INTRODUCTION

Infection with Trypanosoma cruzi, the agent of Chagas disease, is largely spread over Latin America. Congenital T. cruzi infection involves, depending on the endemic area, 2–10% of children born to infected mothers. The diagnosis of T. cruzi infection in newborns is essential for the rapid administration of anti-parasitic drugs. Reliable, easy, and rapid diagnostic methods, sensitive enough to give results with a minimum of blood, are needed. The polymerase chain reaction (PCR) is a potentially useful tool that satisfies these criteria. Trypanosoma cruzi contains nuclear and kinetoplast DNAs (nDNA and kDNA), both of which contain many repetitive sequences that are highly suitable for PCR detection. The 195-basepair satellite repeat of nDNA is the target of the primers Tcz1/Tcz2 and Diaz1/Diaz2,3–7 which do not amplify DNA from other Trypanosoma or Leishmania. Other primers have been designed for the amplification of the nDNA repetitive element E13 (O1/O2)8 or the repetitive sequence for the flagellar protein F29 (BP1/BP2).9 The target present on nDNA is directly amplifiable by PCR without any pretreatment of the sample. However, even when only a few (1-10) parasites are present in a sample, the tandem repeated sequences should be sufficiently well dispersed to result in even distribution of the target.

The conserved domains of minicircles were the target used for the PCR of kDNA with primers S35/S36,6,10–12 121/122,13 CV1/CV2,14 and Tc1/Tc2,15 and with unnamed primers.16 However, such PCRs require partial disruption of the dense kinetoplast minicircle with either a nuclease11 or by boiling the sample in the presence of strong chaotropic agents such as guanidine chloride17 to release linear and amplifiable kDNA fragments. In addition, the quantitative reproducibility of such treatments in releasing kDNA minicircles of parasites was not assessed. Moreover, the S35/S36 primers also amplify kDNA of other parasitic Kinetoplastidae such as Leishmania species and T. rangeli, which are found in the same geographic areas as T. cruzi.18

The first T. cruzi PCRs were able to detect only large quantities of DNA and required extraction of large volumes of blood and a supplementary hybridization step or the use of a hot start PCR to increase their sensitivities and specificities.5,13,14,16,19–21 Moreover, the diagnostic application of a PCR also requires the detection of all T. cruzi strains that can infect patients. Recently, T. cruzi was divided in two major lineages. The T. cruzi lineage II has been further divided into five sublineages.22 However, the similarities of the conserved domains of kDNA in all T. cruzi isolates and the capacity of nDNA primers to detect all lineages of the parasite have not been verified.

The goal of our study was to compare different PCR primers to select those that would allow highly sensitive detection of all T. cruzi lineages in the diagnosis of congenital Chagas disease, as well as to improve the PCR protocol to obtain a reliable, robust, and inexpensive test that was easy to perform in developing countries.

MATERIALS AND METHODS

Blood and parasite samples. Umbilical cord blood samples of newborns from T. cruzi-infected or uninfected mothers were collected at the German Urquidi Maternity Hospital (Universidad Mayor de San Simon, Cochabamba, Bolivia). Maternal infection was assessed using classic parasite-specific serologic tests (enzyme-linked immunosorbent assay, hemagglutination, and immunofluorescence). We analyzed congenital infection with T. cruzi by direct microscopic examination of theuffy coat fraction of cord blood samples in heparinized microhematocrit tubes and/or by hemoculture. Cord blood samples were divided into three groups: 1) a con-
trol group of uninfected newborns from uninfected mothers (n = 30), 2 congenitally infected newborns (n = 18), and 3 uninfected newborns from chagasic mothers (n = 263). Blood was also obtained from one acutely infected patient with a patent parasitemia observed by direct microscopic examination. For each sample, 1 mL of blood was immediately mixed with the same volume of 6 M guanidine-HCl, 0.1 M EDTA, pH 8, boiled for 15 minutes, and kept at 4°C until use. This study was reviewed and approved by the scientific/ethic committees of the Universidad Mayor de San Simon and the Université Libre de Bruxelles. Written consent of the informed mothers was obtained before blood collection.

