**60.4◊ (227)**	46.9 (358) [42.5-51.3]
Single TcVI	7.0 (57)	6.0 (84)	0.9 (231)	3.0 (372) [1.2-4.7]
Total TcVI	**50.9‡ (57)**	17.9 (84)	17.3 (231)	22.6 (372) [18.7-26.4]
Mix TcI-TcVI	**43.9€ (57)**	11.9 (84)	16.5 (231)	19.6 (372) [15.6-23.7]
Total TcII	1.8 (57)	0 (84)	0 (231)	0.3 (372) [0.0-0.8]
Total TcIV	0 (57)	0 (84)	2.2 (231)	1.3 (372) [0.2-2.5]

Bonferroni adjusted X^2 significance in bold greater: * p< 1.0E-6 (lower); ‡ p< 1.0E-6 (greater); § p< 0.001 (lower); # p< 0.05 (greater); & p< 0.01 (lower); ᵠ p< 0.01 (greater); ∞ p< 0.05 (lower); ø p< 0.0001 (lower); ◊ p< 0.0001 (greater); € p< 1.0E-5 (greater)

In contrast to the LI-HG, there was significantly low genotype success (41.2%; χ^2=45.74, df=2, p<1.3E-11), ME sensitivity (36.3%; χ^2=53.22, df=2, p<3.1E-13), kDNA specificity (57.4%; χ^2=10.35, df=2, p<0.001) and proportion of TcIA (24.1%; χ^2=15.88, df=2, p<0.0001) of *T. cruzi* populations in the HI-MG mammal subgroup 2 (wildlife and human).

Genotype success of parasite populations in triatomines (78.8%; χ^2=28.29, df=2, p<1.0E-7), ME sensitivity (77.1%; χ^2=31.44, df=2, p<2.1E-8), kDNA specificity (74.1%; χ^2=5.15, df=2, p<0.02), and 18S specificity (86.3%; χ^2=7.08, df=2, p<0.01) were significantly high in contrast to both mammal subgroups. The proportion of the TcIA subtype was also significantly high in triatomines (60.4%; χ^2=16.43, df=2, p<0.0001).

Discussion

Trypanosoma cruzi and DTU profiles in vector species

Over a decade, we have conducted landscape level sampling and analyses to understand the complex ecological, epidemiological and demographic components driving *T. cruzi* dispersal/transmission to human population. We have developed an inventory of *T. cruzi* host assemblages from the neotropical region (south and southeastern Mexico) in order to analyze transmission dynamics and *T. cruzi*/DTU haplotype dispersal at regional, ecotype, landscape and in habitat subtypes. Initial studies demonstrated variable failure

to amplify, align sequences and genotype with registered (GenBank) *T. cruzi* sequences using common primers and genotype algorithms,[6,8] which prompted the present use of a multi-locus approach avoiding competitive amplification using individual multiple gene fragments for parasite detection and genotyping, and to score parasite identification based solely on sequence identity.[42-44] Herein, the kDNA (S34/S67) was chosen over SAT since our focus was to use standardized markers to detect parasite populations across all assemblages (vectors, mammals), not just human population, we expected far greater proportion of TcI (for which SAT has 10% of repeats)[45] and high non-specific amplification of *T. cruzi* SAT DNA from most mammal reservoir DNA.[18,39]

Trypanosoma cruzi infection prevalence in the six principal neotropical vector species/haplogroups was not uniform, although capacity to genotype these parasite populations was relatively high in most species (> 80%). Although ME sensitivity was high (77%), overall 18S sensitivity was low (24%) and 24Sα sensitivity even lower (10%), indicating a high level of gene fragment polymorphism both at primer binding sites and internally to sequences in vector parasite populations. Unexpectedly, only 74% of amplified kDNA in triatomines had identity to sequences registered in GenBank, significantly lower in *T. dimidata* Hg2 parasite populations, also indicating important but previously undocumented high diversity for mitochondrial gene haplotypes in the region, recently highlighted in several studies using isolates.[25,46] The *T. dimidiata* complex of haplogroups, previously reported to have significant genetic and infection prevalence differentiation, also have different DTU profiles (DTUVI and DTUIV) and TcI subtype proportions (DTUIA) and genotyping success overall.[36] Only TcI was identified in *T. dimidiata* Hg3 and in *T. phyllosoma*, both with low infection prevalence, which coincided with lower ME and nil 24Sα sensitivity. It is unclear whether this reflects methodological failure (marker insensitivity), or true absence or reduced presence of other DTUs (particularly TcIII, TcIV and both hybrids) along the southern Mexican Pacific coast (Chiapas, Isthmus Oaxaca) where both vector species are exclusively distributed. It is noteworthy that TcI from bats and rodents from the same landscapes as the two vector populations were also not genotyped using either the ME or 24Sα.

