Comparison of PCR and Microscopic Methods for Detecting *Trypanosoma cruzi*

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The diagnosis of acute infection with *Trypanosoma cruzi*, the protozoan parasite that causes Chagas' disease, is generally made by detecting parasites by microscopic examination of fresh blood. Although highly specific, this approach often lacks sensitivity. Several years ago, PCR assays for the detection of *T. cruzi* were described, but the sensitivities and specificities of these tests have not yet been defined precisely. In the present study, we first compared the sensitivities of PCR methods that differ in sample processing as well as in the target sequences that are amplified. Then, we challenged eight mice with *T. cruzi*, and on 31 days over a 380-day period, we compared the ability of the PCR method with the highest sensitivity to detect parasites in blood with that of microscopic examination. During the acute phase of the infections, parasites were detected on average 3.9 days earlier by the PCR method than by microscopy. Furthermore, the infected mice were consistently positive by the PCR method during the chronic phase, while parasites were intermittently detected by microscopic examination during that period. Overall, among the 248 comparisons, in 84 the PCR method was positive and no parasites were seen by microscopic examination, whereas the reverse was true in only 1 case, a difference that is highly significant. These findings suggest that this approach should be used in patients suspected of having acute Chagas' disease. Moreover, the higher sensitivity of the PCR method observed in both the acute and chronic phases of the *T. cruzi* infections in the mice that we studied indicates that this approach should be useful in evaluating experimental drugs in *T. cruzi*-infected laboratory animals.

American trypanosomiasis (Chagas' disease), caused by the protozoan parasite *Trypanosoma cruzi*, can result in serious morbidity and death in both its acute and chronic forms (6). Since early drug treatment of acute *T. cruzi* infections in humans is presumed to be beneficial, there is a need for a sensitive, specific, and rapid diagnostic assay for acute Chagas' disease. Moreover, in drug research there is a similar need for detecting *T. cruzi* in laboratory animals treated with experimental agents. Acute *T. cruzi* infection in humans as well as in experimental animals is generally diagnosed by detecting parasites by microscopic examination of fresh blood. Although highly specific, this approach often lacks sensitivity. An alternative approach is to test for anti-*T. cruzi* immunoglobulin M, but this assay has not been standardized and is not widely available.

Several years ago, PCR assays for detecting *T. cruzi* that are highly sensitive in contrived experiments and with small numbers of persons with chronic *T. cruzi* infections were described (9, 16). Although several reports describing the use of these tests have been published, their sensitivities and specificities in laboratory animals and humans with acute and chronic *T. cruzi* infections have not been defined precisely (1, 3, 4, 15, 17). Direct assessment of the sensitivities of PCR *T. cruzi* detection assays is difficult because there is no standardized test for detecting low numbers of parasites in blood that could be used as a basis for comparison. Moreover, this problem cannot be easily circumvented experimentally, since there is no practical way to count very small numbers of parasites which then could be added to measured amounts of blood for testing.

The goal of the present study was to compare the sensitivities of microscopic examination of fresh blood and a PCR assay for the detection of *T. cruzi* in mice. To this end, over a 380-day period we studied by microscopy and a PCR assay paired blood specimens obtained from eight mice challenged with *T. cruzi*. We found that during the early part of the acute phase and throughout the chronic phase of the infection, the sensitivity of the PCR assay is greater than that of microscopy.

MATERIALS AND METHODS

Production of parasites and infection of mice. *T. cruzi* trypomastigotes (Tulahuén strain [12]) were produced in human renal adenocarcinoma cells as described previously (10). Eight BALB/c mice were given 2,000 trypomastigotes under methoxyflurane anesthesia either orally (mice PO1 to PO4) or by intraperitoneal injection (mice IP1 to IP4). The oral route was chosen to mimic contaminative vector-borne transmission that accounts for a large proportion of cases of *T. cruzi* infection in humans and other mammals, while intraperitoneal injection is similar to what occurs in transfusion-associated transmission of the parasite and laboratory accidents.

Microscopic examination of fresh blood for parasites. For microscopic examination of fresh blood, 1.5 μl was collected by tail snip and was examined for motile trypomastigotes under a 12-mm-diameter coverslip at ×400 magnification. Immediately after each blood sample was drawn, 200 fields were examined by a single observer (L.V.K.) over approximately 20 min.

