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Specific primers design based on the superoxide dismutase b gene for *Trypanosoma cruzi* as a screening tool: Validation method using strains from Colombia classified according to their discrete typing unit

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ABSTRACT

Objective: To classify 21 new isolates of *Trypanosoma cruzi* (*T. cruzi*) according to the Discrete Typing Unit (DTU) which they belong to, as well as tune up a new pair of primers designed to detect the parasite in biological samples. **Methods:** Strains were isolated, DNA extracted, and classified by using three Polymerase Chain Reactions (PCR). Subsequently this DNA was used along with other isolates of various biological samples, for a new PCR using primers designed. Finally, the amplified fragments were sequenced. **Results:** It was observed the predominance of DTU I in Colombia, as well as the specificity of our primers for detection of *T. cruzi*, while no band was obtained when other species were used. **Conclusions:** This work reveals the genetic variability of 21 new isolates of *T. cruzi* in Colombia. Our primers confirmed their specificity for detecting the presence of *T. cruzi*.

1. Introduction

Chagas disease, also called American Trypanosomiasis, is a tropical parasitic disease caused by the flagellate protozoan *Trypanosoma cruzi* (*T. cruzi*). It is commonly transmitted to humans and other mammals by an insect vector [1, 2], the blood-sucking bugs of the subfamily Triatominae (family Reduviidae) and with the most of its commonly species belonging to the *Triatoma*, *Rhodnius*,

and *Panstrongylus* genera. The disease may also be spread through blood transfusion and organ transplantation and from a mother to her fetus. This disease is an endemic zoonosis in the Americas [3] and it is considered as the most important parasitic disease in Latin America, because the morbidity and the economic losses that it can produce. Currently, about 8 million people are infected with this systemic disease and another 28 million are currently at risk of acquiring the infection. In America, the incidence of infection is approximately 41 200 new cases, and an average of 12 500 deaths occur annually [4]. The nomenclature of *T. cruzi* has been recently modified and six DTUs have been proposed within *T. cruzi* being: *T. cruzi* I (Tc I), *T. cruzi* II (Tc II), *T. cruzi* III (Tc III), *T. cruzi* IV (Tc IV), *T. cruzi* V (Tc V) and *T. cruzi* VI (Tc VI) to reflect the high genetic variability of the species [5]. Since this classification is one of the

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most currently accepted, we decided to follow it in order to homogenize the knowledge albeit to have performed some other analysis as biochemical and Restriction Fragment Length Polymorphism of the same group of trypanosomes (data not shown).

The proper characterization of the isolates of *T. cruzi* is essential to determine the possible role that the different strains play in the disease evolution, the geographic variability, the clinic stage, disease evolution or prognosis and the morbidity caused by the disease but also the resistance to specific drug chemotherapy. The introduction of new techniques, have increased the ability to characterize the parasites through isoenzymatic analysis, chromosomes migration, restriction enzymes, sequencing, DNA synthesis, DNA probes and the PCR[6]. The kDNAminicircles are a very useful target for PCR since there are present in 10–20 000 copies/parasite, so assays based on the PCR in amplification of these minicircles are especially sensitive[7]. Another advantageous strategy is the amplification of the conserved sequence that includes a hyper variable region that can be used to typify strains[8].

Superoxide dismutases (SOD) are a group of metalloenzymes that have a central component in antioxidant defense in most organisms by their ability of removes excess of superoxide radicals (O_2^-) by converting them to oxygen (O_2) and hydrogen peroxide (H_2O_2). According to their metal cofactor, SODs can be classified into four isoform types: iron (FeSOD), copper/zinc (Cu/ZnSOD), manganese (MnSOD) and nickel (NiSOD)[9]. However, in a number of protozoan organisms, only FeSOD has been found[10]. The enzymatic activity of the FeSOD in *T. cruzi* was detected first by Boveris and Stoppani[11]. Two SOD genes, Fesod–A and Fesod–B, were later cloned and characterized[12, 13]. Recently, new FeSOD–C and two closely related FeSOD–B genes (FeSOD–B1 and FeSOD–B2) have been identified in *T. cruzi* and in other trypanosomatids[10, 14].

