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Trypanosoma cruzi Infection of Squirrel Monkeys: Comparison of Blood Smear Examination, Commercial Enzyme-linked Immunosorbent Assay, and Polymerase Chain Reaction Analysis as Screening Tests for Evaluation of Monkey-Related Injuries

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***Background and Purpose*: Wild-caught New World monkeys (NWM) from Central or South America are often infected with *Trypanosoma* species, including *T. cruzi*. In humans, *T. cruzi* causes Chagas' disease. Even in closed monkey colonies, *T. cruzi* can be propagated by blood-to-blood exposure, sexual activity, and transplacental transmission. Animal handlers and laboratory staff who deal with blood and tissues from infected NWM are at risk for acquiring Chagas' disease via accidental exposure.**

***Methods*: We screened 162 blood samples from wild-caught *Saimiri* sp. monkeys for *Trypanosoma* species infections by use of blood smear examination, ELISA, and polymerase chain reaction (PCR) analysis. Blood samples from 19 employees with recent history of monkey-associated injuries also were tested.**

***Results*: Six percent (10/162) of the monkey samples were *T. cruzi* positive on the basis of blood smear examination results, 10.4% (17/162) were positive by ELISA results, and 26.5% (43/162) were positive by PCR results. Other organisms identified by PCR analysis included *T. rangeli* in two animals, *Plasmodium* spp. in two animals (*P. malariae* confirmed by PCR results) and microfilariae in one animal (morphologically, *Mansonella perstans*). Evidence of trypanosome infection was not found in the 19 employee samples on the basis of results of any of the three aforementioned tests.**

***Conclusions*: Close attention must be paid to worker safety where wild-caught NWM are used. The PCR analysis has a clear advantage over conventional techniques (ELISA, blood smear) for screening NWM for trypanosome infections during quarantine and after employee injury.**

Chagas' disease is caused by the protozoan parasite *Trypanosoma cruzi*. This disease is endemic in many parts of the Amazon basin of South America where it is spread by the bite of several species of reduviid bug (1). It is estimated that 16 to 18 million Latin Americans are currently infected with *T. cruzi* and that about 43,000 of these individuals die annually as a result of this infection (2). The initial infection is severe in 10 to 20% of patients (e.g., high fever, prostration, shaking chills) (3), but most individuals report little more than flu-like symptoms that disappear within 7 to 21 days. The infection persists for life however, and 25 to 40% of infected individuals develop serious and life-threatening complications 20 to 30 years later (e.g., cardiopathy, megacolon, and/or megaesophagus) (4, 5).

Central or South America primates are often infected with *Trypanosoma* species, including *T. cruzi*, *T. rangeli*, *T. evansi* , *T. simiae*, *T. minasense*, *T. saimirii*, *T. lambrechti*, *T. devei*, *T. sanmartini*, and *T. diasi* (6–8). Although *T. cruzi* and *T. rangeli* can infect people, the latter is believed to be non-pathogenic. Many aspects of *T. cruzi* infection in monkeys are similar to Chagas' disease in humans (parasitemia, fever, chronic heart involvement pathologic and histopathologic changes) (9, 10). As is the case for human *T. cruzi* infection, monkey infection appears to be lifelong. Although *T. cruzi* is most commonly spread between monkeys in the wild by reduviid bugs, this infection can also be propagated in open monkey colonies by trauma, blood-to-blood exposure, saliva, sexual activity, and transplacental transmission (11–13). New World monkeys (NWM) are widely used in biomedical research as models for human malaria (14–16), cardiovascular disease (17), and other diseases (18). *Trypanosoma cruzi* can also infect Old World monkeys, and at least one outbreak in a rhesus macaque research colony has been reported (19). Animal handlers and laboratory staff who deal with blood and tissues from NWM are at risk for acquiring Chagas' disease via accidental exposure.

The diagnosis of Chagas' disease is problematic even in humans. Tests that can be used include microscopic detection of parasites in blood (thick and thin smears) or tissue/body fluid aspirates, culture in liquid media, xenodiagnosis in living reduviid bugs, various serologic tests (e. g., complement fixation, immunofluorescence, enzyme linked immunosorbent assay [ELISA]) and polymerase chain reaction (PCR) (20–23). In humans, the period during which parasites can be detected in the blood by microscopy is limited and this test is not sensitive even during the acute

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phase of the illness (approx. 80%) (24). Culture and xenodiagnosis are better than microscopic examination at later stages of the infection, but still achieve only approximately 50% sensitivity. A large number of serologic tests have been developed that tend to be sensitive (> 90%) but not specific due to cross-reactivity with other parasites, such as *Leishmania* sp., *Plasmodium* sp., and *T. rangeli* (25–29). Although PCR is technically complex, nucleic acid-based assays are thought to offer high sensitivity and specificity (96 to 100% and approx. 100%, respectively) (20, 21, 30).