Proportionally, the TcIA subtype was significantly higher in triatomines as compared to mammal reservoirs, indicating either its selective proportional amplification in triatomines or deselection of other subtypes in vectors or in mammals. It is surprising that no other TcI subtype was identified in the vectors, although they may have been present in lower proportions and ineffectively amplified, or not amplified, once again due to binding sequence polymorphisms.[10]

Trypanosoma cruzi prevalence and DTU profiles in major landscape reservoirs

Comparative analyses of parasite populations from mammal taxa have been interpreted with caution since parasite populations identified from wildlife heart tissue (exception one endangered bat species) would be expected to have a minimum of one (10-100 parasites, 1-10pg DNA/sample)[47] to five amastigote nests.[48] Parasite detection and genotyping from livestock, pets, and human populations were identified from blood samples, which represent a bias potentially due to low circulating parasitemia, despite infectious potential.[49] The endpoint PCR method used herein with ME and 24Sα (titrated in human blood) was sensitive for >50 parasites/ml, while that for 18S >5 parasites/ml (Ramsey, personal communication). Lowest infection prevalence in this study was in fact identified from synanthropic reservoirs, i.e. from blood samples (livestock and pets), but it was also significantly low in rodents, for which tissue was analyzed. Therefore, although a sensitivity bias may exist comparing parasite populations from tissue and blood samples, differences observed herein are also taxa-specific.

The HI-MG mammal subgroup 2, which included most widely-dispersing wildlife, had highest parasite prevalence, although these parasite populations were least successfully genotyped and hence, potentially heterogeneous as expected, particularly least amplified using the ME (TcIII, TcIV). Single TcI infections were highly prevalent in these taxa (low TcVI), a similar pattern to that in triatomines. Although didelphid infections were all identified using the kDNA, parasites from other wildlife were poorly identified using the same marker, indicating potential of alternative haplotypes in most wildlife. Parasite populations from humans had a similar pattern to sylvatic wildlife, in contrast to the synanthropic species, despite prevalent assumptions that the latter are the primary source of human infection.[50] Brisse and colleagues[42] have discussed primer-binding sequence polymorphisms even in TcI culture stocks and we interpret the low sensitivity to amplify TcI, and TcIA in some parasite populations (variable haplotypes) and lack of detecting alternative TcI subtypes, a result of these polymorphisms.[43] Triatomines have a greater DTU and haplotype diversity, which may explain less evidence for polymorphic populations.[29]

Trypanosoma cruzi infection prevalence in the synanthropic and less-dispersing LI-HG reservoir group of

domesticated mammals (livestock and pets) and rodents (dispersal capacity <10 m) was significantly lower than in the HI-MG. Their parasite populations were successfully, although not completely genotyped with current nuclear gene markers such as the ME, and particularly using the 24Sα, indicating greater *T. cruzi* homogeneity (similar to isolates and culture-adapted strains). Sensitivity and specificity of 18S amplicons (561bp) for *T. cruzi* in the LI-HG reservoir group was low, although its use herein has evidenced its potential to simultaneously amplify multiple gene-specific amplicons from *T. cruzi* and other co-infecting *Trypanosoma* species, and spillover of *T. dionisii* and T. sp. in humans and synanthropic reservoirs (data to be published elsewhere). No evidence for *T. rangeli* or TcBat has been found in bugs, wildlife, livestock, pets or human population in these Mexican neotropical landscapes. Since the Rhodniini are not autochthonous to Mexico, neither were expected in southern Mexico, despite the brief presence of invasive domestic *R. prolixus* from Central America in regions included in this study (Nopala, Palenque).