The purpose of this work is to classify 21 new isolates in Colombia according to DTU to which they belong, this will be done based on the three molecular markers by PCR: SL, 24S rDNA[15, 16] and 18S rDNA[17]. Once classified, these strains were used for the tuning of a pair of primers designed according with the superoxide dismutase (sod–b) nucleotide sequence of the parasite to detect the presence of parasite DNA in biological samples no matter which strain of *T. cruzi* the sample has. Finally, the amplified fragments were studied by sequencing to obtain more complete information about them specially to let us know which are the differences at this level.

2. Material and methods

2.1. *T. cruzi* new stocks isolation and in vitro culture

The new 21 stocks from different biologic origins and geographical areas from Colombia and 3 different *T. cruzi* reference strains from South America. Epimastigotes were grown in the biphasic culture NNN (Novy–McNeal–Nicole), supplemented with minimal essential medium, and 20% of Foetal Bovine Serum Inactivated (FBSI) at 56 °C for 30 minutes. The isolates were then seed and cultured in vitro using Grace's insect medium supplemented with 10% of FBSI in 75 cm² flask (Roux) starting with a concentration of 5×10^4 parasites/mL at 28 °C. The parasites were removed by centrifugation at 600 g for 10 min at room temperature.

Eleven other trypanosomatids, 6 species belonging to *Leishmania* genera [*Leishmania amazonensis* (*L. amazonensis*), *Leishmania braziliensis* (*L. braziliensis*), *Leishmania guyanensis* (*L. guyanensis*), *Leishmania infantum* (*L. infantum*), *Leishmania mexicana* (*L. Mexicana*) and *Leishmania peruviana* (*L. peruviana*)] and 5 different *Trypanosoma rangeli* (*T. rangeli*) strains (*T. rangeli* 605, *T. rangeli* 606, *T. rangeli* 706, *T. rangeli* Pa and *T. rangeli* Pe) were cultured in similar conditions as described above at 28 °C in tissue–culture flasks until the cultures reached a density of approximately 2×10^7 parasites/mL. Cells were collected at the logarithmic growth phase by centrifugation (600 g for 10 min at room temperature). Similarly, mammalian cells (J774.2 macrophage stable cell line from mouse) were grown in a humidified 95% air, 5% CO₂ atmosphere at 37 °C, using in Minimal Essential Medium plus glutamine (2 mM) and 20% of FBSI. Finally the cells are collected by centrifugation at 100 g for 10 min and washed twice with phosphate buffered saline and about 10 mg of fresh or thawed pellet was used to isolate the DNA of each sample, following the manufacturer instructions of the kit mentioned below.

2.2. DNA extraction and purification

Trypanosomatids were collected by centrifugation of 300 mL of culture medium, when their concentrations had reached about 2×10^7 cells/mL, after about 5 days. They were washed twice in 50 mL of 0.15 M NaCl, 0.015 M sodium citrate, and once with SE buffer (0.15M NaCl, 0.1M EDTA, pH 8.0). Trypanosomatids, genomic DNA from cultured mammalian cells and human cells obtained by scraping the buccal mucosa were isolated following the purification procedure of Wizard® Genomic DNA Purification Kit (Promega).

2.3. PCR–fragments to DTU analysis

Once the DNA has been extracted and purified, the different samples were amplified for certain sequences using PCR. Firstly, the three primer reaction mix used were able to amplify the non–transcribed spacer of the minixon genes (SL) of the *T. cruzi* DNA, the primer sequences were: Tc1 (5′–GTGTCCGCCACCTCCTTCGGGCC–3′), Tc2 (5′–CCTGCAGGCACACGTGTGTGTG–3′) and TcC (5′–CCCCCTCCCAGGCCACACTG–3′). These primers amplified a fragment of 350 bp for the group *T. cruzi* I, 300 bp for the groups *T. cruzi* II, V and VI and no band was obtained for the groups *T. cruzi* III and IV[5]. Then, the amplification products were subjected to electrophoresis on 1.5% agarose gel containing 1:10000 of GelRed™ Nucleic Acid Gel Stain, during 90 minutes at 90 V. Finally the results were visualized in a UV–transilluminator and photographed with a digital camera Olympus Camedia, C–4000 Zoom.

The next PCR performed was done over a divergent domain from 24S α ribosomal RNA gene. The corresponding fragment was achieved with the primers: D71 (5′–AAGGTGCGTCCGACACTGTGG–3′) and D72 (5′–TTTTTCAGAAATGGCCGAACAGT–3′). The amplification products of these pair of primers was a fragment of 110 bp for the group *T. cruzi* I, II and V, 120 bp for the group *T. cruzi* IV or 125 bp length for the group *T. cruzi* VI[15,16]. Once again, the amplification products were subjected to electrophoresis on 3.0% agarose gel containing 1:10000 of GelRed™ Nucleic Acid Gel Stain, during 120 minutes at 80 V and carried out as described above.