Blood sample collection and preparation of DNA samples. Samples of 25 μl of mouse blood were collected by tail snip and were added to tubes containing 2 μl of 0.2 M EDTA (final concentration, 16 mM) to prevent coagulation. The samples were processed immediately or were stored at −20°C. To process by the sodium dodecyl sulfate (SDS)-proteinase K method (9), 5 volumes of lysis buffer (10 mM Tris-HCl [pH 7.6], 10 mM EDTA, 0.1 M NaCl, 0.5% SDS, 300 μg of proteinase K per ml) was added to the samples, and the mixtures were incubated for 2 h at 50°C. The samples were then extracted twice with a mixture of phenol-chloroform-isoamyl alcohol (25:24:1). Nucleic acids were precipitated with 2 volumes of chilled absolute ethanol. After centrifugation in a microcentrifuge for 30 min at 4°C, the pellet was rinsed with 70% ethanol, air dried, and suspended in 50 μl of water.

To process by the guanidine-phenanthroline method (2), 4 volumes of 2× lysis

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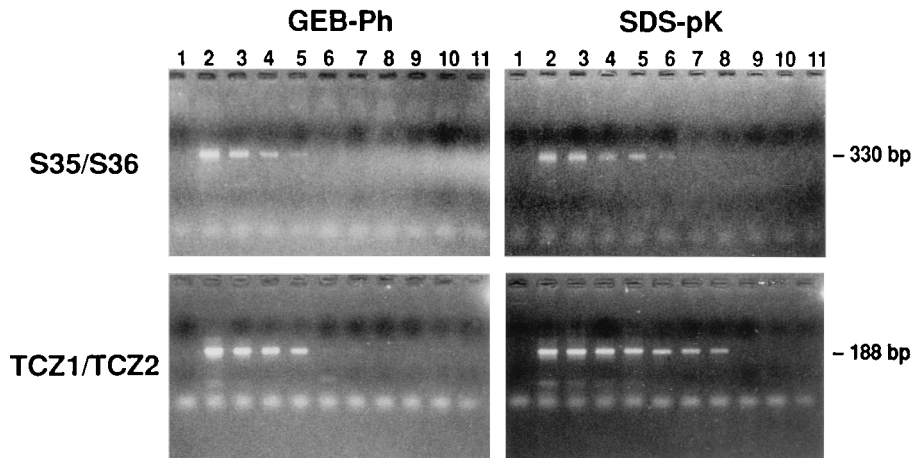† Present address: Yale University School of Medicine, New Haven, CT 06510.

FIG. 1. Results obtained by PCR assays for *T. cruzi* detection in which two DNA extraction methods and two primer sets were compared. A blood sample containing 810 parasites in 25 μl was serially diluted threefold with uninfected mouse blood. The GEB-phenanthroline (GEB-Ph) (2) and SDS-proteinase K (SDS-pK) (9) procedures were then used to process 25-μl samples of each dilution. DNAs obtained by these two methods were then used as templates in PCRs in which either the S35-S36 primers or TCZ1-TCZ2 primers had been added. Electrophoresed amplification products are shown in ethidium bromide-stained agarose gels. In each panel lanes 1 to 11 represent the results obtained with 25-μl blood samples containing the following calculated number of parasites: 0, 810, 270, 90, 30, 10, 3, 1, 1/3, 1/9, and 1/27, respectively.

buffer containing 6 M guanidine-HCl and 0.15 M EDTA (pH 8.0) (GEB) and 3 volumes of water was added to the 25-μl blood samples for a total reaction volume of 200 μl. A total of 0.1 volume of each of the following solutions was then added: 1 M $MgCl_2$, 200 mM $CuSO_4$, 20 mM 1,10-phenanthroline, and 7.5% H_2O_2 (diluted fresh from 30% stock). The reaction was initiated by the addition of 0.1 volume of 50 mM 3-mercaptopropionic acid, and digestion of DNA was allowed to proceed for 30 min at 37°C. The reaction was stopped by the addition of 0.1 volume of 1.5 M 2,9-dimethyl-1,10-phenanthroline. Immediately following the digestion, each sample was extracted once with 1/6 volume of chloroform and once with 1/6 volume of phenol and chloroform (1:1). The aqueous phase (about 250 μl) was added to a solution containing 2 volumes of absolute ethanol, 20 μg of glycogen, and 25 μl of 3 M sodium acetate to precipitate the nucleic acids. After centrifugation in a microcentrifuge for 20 min at room temperature, the pellet was suspended in 100 μl of water and the tube was again rinsed with 100 μl of water. The 200-μl sample was transferred to Centricon-100 microconcentrators (Amicon) containing 1.8 ml of water. The Centricon-100 unit was centrifuged at 1,000 × g for 15 min, and the retentate was washed again with 2 ml of water. After the second 15-min centrifugation, the 100 μl of concentrated retentate was collected as suggested by the manufacturer.