Despite significant differences to genotype *T. cruzi* populations, the proportion of TcVI and mixed TcI-TcVI infections in the LI-HG synanthropic subgroup (livestock and pets) was similar to that in human population (HI-MG). Lewis and colleagues[51] have suggested that TcVI most likely originated as a result of human activities that promoted mixing in domesticated vectors, while several recent studies suggest introgression between TcIII and TcIV prior to hybridization with TcII.[52] However, TcIII has not been sequenced from any reservoir specimen in Mexico, although despite inconclusive evidence using qPCR from two specimens from *T. pallidipennis*,[5] TcIV has been previously sequenced from *T. dimidiata* (unspecified haplogroup)[53] and in the present study. TcII has also not been previously sequenced from reservoirs in Mexico, and in this study we report its presence based on a sequence from only one rodent species *Heteromys desmarestianus* (Heteromydae) but not from 36 co-collected *T. dimidiata* Hg2, *T. dimidata* Hg1 or Hg3,[36] or 156 other co-collected mammal specimens (79 rodents of three species, 28 specimens of the same species, 12 didelphids of three species, 37 bats of six species) from the same landscape directly east of the biogeographic barriers of the Selva El Ocote Biosphere Reserve and the Tehuantepec Isthmus. In North America (NA), TcII has been sequenced only from two rodent specimens from Louisiana, USA, although that study did not identify whether the sequences were identified from *Peromyscus gossypinus* or *Mus musculus*.[27] Identification of TcII in rodents in both the former and present study and its absence in sympatric vector samples will require further analyses to characterize association with the rodent taxon and potential infectious barrier in Mexican *T. dimidiata* haplogroups Hg1, Hg2 and Hg3. It is noteworthy that *T. cruzi* populations from three of seven co-collected infected mammal specimens (bats, didelphids, rodents) where TcII was identified in the present study, were not successfully genotyped, although both TcI and TcVI were identified in vectors and other reservoirs from the same landscape.[36]

Secondary DTUs and regional distributions

Geographic prevalence of TcVI increases from south to north in Mexico, similar to territorial coverage of livestock, large-scale agriculture (and rodent pests), and human population density, with clear geographic shift north and northwest of the Tehuantepec Isthmus, an important biogeographic transition barrier in Mexico (northern limit of the Mesoamerican region).[54] Antibody-mediated lytic immune responses in domesticated livestock and pets to the TcI may affect subtypes circulating by limiting their presence in blood samples, thereby providing an "apparently" high proportion of TcVI in these reservoirs. These patterns could also be related to infrapopulation interactions of *T. cruzi* lineages within hosts, since these inter-DTU interactions could be density and tissue dependent such that the abundance of one parasite DTU may affect the fitness of a co-infecting DTU, and immune-mediated interactions may be temporally delayed, or dependent on host condition.[55] Lower TcI prevalence in rodents and the higher prevalence of the TcVI hybrid in multiple rodent species (agricultural pests of Cimetidae) and in invasive domestic species of the Muridae (*Rattus rattus, Mus musculus*) may provide a continuous "sylvatic" but synanthropic source of this hybrid DTU across modified landscapes. Lopez-Cancino and colleagues[6] found that TcI decreased proportionally between sylvatic and domestic habitats in wildlife, although to a lesser degree in livestock (from blood), despite consistent prevalence in vectors in all habitats. Additionally, although certain DTUs (such as TcI) may have reproductive advantage in specific vectors as indicated in the present study, thereby reducing potential infective sources of TcVI, other vectors may be refractory to TcVI (also TcIV and TcII) such as in the two vector species (*T. dimidiata* Hg3, *T. phyllosoma*) exclusively found along the southern Mexican Pacific coast.

TcIV has been previously identified in a cultured *T. cruzi* isolate from a Mexican opossum from Veracruz state (host species and collection site not reported)[56] and one *T. dimidiata* specimen from Quintana Roo[53] using the V1/V2 primers of 18S.[42] We have not identified TcIV in any of 47 didelphid specimens (four