The last PCR was performed over the small subunit ribosomal DNA (18S rDNA), so the primers used were: V1 (5′–CAAGCGGCTGGGTGGTTATTCCA–3′) and V2 (5′–TTGAGGGAAGGCATGACACATGT–3′). The amplification products of these pair of primers were fragment of 155 bp for the group *T. cruzi* IV, 165 bp for the group *T. cruzi* II, III and V or 175 bp for the group *T. cruzi* I, while no band was detected for DTU VI[17]. Further observation and documentation of the products was done equally as above.

2.4. Sod gene PCR

Finally, two primers were designed in our laboratory, based on the published sequence of the enzyme superoxide dismutase *T. cruzi* CL Brenner (GenBank accession No. XM_808937): iSODTc–r (5′–GTTGTCCGATGGCAACCTCTT–3′) and iSODTc–d (5′–ATGAGCTTCGTCATTCCTCC–3′). These two nucleotide sequences have been deposited in the GenBank database with accession number DQ441589.

This pair of primers amplify a fragment of approximately 270 bp belonging to superoxide dismutase gene b in all strains of *T. cruzi*. The PCR was performed in a total volume of 20 μ L. The reaction mixture: 10% of DMSO, 200 nM of iSODd, 200 nM of iSODr, 10 mM Tris–HCl (pH 9.0), 1.5 mM MgCl₂, 50 mM KCl, 0.01% gelatin, 0.1% triton X–100, 10 mM of each dNTP, 0.5 U of *Taq* DNA polymerase, 1–20 ng of kDNA and HPLC water to complete a final volume of 20 μ L. The amplification was done in Thermal Cycler™ MyCyclerthermocycler (BioRad) with the following routine temperature: 95 °C/3 min, 30 cycles of 95 °C/30 s, 55.5 °C/45 s, 72 °C/30 s and 72 °C/7 min. Next, the amplification products were subjected to electrophoresis on 1.5% agarose gel containing 1:10 000 of GelRed™ Nucleic Acid Gel Stain, during 90 minutes at 90 V.

2.5. Sequencing

The fragments amplified by PCR–SOD of the 24 strains, were migrated in low melting point agarose gel and then purified with a column (Wizard SV gel purification system; Promega, Madison, USA) and then sequenced. The sequencing was carried out, for the 300 bp fragments, using a capillary electrophoresis sequencer ABI3100 Avant (AppliedBiosystems). Finally, the chromatograms obtained were treated with the program MEGA4 getting a size sequence approximately 270 bp for each strain after removing the primers, where the variable nucleotides or polymorphism in the sequence were observed of each strain.

3. Results

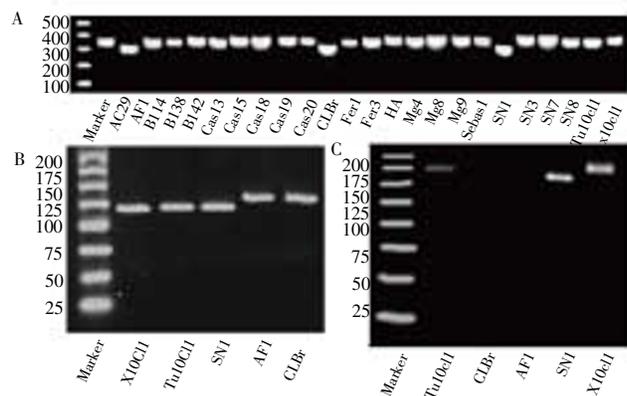
After performing the above three PCRs mentioned, a series of bands of the *T. cruzi* strains were obtained that allowed us to identify those DTUs which the new isolates belong to. Table 1 lists the identification names of the strains together with their geographical origin and the species from they were isolated. The next three columns show the results according to the three PCRs: SL, 24S α 18S ribosomal RNA gene and rDNA (Figure 1). The classification could be sufficiently clear only through PCR#1: SL (Figure 1A), with exception of AF1, SN1 and CLBr isolates which were submitted to PCR#2: 24S α rDNA (Figure 1B) and PCR#3: 18S rDNA (Figure 1C). The last column shows the final results obtained by assigning the DTU to which each strain belongs. As reference strains were selected two DTU I: Tu10cl1 and X10cl1[19], also one of the most studied strains, CLBr, representative of the DTU VI[20]. All new isolated belong to DTU I except for AF1 and SN1

Table 1Origin and classification of isolates studied of *T. cruzi*.