Culture-derived T. cruzi trypomastigotes (Tulahuen strain) were collected from the supernatants of fibroblasts from 3T3 rats infected 6–8 days earlier. They were washed three times in cold Dulbecco’s minimal essential medium (Life Technologies/Gibco/BRL, Gaithersburg, MD) and counted before being mixed with parasite-negative human blood samples. Pure DNA was isolated from epimastigotes of the T. cruzi lineages 1, 2a, 2b, 2c, 2d, and 2e and from T. rangeli (kindly provided by Drs. C. Barnabé and M. Tibayrenc, Centre National de Recherche Scientifique, Institut de Recherche pour le Développement, Montpellier, France) that were grown in liver infusion tryptose medium.

**Extraction of DNA.** Preliminary experiments using blood of the acutely infected patient allowed us to assess a simple and reliable method for extraction of DNA. Two-hundred microliters of blood/guanidine solution previously boiled for 15 minutes was extracted once with the same volume of phenol-chloroform, followed by a single extraction with 200 μL of chloroform. One microliter of GenElute-LPA, a soluble polyacrylamide (Sigma, St. Louis, MO), was added to the DNA mixture in the presence of 0.5 mM Tris-HCl, 1 mM EDTA. It was verified that the presence of GenElute-LPA in the sample did not interfere with the PCR amplification. The DNA extracted under such conditions was stable for at least three months at 4°C.

**PCR amplification.** The nuclear and kinetoplastic primers used in the present comparative study are shown in Table 1. This includes the previously described primers and primers Tca1/Tca2, which we designed to target the recently described repetitive element TclRE. To perform a maximal number of cycles without generation of secondary or non-specific products due to over-cycling, we used a lower amount (0.12 units) of Taq polymerase (Goldstar PCR kit; Eurogentec, Seraing, Belgium) than is usually recommended. Amplifications were performed using the GeneAmp 2400 apparatus (Perkin Elmer, Norwalk, CT) and 1 μL (10–15 ng) of extracted DNA in 0.2-mL thin-walled tubes, the buffer provided with the Taq polymerase by the manufacturer, 1.9 mM MgCl2, 0.2 mM of each dNTP, and 0.5 μM of each primer. The final volume of the reaction mixture was 20 μL. Optimal cycling parameters in this apparatus using thin-walled tubes resulted in shorter denaturation and annealing times and a higher programmed temperature than those used in other thermocyclers. Unless indicated otherwise, the cycling program included an initial denaturation at 94°C for 5 minutes, 30–40 amplification cycles at 94°C for 20 seconds, 57°C for 10 seconds, and 72°C for 30 seconds, and a terminal extension at 72°C for seven minutes. Three other PCR kits with automatic hot starts (FastStart; Roche, Basel, Switzerland; Platinum, Bethesda Research Laboratories, Rockville, MD; and Accuprime; Invitrogen, Carlsbad, CA) were also tested in some comparative experiments using the conditions recommended by the manufacturers or with a 1:10 dilution of the recommended amount of Taq polymerase.

Negative controls were included in each PCR. A PCR amplification of a fragment of the human β-globin gene was systematically performed with DNA extracted from blood samples to assess the integrity of extracted DNA.

Gel electrophoresis was performed using 1% agarose gels and TAE buffer (40 mM Tris, 40 mM acetate, 1 mM EDTA) in the presence of 0.5 μg/mL of ethidium bromide. A 1-kb

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primers used for the amplification of Trypanosoma cruzi and human β-globin DNA*</th>
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</thead>
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<tr>
<td>Primer</td>
<td>Sequence</td>
</tr>
<tr>
<td>---------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>I21</td>
<td>AAATAAATGTACGGGKGAGATGCATGA</td>
</tr>
<tr>
<td>I22</td>
<td>GGTTCGATTGGGGTTGGTGATATATA</td>
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<td>BP2</td>
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<td>Diaz2</td>
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<tr>
<td>O2</td>
<td>AGGAGTGACGGGGTTGAGCT</td>
</tr>
<tr>
<td>Pon1</td>
<td>TGGCTTGGAGGAGTATTG</td>
</tr>
<tr>
<td>Pon2</td>
<td>AGGAGTGACGGGTTCGAGTCAGT</td>
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<td>S35</td>
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</tr>
<tr>
<td>Tcz2</td>
<td>CTCACCTTTCTCCATGGAG</td>
</tr>
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</table>

* The origin (N = nuclear or K = kinetoplastic) and the approximate size of the amplicons are indicated.
(Life Technologies/Gibco/BRL) ladder was used as a standard. The gels were examined using the Appitek transiluminator (300 nm) (Ultra-Violet Products, Inc., San Gabriel, CA) and bands were visualized using Bio1D software (Wilber-Lourmat, Marne la Vallee, France). The same program was used to calculate the relative fluorescent intensity of the bands.