species) in the present study but have identified it in two co-collected vector species. TcIV was amplified and sequenced using all three nuclear genes, albeit differentially according to geographic region. Two specimens of *T. pallidipennis* (N=38) from Morelos amplified TcIV using the ME, while four specimens, one of *T. dimidiata* Hg2 and three of either *T. dimidiata* Hg1 or Hg2 (not genotyped) from southeast Campeche amplified using the 18S; the population from *T. dimidiata* Hg2 also sequenced using the 24Sα. Three rodent specimens from Morelos (N=45, *Baiomys musculus, Peromyscus melanophrys, Neotoma mexicana*) co-collected with *T. pallidipennis* were *T. cruzi* infected (kDNA), but none of their parasite populations amplified with any nuclear marker, and no specimen among 30 sympatric bats were apparently infected. Similarly, although TcIV was identified using the 18S in *T. dimidiata* Hg2 and Hg1 from Calakmul, Campeche, it was not identified in co-collected TcI and TcVI-infected livestock and pets (21) or 32 infected wildlife specimens (from all taxa). Curiously, of the four bugs from Campeche amplifying TcIV using 18S, three did have TcI sequenced using ME and one additionally had TcVI. The single specimen (genotyped as *T. dimidata* Hg2) that amplified TcIV using both 18S and 24Sα, also amplified TcI using ME, with identity for the IA subtype.

Present analyses highlight important heterogeneity of mitochondrial and nuclear genes in *T. cruzi* populations of most natural hosts in southern Mexico and significant differences in these parasite populations among mammal reservoir taxa, and even among vector species. Differential population selection is not a novel concept, but there has been little evidence for differential selective or deselective processes among multiple reservoir species/taxa from landscape assemblage studies in the continent. Current molecular tools (primers) are insufficient to detect or genotype all parasite populations across the Mexican neotropical region, which highlights the need for different approaches to reduce uncertainty and analyze landscape genetics and *T. cruzi* transmission dynamics. Despite 10 to 200 copies of the ME gene in the parasite genome, current primers based on the conserved region can amplify dominant populations in 90% of vectors, but only 60% of parasite populations across mammal hosts. Similarly, no more than 20% of parasite populations were amplified using primers for either the 18S or 24Sα gene fragments, in vectors and mammals. Independent of whether this can be overcome by redesigning primers or fragments analyzed or using next generation sequencing (NGS)[29,57] or other novel methods,[58,59] it is evident that most studies have underestimated parasite detection and diversity in mammal reservoirs and vectors in Mexico, and perhaps in other regions. Reservoir-specific and regional analyses of *T. cruzi* populations and haplotypes, as well as other *Trypanosoma* spp. reported herein will be presented in separate publications. Without appropriate sampling design and improved parasite detection and genotyping tools, however, the complexity of the parasite´s population dynamics will continue to evade representative and appropriate analyses which could better inform current failure to sensitively diagnose human infections.

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References

1. Noireau F, Diosque P, Jansen AM. *Trypanosoma cruzi*: Adaptation to its vectors and its hosts. Vet Research. 2009;40:26. https://doi.org/10.1051/vetres/2009009

2. Izeta-Alberdi A, Ibarra-Cerdeña CN, Moo-Llanes DA, Ramsey JM. Geographical, landscape and host associations of *Trypanosoma cruzi* DTUs and lineages. Parasite Vector. 2016;9:1-20. https://doi.org/10.1186/s13071-016-1918-2

3. Lidani KCF, Andrade FA, Bavia L, Damasceno FS, Beltrame MH, Messias-Reason IJ, Lucas-Sandri T. Chagas Disease: From Discovery to a Worldwide Health Problem. Front Public Health. 2019;7:166. https://doi.org/10.3389/fpubh.2019.00166

4. World Health Organization. Chagas disease in Latin America: an epidemiological update based on 2010 estimates. Wkly Epidemiol Rec. 2015;6:33-44.

5. Ibáñez-Cervantes G, Martínez-Ibarra A, Nogueda-Torres B, López-Orduña E, Alonso AL, Perea C, *et al*. Identification by Q-PCR of *Trypanosoma*