Code	Geographical origin	Biological origin	PCR#1:SL	PCR#2: 24S rDNA	PCR#3: 18S rDNA	DTU
AC 29	Acandí–Choco (Colombia)	<i>Rhodnius pallescens</i>	350	–	–	Tc I
AF 1	Amalfi–Antioquia (Colombia)	<i>Panstrongylus geniculatus</i>	300	125	no band	Tc VI
B 114	Cordoba (Colombia)	<i>Triatoma dimidiata</i>	350	–	–	Tc I
B 138	Cordoba (Colombia)	<i>Triatoma dimidiata</i>	350	–	–	Tc I
B 142	Cordoba (Colombia)	<i>Triatoma dimidiata</i>	350	–	–	Tc I
Cas 13	Casanare (Colombia)	<i>Rhodnius prolixus</i>	350	–	–	Tc I
Cas 15	Casanare (Colombia)	<i>Rhodnius prolixus</i>	350	–	–	Tc I
Cas 18	Casanare (Colombia)	<i>Didelphis marsupialis</i>	350	–	–	Tc I
Cas 19	Casanare (Colombia)	<i>Rhodnius prolixus</i>	350	–	–	Tc I
Cas 20	Casanare (Colombia)	<i>Rhodnius prolixus</i>	350	–	–	Tc I
*CL Br	Brazil	<i>Triatoma infestans</i>	300	125	no band	Tc VI
Fer 1	Bolivar (Colombia)	<i>Rhodnius pallescens</i>	350	–	–	Tc I
Fer 3	Bolivar (Colombia)	<i>Rhodnius pallescens</i>	350	–	–	Tc I
HA	Casanare (Colombia)	<i>Homo sapiens</i>	350	–	–	Tc I
Mg 4	Magdalena (Colombia)	<i>Rhodnius pallescens</i>	350	–	–	Tc I
Mg 8	Magdalena (Colombia)	<i>Triatoma dimidiata</i>	350	–	–	Tc I
Mg 9	Magdalena (Colombia)	<i>Triatoma dimidiata</i>	350	–	–	Tc I
Sebas 1	Magdalena (Colombia)	<i>Rhodnius pallescens</i>	350	–	–	Tc I
SN 1	Guajira (Colombia)	<i>Rhodnius prolixus</i>	300	110	165	Tc V
SN 3	Guajira (Colombia)	<i>Rhodnius prolixus</i>	350	–	–	Tc I
SN 7	Guajira (Colombia)	<i>Triatoma dimidiata</i>	350	–	–	Tc I
SN 8	Guajira	<i>Rhodnius prolixus</i>	350	–	–	Tc I
*Tu10cl 1	Bolivia	<i>Triatoma infestans</i>	350	110	175	Tc I
*X10cl 1	Brazil	<i>Homo sapiens</i>	350	110	175	Tc I

*Reference strains

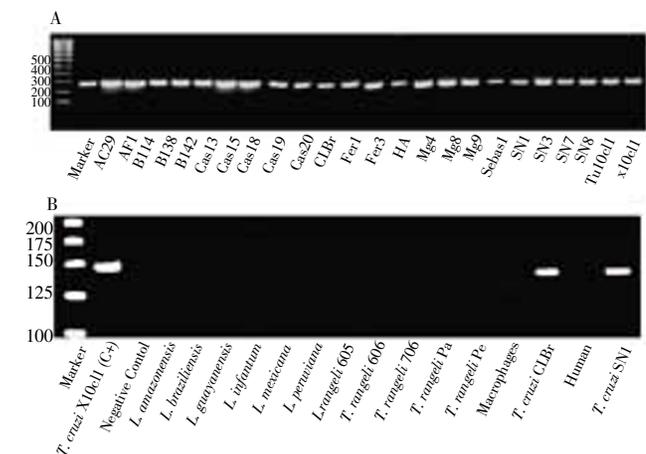
that belong to DTU VI and V, respectively.

**Figure 1.** DNA fragments obtained by the different PCR assays.

A, Shows fragment obtained after SL PCR; B, Shows fragment obtained after 24S rDNA PCR and C, Shows fragment obtained after 18S rDNA PCR.