RESULTS

Comparison of primers in the PCR detection of T. cruzi infection. The robustness of our PCR system to variations in fundamental parameters was tested in preliminary experiments. We observed that an increase in the previously described annealing temperature (55°C) up to 60°C for the Tcz1/Tcz2 primers and a reduction in the time did not modify the amplification yield. In addition, only limited variations of the amplification yield was observed for magnesium concentrations between 1.5 mM and 2.5 mM. To increase the sensitivity of our PCR system, we amplified the DNA extracted from either T.cruzi epimastigotes or from blood of an acutely infected patient using the common nDNA primers Tcz1/Tcz2 and Diaz1/Diaz2 and 30, 35, and 40 amplification cycles. As shown in Figure 1, primers Tcz1/Tcz2 showed a visible amplicon of 200 basepairs after 30 cycles. Increasing the number of cycles led to a slight intensification of the 200-basepair band and the occurrence of an additional primer-dimer band. This primer-dimer band amplified during overcycling and was observed under all conditions tested. Thus, its presence is useful as an internal control of amplification. With the primers Diaz1/Diaz2, weak amplification of a 200-basepair was observed after 30 cycles. Its intensity increased after 35 and 40 cycles and no additional band was observed (Figure 1). However, the intensity of the Diaz1/Diaz2 amplicon was lower than that of Tcz1/Tcz2 amplicon. Based on these results, we have continued to use 40 cycles in all our subsequent experiments.

We also tested the other primers shown in Table 1. All resulted in amplicons of the expected sizes with high concentrations (100 fg/assay) of DNA purified from cultured parasites. However, depending on the sample, the kinetoplastid primers S35'/S36' and 121/122 amplified fragments that showed variable intensity, ranging from 10 times lower to similar to that of Tcz1/Tcz2 (see Discussion). The nuclear primers Pon1/Pon2 amplified fragments that showed at least 100 times lower intensity than those amplified with Tcz1/Tcz2. When the amplification was performed with DNA extracted from blood of an acutely infected patient, primers Tca1/Tca2 amplified a 250-basepair amplicon of similar intensity to that obtained with Tcz1/Tcz2 and Diaz1/Diaz2, whereas the primers BP1/BP2 and O1/O2 did not amplify any DNA fragments under these conditions (Figure 2). This low intensity was observed even when annealing temperature was decreased to 53°C.

Trypanosoma cruzi lineages detected by the PCR. Since the differential amplifications observed with the tested primers could arise from primer specificities restricted to some T. cruzi lineages, we tested the primers with aliquots of purified
DNA representing all lineages of *T. cruzi* (1, 2a, 2b₁, 2b₂, 2c, 2d, and 2e) at DNA concentration of 0.2 pg/assay. Primers Tcz1/Tcz2 (Figure 3A) and Diaz1/Diaz2 (Figure 3B) showed strong amplification of similar intensity with all lineages. The Tca1/Tca2 primers also amplified the DNA from all *T. cruzi* lineages, but the band intensity was stronger with the lineages 2c, 2d, and 2e (Figure 3C). The S35/S36 primers showed a more intense 330-basepair amplicon with the lineages 2d and 2e, whereas the primers O1/O2 amplified only a 220-basepair band with DNA extracted from lineages 1, 2a, 2d, and 2e, and the primers BP1/BP2 showed negative results with all lineages. At a DNA concentration of 20 pg/assay, primers BP1/BP2 amplified all lineages (amplicon of 700 basepairs), except 1 and 2b₁. Based on these results, we selected Tcz1/Tcz2 and/or Diaz1/Diaz2 primers, which under our experimental conditions, showed amplification of all *T. cruzi* lineages and were easier for routine use. Moreover, we also confirmed that the Tcz1/Tcz2 and Diaz1/Diaz2 primers were not reactive with *T. rangeli* DNA (Figure 3).

**Sensitivity of the PCR in detecting *T. cruzi***. The sensitivity of the Tcz1/Tcz2 PCR system was estimated by assessing the amplification of DNA extracted from either *T. cruzi* epimastigotes or blood artificially infected with *T. cruzi* trypomastigotes. Both samples were serially diluted in the DNA solution.