cruzi lineage and determination of blood meal sources in triatomine gut samples in México. Parasitol Int. 2013;62(1):36-43. https://doi.org/10.1016/j.parint.2012.09.003
6. López-Cancino SA, Tun-Ku E, De la Cruz-Felix HK, Ibarra-Cerdena CN, Izeta-Alberdi A, Pech-May A, *et al.* Landscape ecology of *Trypanosoma cruzi* in the southern Yucatan Peninsula. Acta Trop. 2015;151:58-72. https://doi.org/10.1016/j.actatropica.2015.07.021
7. 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;12:1341-3. https://doi.org/10.1016/j.meegid.2012.04.024
8. Ramsey JM, Gutiérrez-Cabrera AE, Salgado-Ramírez L, Peterson AT, Sánchez-Cordero V, Ibarra-Cerdeña CN. Ecological Connectivity of *Trypanosoma cruzi* Reservoirs and *Triatoma pallidipennis* Hosts in an Anthropogenic Landscape with Endemic Chagas Disease. PLoS One. 2012;7:e46013. https://doi.org/10.1371/journal.pone.0046013
9. Tamay-Segovia P, Alejandre-Aguilar R, Martínez F, Villalobos G, Zavala-Díaz De La Serna FJ, De La Torre P, *et al.* Two *Triatoma dimidiata* clades (chagas disease vector) associated with different habitats in Southern Mexico and Central America. Am J Trop Med Hyg. 2008;78:472-8. https://doi.org/10.4269/ajtmh.2008.78.472
10. Zumaya-Estrada FA, Messenger LA, Lopez-Ordonez T, Lewis MD, Flores-Lopez CA, Martínez-Ibarra AJ, *et al.* North American import? Charting the origins of an enigmatic *Trypanosoma cruzi* domestic genotype. Parasite Vector. 2012;5:226. https://doi.org/10.1186/1756-3305-5-226
11. Martínez-Hernández F, Rendon-Franco E, Gama-Campillo LM, Villanueva-García C, Romero-Valdovinos M, Maravilla P, *et al.* Follow up of natural infection with *Trypanosoma cruzi* in two mammals species, Nasua narica and Procyon lotor (Carnivora: Procyonidae): Evidence of infection control? Parasite Vectors. 2014;7:405. https://doi.org/10.1186/1756-3305-7-405
12. Cura CI, Duffy T, Lucero RH, Bisio M, Péneau J, Jimenez-Coello M, *et al.* Multiplex real-time PCR assay using TaqMan Probes for the Identification of *Trypanosoma cruzi* DTUs in biological and clinical samples. PLoS Negl Trop Dis. 2015;9:e0003765. https://doi.org/10.1371/journal.pntd.0003765
13. Martínez I, Nogueda B, Martínez-Hernández 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 Dis. 2013;13:181-7. https://doi.org/10.1089/vbz.2012.1072
14. Zingales B, Miles MA, Campbell DA, Tibayrenc M, Macedo AM, Teixeira MMG, *et al.* The revised *Trypanosoma cruzi* subspecific nomenclature: Rationale, epidemiological relevance and research applications. Infect Genet Evol. 2012;12:240-53. https://doi.org/10.1016/j.meegid.2011.12.009
15. Margioto Teston AP, Paula de Abreu A, Gruendling AP, Bahia MT, Gomes ML, Marques de Araújo S, *et al.* Differential parasitological, molecular, and serological detection of *Trypanosoma cruzi* I, II, and IV in blood of experimentally infected mice. Exp Parasitol. 2016;166:44-50. https://doi.org/10.1016/j.exppara.2016.03.013
16. Britto CC. Usefulness of PCR-based assays to assess drug efficacy in Chagas disease chemotherapy: Value and limitations. Mem Inst Oswaldo Cruz. 2009;104(suppl 1):122-35. https://doi.org/10.1590/S0074-02762009000900018
17. Schijman AG, Bisio M, Orellana L, Sued M, Duffy T, Mejia-Jaramillo AM, *et al.* International study to evaluate PCR methods for detection of *Trypanosoma cruzi* DNA in blood samples from Chagas disease patients. PLoS Negl Trop Dis. 2011;5. https://doi.org/10.1371/journal.pntd.0000931
18. Lewis MD, Llewellyn MS, Gaunt MW, Yeo M, Carrasco HJ, Miles MA. Flow cytometric analysis and microsatellite genotyping reveal extensive DNA content variation in *Trypanosoma cruzi* populations and expose contrasts between natural and experimental hybrids. Int J Parasitol. 2009;39:1305-17. https://doi.org/10.1016/j.ijpara.2009.04.001
19. Rusman F, Tomasini N, Yapur N-F, Puebla AF, Ragone PG, Diosque P. Elucidating diversity in the class composition of the minicircle hypervariable region of *Trypanosoma cruzi*: New perspectives on typing and kDNA inheritance. PLoS Negl Trop Dis. 2019;13:e0007536. https://doi.org/10.1371/journal.pntd.0007536