PCR amplifications. PCRs were performed with the TCZ1 (5′-CGAGCTCT TGCCCACACGGGTGCT) and TCZ2 (5′-CCTCCAAGCAGCGGATAGTTC AGG) primer pair, which amplifies 188 bp of a 195-bp repetitive nuclear sequence (~100,000 copies per parasite) (9), or the S35 (5′-AAATAATGTACG GGTGAGATGCATG) and S36 (5′-GGGTTCGATTGGGGTTGGTG) primer pair, which amplifies a 330-bp minicircle sequence (~120,000 copies per parasite; 4 in each of ~30,000 minicircles) (16), by using conditions described previously for the two primer sets. After thermal cycling, 10 μl of each reaction mixture (1/5 of the reaction volume) was electrophoresed in a 3% NuSieve GTG agarose gel containing 1 μg of ethidium bromide per ml.

To minimize the possibility of contamination of reaction mixtures with *T. cruzi* DNA or amplification products from previous runs, blood samples were processed and PCRs were set up in a laboratory in one building, and PCRs were run and electrophoresis was performed in a second facility. Aerosol-free barrier tips were used in the first laboratory, and separate supplies obtained directly from outside sources were used in the two work areas. To assess the efficacies of these procedures in avoiding contamination, negative control mice were bled under the same conditions as the test mice, and these samples were processed in parallel with the test specimens. Other controls included blood from a *T. cruzi*-infected mouse and distilled water as templates.

Radioimmune precipitation assay. As a confirmatory assay for chronic *T. cruzi* infection, mouse sera were tested in a previously described radioimmune precipitation assay (7, 8).

RESULTS

The goal of our first set of experiments was to determine which combination of the two primer sets and the two blood sample processing methods described previously would give the most sensitive PCR assay for *T. cruzi* detection. To this end

we took blood from an infected mouse (~810 parasites per 25 μl), did nine serial 1:3 dilutions with blood from an uninfected mouse, divided the resulting dilutions into eight aliquots, and then ran the two sets of four aliquots and negative controls through PCRs based on the four possible combinations of primer sets and blood processing methods. Similar results were obtained in the two runs. As can be seen by comparison of the two horizontal pairs of panels shown in Fig. 1, in both cases SDS-proteinase K processing gave positive results in two more dilutions than did GEB-phenanthroline processing (four versus six positive results with S35-S36; five versus seven positive results with TCZ1-TCZ2). Comparison of the two vertical pairs of panels shows that in both instances the TCZ1-TCZ2 primer set gave positive results in one more dilution than did the S35-S36 primer pair (four versus five positive results with GEB-phenthroline; six versus seven positive results with SDS-proteinase K). Since these findings suggested that the PCR with SDS-proteinase K and TCZ1-TCZ2 is the most sensitive of the four PCR assays evaluated, we used it in the remaining experiments in the study.

To compare directly the sensitivities of the PCR assay and microscopic examination, we studied paired blood samples from the eight *T. cruzi*-challenged mice obtained on 31 days over a 380-day period. Representative results obtained by the PCR assay are presented in Fig. 2, which shows the amplification products obtained on day 51 of the experiment, when all eight mice were negative by microscopy. As a basis for comparison, lane 4 shows the expected 188-bp band obtained when blood from a *T. cruzi*-infected mouse was used as a positive control. Similar bands are present in lanes 5 to 7 (mice IP1 to IP3, respectively) and lanes 9 to 12 (mice PO1 to PO4, respectively), thus indicating that these seven mice had *T. cruzi* parasitemias detectable by the PCR test at that time. Lane 8, representing the IP4 mouse, is negative, lacking the 188-bp band, as are the negative control lanes, lanes 2 and 3, which represent water and uninfected mouse blood templates, respectively.