Then the pair of primers designed in our laboratory was set up, using the same set of DNA samples. As it can be seen in Figure 2A, all strains originate a band near the 300 bp that corresponds to a fragment within the *sod* gene of the parasite. After obtaining these fragments, they were purified and sequenced as it has been described above; Table 2 shows the variations that appear within this fragment,

and the positions where the variations occur. Within the sequence of 270 bp, the variable positions were 20. All these DNA sequences were deposited into the NCBI/GeneBank database under the Accession numbers ET064919 to ET064942 (GSS category).

**Figure 2.** Tune up of new primers designed based on *sod* gene b primers.

A, Result of PCR amplification of different strains with the *T. cruzi*. B, Result of PCR amplification of DNA samples belonging to different species.

In order to confirm the *T. cruzi* *sod*–PCR specificity, this pair of primers was assayed with several DNA isolates of

Table 2Variable positions within a sequence of 270 bp belonging to the *sod-b* gene of the *T. cruzi* strains studied.

STRAIN CODE	Variable positions																			
	20	110	122	143	146	155	186	188	189	190	191	218	219	220	221	222	224	226	230	242
CLBr	C	C	C	T	C	G	T	C	T	T	G	–	G	A	G	A	G	C	–	A
AF1	G	A	–	–	.
Tu10cl 2	G	T	N	G	A	.	.	T	.	G	–	T	–	.
AC29	G	T	T	G	A	.	.	G	A	.	.	–	–	.
B114	G	T	T	G	A	.	.	–	T	.
B138	G	T	T	T	.	.	.	–	–	.
B142	G	T	T	T	.	.	.	–	–	.
Cas13	G	T	T	T	.	.	.	–	–	.
Cas15	G	T	T	.	.	.	–	–	.
Cas18	G	T	N	T	.	.	.	G	A	G	A	G	C	T	–	.
Cas19	G	T	N	T	.	.	.	T	–	.
Cas20	G	T	N	T	.	.	.	T	–	.
Fer1	G	T	T	T	.	.	.	–	–	.
Fer3	G	T	T	T	.	.	.	–	–	.
HA	G	T	T	T	.	.	.	T	–	.
Mg4	G	T	T	T	.	.	.	–	–	.
Mg8	G	T	T	T	.	.	.	–	–	.
Mg9	G	T	T	T	.	.	.	T	–	.
Sebas1	G	T	T	G	.	.	.	–	–	–
SN1	G	T	T	T	.	.	.	–	–	.
SN3	G	T	T	T	.	.	.	T	–	.
SN7	G	T	.	G	A	.	G	T	G	G	.	T	–	.
SN8	G	T	T	T	.	.	.	–	–	.
X10cl1	G	T	T	G	A	.	.	T	A	.	.	T	–	.

Key: G, guanidine; C, cytosine; A, adenine; T, timine; –, Gap; N, any nucleotide; •, repetition

different species of kinetoplastids (*Trypanosoma rangeli* and *Leishmania* spp.) and three mammals (mice, monkeys and humans). The results are shown in Figure 2B, which display only the expected band of around 300 bp in the samples corresponding to *T. cruzi* but not in any other non-*T. cruzi* DNA.

4. Discussion

As its has known, the clonal evolution of this parasite and its several forms found in a lot of mammal reservoirs, arthropod vector and humans has an important role in the behavior of a particular variety of a strain. The correct positioning and identification of its origin is very important for the comprehension and understanding of the epidemiological pattern that a particular clone of this

parasite may represent for a local environment, not only for the human impact in health but also for the economy (cost of treatment or work disabilities).

Thus, the results of this work show a clear representation of *T. cruzi* DTU I in Colombia, saving an isolate identified as DTU V and another as DTU VI. This result confirm the previous reports which showed the high prevalence of DTU I in north of the Amazon where is located Colombia^[19].

The PCR designed based on the sequence of the *sod* gene of *T. cruzi* allows to identify the strains of the parasite in different samples. After more extensive studies, including other kind of samples of different origin, it could become in a new tool to perform epidemiological surveys and genotyping studies, since it is a highly specific technique capable of discriminating the parasite DNA compared to other DNA samples from different species. It also would allow establish a criteria for cure after observing the disappearance of the

parasite DNA in chemotherapy experiments and new drugs design against *T. cruzi* as observed in preliminary studies currently being carried on in our lab (data not shown).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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