20. Fernandes O, Souto RP, Castro JA, Pereira JB, Fernandes NC, Junqueira ACV, *et al.* Brazilian isolates of *Trypanosoma cruzi* from humans and triatomines classified into two lineages using mini-exon and ribosomal RNA sequences. Am J Trop Med Hyg. 1998;58:807-11. https://doi.org/10.4269/ajtmh.1998.58.807
21. 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. https://doi.org/10.1016/j.actatropica.2008.12.003
22. Maia Da Silva F, Noyes H, Campaner M, Junqueira ACV, Coura JR, Añez N, *et al.* Phylogeny, taxonomy and grouping of *Trypanosoma rangeli* isolates from man, triatomines and sylvatic mammals from widespread geographical origin based on SSU and ITS ribosomal sequences. Parasitology. 2004;129:549-61. https://doi.org/10.1017/S0031182004005931
23. Noyes HA, Stevens JR, Teixeira M, Phelan J, Holz P. A nested PCR for the ssrRNA gene detects *Trypanosoma binneyi* in the platypus and *Trypanosoma* sp. in wombats and kangaroos in Australia. Int J Parasitol. 1999;29:331-9. https://doi.org/10.1016/S0020-7519(98)00167-2
24. Souto RP, Zingales B. Sensitive detection and strain classification of *Trypanosoma cruzi* by amplification of a ribosomal RNA sequence. Mol Biochem Parasitol. 1993;62:45-52. https://doi.org/10.1016/0166-6851(93)90176-X
25. Sturm NR, Degrave W, Morel C, Simpson L. Sensitive detection and schizodeme classification of *Trypanosoma cruzi* cells by amplification of kinetoplast minicircle DNA sequences: use in diagnosis of Chagas' disease. Mol Biochem Parasitol. 1989;33:205-14. https://doi.org/10.1016/0166-6851(89)90082-0
26. Charles RA, Kjos S, Ellis AE, Barnes JC, Yabsley MJ. Southern plains woodrats (*Neotoma micropus*) from Southern Texas are important reservoirs of two genotypes of *Trypanosoma cruzi* and host of a putative novel *Trypanosoma* species. Vector-Borne Zoonotic Dis. 2013;13:22-30. https://doi.org/10.1089/vbz.2011.0817
27. Herrera CP, Licon MH, Nation CS, Jameson SB, Wesson DM. Genotype diversity of *Trypanosoma cruzi* in small rodents and Triatoma sanguisuga from a rural area in New Orleans, Louisiana. Parasite Vector. 2015;8. https://doi.org/10.1186/s13071-015-0730-8
28. Vandermark C, Zieman E, Boyles E, Nielsen CK, Davis C, Agustín Jiménez F. *Trypanosoma cruzi* strain TcIV infects raccoons from Illinois. Mem Inst Oswaldo Cruz. 2018;113:30-7. https://doi.org/10.1590/0074-02760170230
29. Maiguashca-Sánchez J, Sueto SOB, Schwabl P, Grijalva MJ, Llewellyn MS, Costales JA. Remarkable genetic diversity of *Trypanosoma cruzi* and *Trypanosoma rangeli* in two localities of southern Ecuador identified via deep sequencing of mini-exon gene amplicons. Parasite Vector. 2020;13:252. https://doi.org/10.1186/s13071-020-04079-1
30. Bontempi IA, Bizai ML, Ortiz S, Manattini S, Fabbro D, Solari A, *et al.* Simple methodology to directly genotype *Trypanosoma cruzi* discrete typing units in single and mixed infections from human blood samples. Infect Genet Evol. 2016;43:123-9. https://doi.org/10.1016/j.meegid.2016.05.026
31. Bosseno MF, Telleria J, Vargas F, Yaksic N, Noireau F, Morin A, *et al.* *Trypanosoma cruzi*: Study of the distribution of two widespread clonal genotypes in Bolivian *Triatoma infestans* vectors shows a high frequency of mixed infections. Exp Parasitol. 1996;83:275-82. https://doi.org/10.1006/expr.1996.0075
32. Yeo M, Lewis MD, Carrasco HJ, Acosta N, Llewellyn M, da Silva Valente SA, *et al.* Resolution of multiclonal infections of *Trypanosoma cruzi* from naturally infected triatomine bugs and from experimentally infected mice by direct plating on a sensitive solid medium. Int J Parasitol. 2007;37:111-20. https://doi.org/10.1016/j.ijpara.2006.08.002
33. Poveda C, Higuera A, Urbano P, Ramírez JD. Ecology of *Trypanosoma cruzi* I genotypes across Rhodnius prolixus captured in Attalea butyra-