The lack of 188-bp bands in lanes 2 and 3 also indicates that the positive results obtained with the *T. cruzi* control and the seven positive test mice are not false-positive results resulting
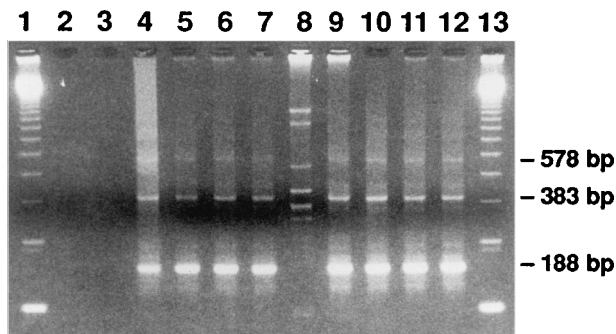
1 2 3 4 5 6 7 8 9 10 11 12 13

– 578 bp

– 383 bp

– 188 bp

FIG. 2. Sensitivity of a PCR assay for detecting *T. cruzi* in mouse blood. Template DNA extracted from 25-μl blood samples by the SDS-proteinase K method and with the TCZ1-TCZ2 primer pair (9) were used in the reactions. Electrophoresed amplification products are shown in an ethidium bromide-stained agarose gel. Lanes 1 and 13, 123-bp ladder; lane 2, negative control, water as template; lane 3, negative control, uninfected mouse blood as template; lane 4, positive control, blood from a *T. cruzi*-infected mouse; lanes 5 to 8, blood from mice IP1 to IP4, respectively; lanes 9 to 12, blood from mice PO1 to PO4, respectively. Processed samples were obtained 51 days after challenge, at which time microscopic examination of fresh blood was negative for parasites in all mice.

TABLE 1. Comparison of rates of detection of *T. cruzi* in eight mice by PCR assay and microscopic examination[a]

Microscopy result	PCR assay result	
	+	−
+	116	1
−	84	47

[a] Paired blood specimens obtained on 31 days over a 380-day period were used ($\chi^2 = 47.2$; $P < 10^{-10}$).

much more sensitive detector of *T. cruzi* parasitemia than microscopic examination of fresh blood.

One of the goals of the present study was to determine if the PCR assay could detect acute *T. cruzi* infection earlier than microscopy could. Examination of the data from the first 10 days of the experiment depicted in Fig. 3 indicates that this is the case. In six of the mice the PCR assay was positive several days before parasites were seen microscopically, in one mouse (mouse IP4) the two approaches were first noted to be positive on the same day, and in one mouse (mouse IP3) microscopy was positive a day before the PCR test. On average, the PCR assay gave the diagnosis of *T. cruzi* infection 3.9 days earlier than microscopic examination.

It is of interest that in the PCR assay three of the mice infected orally were positive on day 4, negative on day 5, and then positive on day 6 and during the remainder of the experiment. Since this observation runs contrary to parasitemia curve patterns measured in other experiments (5, 8), we ran a second set of PCRs using DNAs extracted from the original samples, and we also did PCRs using DNAs extracted from backup samples drawn from the mice on that same day. These experiments confirmed the original results, and control specimens gave the expected outcomes, and thus, we found no technical basis for the unexpected results obtained on day 5.

A final observation of interest in Fig. 3 is that the IP4 mouse was negative in the PCR assay and by microscopy throughout the experiment except on day 6, and we performed an additional experiment to shed light on the infection in this mouse. We tested serum from the IP4 mouse in the radioimmune precipitation assay, and the results are provided in Fig. 4. As a basis for comparison, lane 1 shows the pattern of immunoprecipitated *T. cruzi* antigens obtained with serum from a mouse known to be chronically infected with the parasite. This lane

from contamination of the reaction mixtures with *T. cruzi* DNA or with 188-bp amplicons produced in previous PCR runs in which the TCZ1-TCZ2 primer set was used. Moreover, the result for the negative mouse blood control indicates that the 188-bp bands seen in the positive lanes are not the result of nonspecific amplification of mouse DNA sequences. Finally, it is of interest that in the eight positive lanes, 383- and 578-bp bands are present, in addition to the 188-bp bands. These are also specific products of the PCR that result from amplification of two and three of the 195-bp repeats, which are arranged in tandem arrays.