![Figure 3](image-url)

**Figure 3.** Amplification of DNA extracted from all *Trypanosoma cruzi* lineages using various primers. DNA was purified from the reference lineages of *T. cruzi* (1, 2a, 2b₁, 2b₂, 2c, 2d, and 2e) amplified under the standard conditions described in the Materials and Methods. DNA extracted from *T. rangeli* was used as a control of specificity. **A**, Tcz1/Tcz2 primers; **B**, Diaz1/Diaz2 primers; **C**, O1/O2; S35'/S36', and Tca1/Tca2. Std = standard; Neg. Cont. = negative control; kb = kilobases.
extracted from *T. cruzi*-negative blood. Amplification was observed with epimastigote DNA at a concentration as low as 1 fg/assay (Figure 4A). If one assumes a mean DNA content of 300 fg/parasite, this corresponds to approximately 0.003 parasites/assay.

Amplification of DNA extracted from artificially infected blood was performed in four independent duplicate experiments. Figure 4B shows the relative fluorescent intensity of bands as a function of the number of parasites per assay. A strong amplification was observed in all four experiments using DNA corresponding to one parasite per extracted sample (0.1 mL of blood), i.e., 0.02 parasites/assay, and even a 10 times lower amount of *T. cruzi* DNA was detected (0.002 parasites/assay). Thus, this PCR assay has sensitivity sufficient to detect only one parasite present in a sample. Such sensitivity was similar to that observed using pure parasite DNA (Figure 4).

The limit of detection of the Tcz1/Tcz2 primers was also tested using three hot start PCR kits (Figure 5). When we used the protocols supplied with these kits, we observed smears with bands of approximately 200, 400, and 600 basepairs. These additional bands probably originated from tandem repetitions of target sequences in the *T. cruzi* DNA. Cross-hybridizations of these longer amplicons probably resulted in the smears observed at higher concentration of target DNA. By decreasing the amount of *Taq* polymerase recommended by the manufacturers of these kits by a factor of 10, we were able to improve the intensity of the bands, resulting in a sensitivity similar to (FastStart) or higher than (Platinum) that obtained with the Goldstar kit (Figure 5).

**Application of the PCR to the diagnosis of congenital *T. cruzi* infection.** We used the procedures developed to screen 311 umbilical cord blood samples described in the Materials and Methods. All samples were tested in duplicate with the Tcz1/Tcz2 primers to detect the presence of parasitic DNA, and with β-globin primers to check the integrity of DNA in the extracted sample. Figure 6 shows two series of PCR results obtained with these samples. Both positive and negative samples showed the β-globin amplicons. A 200-basepair band appeared in all 18 newborns with congenital *T. cruzi* infections, whereas all 30 parasitologically negative babies born to uninfected mothers showed negative PCR results. The screening of 263 blood samples from parasitologically negative babies born to chagasic mothers showed negative PCR results, except for one sample that previously classified as negative based on the result of the microhematocrit test. Since it was not possible to collect additional blood samples from this baby and to identify by a parasitologic test a possible false-positive PCR result, we repeated the extraction of DNA and the PCR protocol with the same sample. We again obtained a positive PCR result for the presence of *T. cruzi* DNA.

**DISCUSSION**

Our results show that a simple, reliable, and inexpensive PCR protocol using Tcz1/Tcz2 primers can be used for the diagnosis of congenital Chagas disease with high sensitivity and specificity for all *T. cruzi* lineages.

It was previously reported that the use of the blood-guanidine-HCl mixture carries over substances that could inhibit PCR amplification of *T. cruzi* 195-basepair satellite repeats. For this reason, many investigators use different (more or less complicated) proteinase K digestion steps in the preparation of *T. cruzi* nDNA. Alternatively, Tth polymerase, which is inhibited less by blood components, has been used instead of *Taq* polymerase. The simple phenol/chloroform extraction protocol for blood has been used with other primers, such as O1/O2, or with kinetoplastic primers. Use of glycogen as a precipitant carrier in the simple extraction protocol for isolation of kDNA from blood samples has also been reported. Our results show that, at least for the diagnosis of congenital *T. cruzi* infection, it is...
possible to use a blood-guanidine-HCl mixture and a single phenol/chloroform extraction to isolate nDNA of sufficient purity for further PCR amplification. Moreover, the addition of a soluble polyacrylamide as a precipitant carrier to such mixtures improves the reproducibility and the visual control of the nDNA extraction without affecting the subsequent amplification.