cea palms. Infect Genet Evol. 2017;49:146-50. https://doi.org/10.1016/j.meegid.2017.01.017
34. Sikes RS, Gannon WL. Guidelines of the American Society of Mammalogists for the use of wild mammals in research animal care and use committee 1 of the american society of mammalogists. J Mammal. 2011;92:235-53. https://doi.org/10.1644/10-MAMM-F-355.1
35. Izeta-Alberdi A, Pech-May A, Tun-Ku E, Mazariegos-Hidalgo CJ, López-Cancino SA, Gutiérrez S, *et al. Trypanosoma cruzi* in Mexican Neotropical vectors and mammals: wildlife, livestock, pets, and human population. Dryad, 2022. https://doi.org/10.5061/dryad.kh1893294
36. Pech-May A, Mazariegos-Hidalgo CJ, Izeta-Alberdi A, López-Cancino SA, Tun-Ku E, de la Cruz-Félix K, *et al.* Genetic variation and phylogeography of the *Triatoma dimidiata* complex evidence a potential center of origin and recent divergence of haplogroups having differential *Trypanosoma cruzi* and DTU infections. PLoS Negl Trop Dis. 2019;13:1:e0007044. https://doi.org/10.1371/journal.pntd.0007044
37. Ramsey JM, Townsend-Peterson A, Carmona-Castro O, Moo-Llanes DA, Nakazawa Y, Butrick M, *et al.* Atlas of Mexican Triatominae (Reduviidae: Hemiptera) and vector transmission of Chagas disease. Mem Inst Oswaldo Cruz. 2015;110:339-52. https://doi.org/10.1590/0074-02760140404
38. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press, 1989.
39. Mota J, Chacon JC, Gutiérrez-Cabrera AE, Sánchez-Cordero V, Wirtz RA, Ordoñez R, *et al.* Identification of blood meal source and infection with *Trypanosoma cruzi* of Chagas disease vectors using a multiplex cytochrome b polymerase chain reaction assay. Vector-Borne Zoonotic Dis. 2007;7:617-27. https://doi.org/10.1089/vbz.2007.0106
40. Souto RP, Fernandes O, Macedo AM, Campbell DA, Zingales B. DNA markers define two major phylogenetic lineages of *Trypanosoma cruzi.* Mol Biochem Parasitol. 1996;83:141-52. https://doi.org/10.1016/s0166-6851(96)02755-7
41. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018;35:1547-9. https://doi.org/10.1093/molbev/msy096
42. Brisse S, Verhoef J, Tibayrenc M. Characterisation of large and small subunit rRNA and mini-exon genes further supports the distinction of six *Trypanosoma cruzi* lineages. Int J Parasitol. 2001;31:1218-26. https://doi.org/10.1016/S0020-7519(01)00238-7
43. Segatto M, Rodrigues CM, Machado CR, Franco GR, Pena SDJ, Macedo AM. LSSP-PCR of *Trypanosoma cruzi*: How the single primer sequence affects the kDNA signature. BMC Res Notes. 2013;6:1. https://doi.org/10.1186%2F1756-0500-6-174
44. Westenberger SJ, Sturm NR, Yanega D, Podlipaev SA, Zeledón R, Campbell DA, *et al.* Trypanosomatid biodiversity in Costa Rica: Genotyping of parasites from Heteroptera using the spliced leader RNA gene. Parasitology. 2004;129:537-47. https://doi.org/10.1017/S003118200400592X
45. Elias MCQB, Vargas NS, Zingales B, Schenkman S. Organization of satellite DNA in the genome of *Trypanosoma cruzi*. Mol Biochem Parasitol. 2003;129:1-9. https://doi.org/10.1016/S0166-6851(03)00054-9
46. Callejas-Hernandez F, Herreros-Cabello A, del Moral-Salmoral J, Fresno M, Gironès N. The Complete Mitochondrial DNA of *Trypanosoma cruzi*: Maxicircles and Minicircles. Front. Cell. Infect. Microbiol. 2021;11: 672448. https://doi.org/10.3389/fcimb.2021.672448