The overall results obtained with the two diagnostic approaches during the 13-month course of *T. cruzi* infection in the eight test mice are shown graphically in Fig. 3. As summarized in Table 1, there were 163 (116 + 47) mouse-days on which the results of the two assays were concordant, whereas there were 84 instances in which the PCR test was positive and microscopy was negative, and there was only 1 case in which the reverse was true. This difference is highly significant ($\chi^2 = 47.2$; $P < 10^{-10}$) and indicates clearly that the PCR assay is a

Days After Challenge

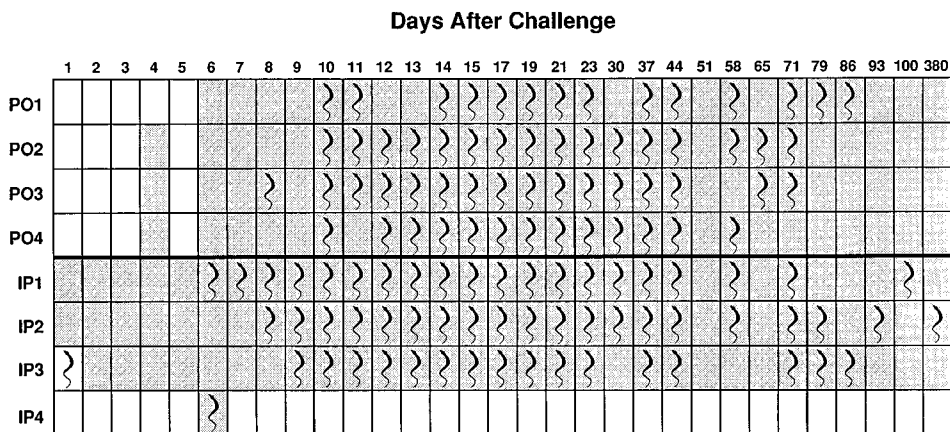|	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	17	19	21	23	30	37	44	51	58	65	71	79	86	93	100	380

FIG. 3. Comparison of results of PCR assays and microscopic examination for *T. cruzi* in mice challenged by mouth or intraperitoneally. A gray background indicates positivity in the PCR assay, and the trypanosome icon denotes positivity by microscopic examination of fresh blood.
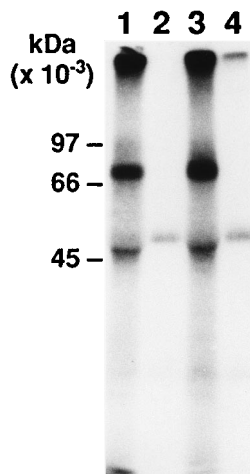
FIG. 4. Detection of antibodies to *T. cruzi* in mouse sera by radioimmune precipitation assay. Immunoprecipitated ^{125}I-labeled *T. cruzi* antigens were detected by autoradiography after electrophoresis. Lane 1, *T. cruzi*-infected control mouse; lane 2, IP4 mouse; lane 3, IP3 mouse; lane 4, uninfected control mouse.

has a broad band centered around 72 kDa and an even broader and less intense band centered around 90 kDa, which is the pattern typically obtained with sera from *T. cruzi*-infected mice. Lane 2, which is the IP4 mouse lane, has neither of these bands and is indistinguishable from lane 4, which is the negative control mouse lane. As an additional internal control, lane 3 contains the antigens immunoprecipitated with serum from the IP3 mouse, which was found by the PCR assay and by microscopy to be chronically infected with *T. cruzi*. When taken together, these results indicate unequivocally that the IP4 mouse was not chronically infected with *T. cruzi*, even though on day 6 of the experiment it clearly had circulating parasites.

DISCUSSION

In the first part of the study we did a direct comparison of the sensitivities of PCR assays that used the four possible combinations of two blood sample processing methods and two primer sets. Our results suggest that the PCR assay with SDS-proteinase K and TCZ1-TCZ2, which is easier to perform than the GEB-phenanthroline procedure, is more sensitive than the others. As noted, we prepared the diluted blood samples that were run in the four assays in a manner that would optimize the likelihood that the four tubes at each dilution contained the same number of parasites. Nonetheless, when the number of parasites is very small, even the approach that we used could result in different numbers of parasites in tubes that are meant to have the same number. The potential importance of this variability in the distribution of whole parasites, however, may be diminished by the presence of free parasite DNA in the liquid portion of the blood samples. Russomando et al. (15) showed that sera from patients with acute and chronic *T. cruzi* infections consistently contained DNA fragments that could be amplified by the TCZ1-TCZ2 primer pair to produce the 188-bp product. Presumably, the free parasite DNA in blood specimens comes from partially degraded organisms, and its presence would increase the accuracy of the dilution method that we used. At any rate, we feel that it is important to keep in mind that our assessment of the four detection methods was limited in scope and does not constitute a definitive comparison of these procedures.