Boiling the blood-guanidine-HCl mixture partially disrupts the dense kDNA minicircle.13,17 Our data indicate that such treatment also disrupts parasitic nDNA. Indeed, such disruption of nDNA is essential if one wants to detect low numbers of parasites in small volumes of blood. In contrast, an absence of or insufficient disruption of DNA containing highly repetitive target sequences may lead to a random distribution of DNA in the PCR samples, resulting in excessive, poor, or no PCR amplification.

The limit of detection of the PCR using primers Tcz1/Tcz2 was at least 0.002 parasites/assay, i.e., in the same range as other more sophisticated protocols such as post-PCR hybridization4, assay of a blood nuclear extract5, proteinase K treatment of samples6,7, hot start PCR13, competitive PCR12, or nested PCR.30,31 Our results are consistent with those of a previous report that indicate that the sensitivity of the Tcz1/Tcz2 PCR is approximately 10 times higher than that of the PCR using the kS35'/H11032/kS36'/H11032 primers,6 despite the theoretically similar number of targets. Such results might be related to an incomplete dispersion of the dense kinetoplast minicircle present in the blood samples.

**Figure 4.** Relative polymerase chain reaction (PCR) amplification intensity of serially diluted *Trypanosoma cruzi* DNA. The limit of detection of *T. cruzi* DNA by PCR amplification with Tcz1/Tcz2 primers was assayed on A, serial dilutions of DNA extracted from epimastigotes or B, from human blood artificially infected with trypomastigotes. B shows the mean ± SEM results of five experiments done in duplicate. The density of each amplified band was measured using Bio1D software. The relative density of each sample was calculated using the highest concentration of DNA as 100%. Std = standard.
Although many PCR systems were previously reported for the detection of *T. cruzi*, their capacity to amplify all lineages of *T. cruzi* at a similar yield was not tested and only the results obtained with few non-typical strains were compared. Among the six primer pairs we have tested, only Diaz1/Diaz2 and Tcz1/Tcz2, which amplify the same repetitive nuclear sequence, detected all *T. cruzi* lineages with a similar intensity. In contrast, we observed that some of the nDNA primers, such as Bp1/Bp2 or O1/O2, which have been recommended for the detection of *T. cruzi*, amplified only some lineages. This suggests that there may be variations in the corresponding repetitive nuclear sequences between lineages. Such primers might be potentially useful in the determination of parasite lineage groups, but not for diagnosis application. Moreover, although kDNA primers might equally amplify all lineages, we observed that the primers S35'/S36' resulted in amplicons of different intensities. Similar results were obtained with the nuclear primers Tca1/Tca2. Nevertheless, since these primers could amplify DNA of all lineages, they might be useful in second-line PCR assays to assess doubtful results when contamination with the Tcz1/Tcz2 amplicon is suspected.

Our protocol is more simple and shorter than the previously published protocol used for detection of congenital *T. cruzi* infection. The sensitivity of our PCR protocol using Tcz1/Tcz2 primers is sufficient for diagnostic applications, since a single parasite can be detected in a reasonable volume of blood (0.1 mL). Such sensitivity should be sufficient to detect congenital infection corresponding to an acute parasitemic phase. All newborns congenitally infected with *T.
cruzi tested positive in our PCR protocol. In contrast, none of newborns born to uninfected mothers were positive, indicating the high reliability of our PCR test. Among the 263 parasitologically negative newborns from T. cruzi-infected mothers, only one was positive for the presence of the T. cruzi DNA. Since this baby did not undergo further medical testing, it has not been possible to confirm this congenital infection. However, comparison of this PCR method with several parasitologic or serologic assays in such neonatal diagnosis is presently under way.

Our results also indicated that comparable results were obtained with the three hot start PCR kits tested only after significant dilution of Taq polymerase. This suggests that the high activity of Taq polymerase is detrimental in the detection of tandem repetitive sequences of 195-basepair satellite repeats. The amplification step in our protocol gave better reproducible results when the Taq polymerase was used at concentrations three times lower than those currently used. Furthermore, use of a small volume of blood requires minimal amounts of reagents, and the simple procedure of DNA extraction requires limited equipment, thus decreasing markedly the cost of each PCR assay. Our study demonstrates a reliable, robust, and cheap PCR protocol that detects all T. cruzi lineages, is easy to perform by persons unskilled in the methods of molecular biology, and will be a useful alternative tool in the diagnosis of congenital Chagas disease.

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PCR FOR DIAGNOSIS OF CONGENITAL T. CRUZI INFECTION

REFERENCES