47. Brisse S, Dujardin JC, Tibayrenc M. Identification of six *Trypanosoma cruzi* lineages by sequence-characterised amplified region markers. Mol Biochem Parasitol. 2000;111:95-105. https://doi.org/10.1016/s0166-6851(00)00302-9
48. Taylor MC, Ward A, Olmo F, Jayawardhana S, Francisco AF, Lewis MD, *et al.* Intracellular DNA replication and differentiation of *Trypanosoma cruzi* is asynchronous within individual host cells in vivo at all stages of infection. PLoS Negl Trop Dis. 2020;14:e0008007. https://doi.org/10.1371/journal.pntd.0008007
49. Ramírez JC, Cura CI, Da Cruz Moreira O, Lages-Silva E, Juiz N, Velázquez E, *et al.* Analytical validation of quantitative real-time PCR methods for quantification of *Trypanosoma cruzi* DNA in blood samples from chagas disease patients. J Mol Diagnostics. 2015;17:605-15. https://doi.org/10.1016/j.jmoldx.2015.04.010
50. Ibarra-Cerdeña CN, Valiente-Banuet L, Sánchez-Cordero V, Stephens CR, Ramsey JM. *Trypanosoma cruzi* reservoir-triatomine vector co-occurrence networks reveal meta-community effects by synanthropic mammals on geographic dispersal. PeerJ. 2017;2017:e3152. https://doi.org/10.7717/peerj.3152
51. Lewis MD, Llewellyn MS, Yeo M, Acosta N, Gaunt MW, Miles MA. Recent, Independent and Anthropogenic Origins of *Trypanosoma cruzi* Hybrids. PLoS Negl Trop Dis. 2011;5:e1363. https://doi.org/10.1371/journal.pntd.0001363
52. Tomasini N, Diosque P. Evolution of Trypanosoma cruzi: clarifying hybridisations, mitochondrial introgressions and phylogenetic relationships between major lineages. Mem Inst Oswaldo Cruz, Rio de Janeiro. 2015;110(3): 403-13. https://doi.org/10.1590/0074-02760140401
53. Dorn PL, McClure AG, Gallaspy MD, Waleckx E, Woods AS, Monroy MC, *et al.* The diversity of the Chagas parasite, *Trypanosoma cruzi,* infecting the main Central American vector, *Triatoma dimidiata,* from Mexico to Colombia. PLoS Negl Trop Dis. 2017;11. https://doi.org/10.1371/journal.pntd.0005878
54. Morrone JJ. Regionalización biogeográfica y evolución biótica de México: encrucijada de la biodiversidad del Nuevo Mundo. Revista Mexicana de Biodiversidad. 2019;90. https://doi.org/10.22201/ib.20078706e.2019.90.2980
55. Pedersen AB, Fenton A. Emphasizing the ecology in parasite community ecology. Trends Ecol Evol. 2007;22(3):133-9. https://doi.org/10.1016/j.tree.2006.11.005
56. Higo H, Miura S, Horio M, Mimori T, Hamano S, Agatsuma T, *et al.* Genotypic variation among lineages of *Trypanosoma cruzi* and its geographic aspects. Parasitol Int. 2004;53:337-44. https://doi.org/10.1016/j.parint.2004.06.001
57. Dumonteil E, Ramirez-Sierra MJ, Pérez-Carrillo S, Teh-Poot C, Herrera C, Gourbière S, *et al.* Detailed ecological associations of triatomines revealed by metabarcoding and next-generation sequencing: Implications for triatomine behavior and *Trypanosoma cruzi* transmission cycles. Sci Rep. 2018;8. https://doi.org/10.1038/s41598-018-22455-x
58. Hamilton PB, Lewis MD, Cruickshank C, Gaunt MW, Yeo M, Llewellyn MS, *et al.* Identification and lineage genotyping of South American trypanosomes using fluorescent fragment length barcoding. Infect Genet Evol. 2011;11:44-51. https://doi.org/10.1016/j.meegid.2010.10.012
59. Gaur P, Chaturvedi A. Mining SNPs in extracellular vesicular transcriptome of *Trypanosoma cruzi:* A step closer to early diagnosis of neglected Chagas disease. PeerJ. 2016;2016. https://doi.org/10.7717/peerj.2693