The central finding of the present study is that the PCR assay is significantly more sensitive than microscopic examination of fresh blood for detecting *T. cruzi* parasitemias in mice. This relatively greater sensitivity to a considerable degree probably results from the larger volume of blood processed in the PCR assay (25 versus 0.44 μl). The greater sensitivity of the PCR assay allowed considerably earlier detection of the parasites as the acute phase developed in the challenged mice, and also resulted in much more frequent detection of parasites during the chronic phase. During the acute phase the PCR assay offered no advantage, since the parasites were easily seen by microscopy. The Tulahuén strain of *T. cruzi* that we used here is known to cause easily detectable parasitemias during the acute phase in laboratory animals, but many other strains and clones of the parasite result in lower acute-phase parasitemias that are difficult to detect by microscopy (13, 14). Thus, in humans and other mammals infected with "low-parasitemia" isolates, the PCR assay may also be advantageous in comparison with microscopy throughout the entire course of the infection.

It is important to note that the method of microscopic examination of fresh blood for *T. cruzi* has not been standardized. Thus, our choosing to examine 200 fields, which in aggregate contain only 0.44 μl of blood in the system that we use, was arbitrary and was based on the time that it took the observer to examine this number of fields (~20 min) and the fact that eight mice needed to be studied every day during the initial days of the study. It is reasonable to expect that increasing the number of fields examined would increase the likelihood of seeing parasites, but since observer fatigue is an important element in microscopy, looking at a greater number of fields might lead to failing to notice some parasites. Obviously, this is not an issue in the PCR assay. It is also worthy of mention that microscopy must be done when the blood is fresh, and it cannot be stored for examination hours or days later, whereas the blood processing and the PCR assay can be interrupted at several points without any deleterious effects.

One persistent problem with PCR detection assays for *T. cruzi* and other pathogenic microorganisms is the occurrence of false-positive results caused by contamination of reaction mixtures with amplification products of previous runs in which the same primer set was used. The consistently negative results that we obtained with the numerous negative controls included in our runs indicate that false-positive reactions were not a problem in the PCRs that we did. To accomplish this, however, we used laboratories in different buildings to do the two major phases of the PCRs. There are several other ways to avoid the problem of false-positive reactions caused by contamination, but these involve additional procedures that require added expenses in terms of reagents and personnel time (11).

The course of *T. cruzi* infection in the IP4 mouse is of interest. As can be seen in Fig. 3, this mouse was negative in the PCR assay and by microscopy throughout the experiment, except on day 6, when both tests were positive. Moreover, the results presented in Fig. 4 indicate that this mouse did not have detectable antibodies to *T. cruzi* and thus had not developed a chronic infection. These findings indicate that the mouse was acutely infected with the parasite, but then self-cured the infection. Whether or not this is an extraordinary occurrence is not known, since to our knowledge the issue has not been studied in laboratory animals. As far as humans are concerned, there has been only one report in which patients who appeared to have self-cured *T. cruzi* infection are described (18). This latter article is of limited relevance, however, because it deals with persons with chronic *T. cruzi* infection who were noted, years after infection with the parasite had been first diagnosed,

to no longer have anti-*T. cruzi* antibodies, and subsequent xenodiagnoses were negative. There is no context in which spontaneous self-cure of acute *T. cruzi* infection in humans would be noted, since drug treatment is available and would be given whenever a specific diagnosis is made.

In summary, we have shown that a PCR assay for the detection of *T. cruzi* in mice that is based on SDS-proteinase K blood processing and the TCZ1-TCZ2 primer set is far more sensitive than microscopic examination of fresh blood. This finding suggests that this assay should be used for patients who are suspected of having acute *T. cruzi* infections that may have resulted from vector-borne, congenital, transfusion-associated, or accidental laboratory transmission. Moreover, our results indicate that the assay should be useful in detecting *T. cruzi* in laboratory animals, including those in which experimental drugs are being tested.

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