The study reported here was initiated when routine blood smear screening in a large squirrel monkey colony revealed high-density trypanosome infections in some animals. On morphologic grounds, these parasites were thought to be *T. cruzi*. A PCR protocol was developed for diagnosis and differentiation of *Trypanosoma* species infections, and we compared microscopic examination, a commercial ELISA kit, and PCR analysis for detection of *T. cruzi* in squirrel monkeys. The implications of these findings for the screening of NWM and the evaluation of laboratory staff injuries/exposures are discussed.

Materials and Methods

Samples and sample handling: One-hundred sixty-two wild-caught squirrel monkeys (*Saimiri sciurea*) brought to Canada from Guyana and Peru between 1985 and 1998 were screened for blood-borne parasitic infections (Table 1). A single venous blood sample from each monkey was collected in an EDTA-containing tube. After thin and thick blood smears were prepared (31), the samples were centrifuged (300 ×*g* for 10 minutes) and the plasma was aliquoted and stored at -70°C until used. Single drops (approx. 40 μl) of the concentrated EDTA-treated blood samples were spotted on Whatman No. 4 filter paper and left to air dry. The samples were stored at 4°C or -20°C in sealed plastic bags containing silica gel. Blood samples from 19 employees with recent history of monkey-associated injuries were processed as described above.

Microscopic examination: Giemsa-stained thin and thick blood smears were prepared at the McGill Center for Tropical Diseases as described (31). Thick smears were systematically scanned by a technologist until at least one parasite had been observed or up to a maximum of 20 minutes (reported as 'positive' or 'negative'). Thin blood smears were reviewed for samples with large numbers of organisms to obtain a more accurate parasite count (expressed as organisms per high-power field [hpf]).

Enzyme-linked immunosorbent assay: Serum antibodies directed against *T. cruzi* were measured by use of a commercial ELISA kit (Chagas' antibody EIA, Abbott Laboratories, Mississauga, Ontario, Canada), which was performed according to the manufacturer's instructions. Results are reported as positive (OD = 0.3) equivocal (0.3 < OD < 0.1) or negative (OD ≤ 0.1). Although, this assay has not been validated for use with non-human primate samples, preliminary results indicated high optical density readings in samples from monkeys with high parasite counts.

Polymerase chain reaction

Reference DNA and PCR primers: Trypanosome isolates and oligonucleotides used as primers for the amplification of DNA are detailed in Table 2 (30, 32, 33). Although many trypanosome species can infect wild-caught NWM (6–8), we screened for the four most common parasites: *T. cruzi*, *T. rangeli*, *T. simiae*, and *T. evansi*. After optimization of the individual PCR assays for the four trypanosome species, the specificity of each amplification reaction was confirmed by use of heterospecific control DNA. Since *T. minasense* has been described in wild-caught NWM in Peru (34), we also tested the smear-positive specimens (n = 10) and genomic DNA prepared from the trypanosome isolates described in Table 2, using *T. minasense* primers.

Table 1. Geographic distribution and mean ± SEM age of imported squirrel monkeys tested for *Trypanosoma cruzi*

Origin	No. examined	Age (yr)
Peru		
Captive-bred	18	6.5 ± 0.9
Wild-caught	130	16 ± 0.3
Guyana	13	~20
Born in Canada	1	19

Extraction, amplification, and detection of DNA: Extraction of DNA for PCR was performed as described (32). In brief, two 6-mm-diameter confetti were obtained from each blood spot using a chromium-plated paper punch and were eluted in 1 ml of double-distilled water for 30 minutes at room temperature. After centrifugation (7,800 ×*g* for 10 minutes), the supernatant was discarded and 200 μl of a Chelex® 100 (BioRad, Hercules, CA) suspension was added to the "pellet." The mixture was incubated at 56°C for 30 minutes, then boiled for 8 minutes. The sample was then vortexed for 2 minutes. After a final centrifugation (7,800 ×*g* for 5 minutes) the supernatant was used immediately for the PCR reaction or stored in aliquots at -20°C.

The PCR analyses were conducted, using a programmable thermal cycler (MJC Research PTC 200, Watertown, MA). Each 50-μl reaction cocktail consisted of 2 U of *Taq* DNA polymerase (Promega, Madison, WI), 200 μ*M* dNTPs (Promega, Madison, WI), 2 m*M* MgCl2, 1 μ*M* primers, and 20 μl of sample. Control positive and negative DNA samples were included with each series of samples to verify that carryover DNA contamination had not occurred. Details of the optimized PCR conditions for the individual trypanosome species are presented in Table 3. Detection of amplified DNA was accomplished by electrophoresis of 10 μl of PCR product on 2% agarose gels previously stained with ethidium bromide (1 mg/L). Fluorescent bands were visualized, using UV-illumination, and were photographed, using Polaroid film.

Statistical analysis: All statistical analysis was performed using a statistical software package (StatView version 5, SAS Institute Inc., Cary, NC). A statistical comparison of the test results for all the methods used was determined using Cochran's Q-test. Values of $P < 0.01$ were considered significant.

Results

Microscopic examination: The parasitologic, serologic, and PCR results are summarized in Table 4. Cochran's Q-test indicated significant difference ($P < 0.001$) between the three tests. Ten of the 162 (6.1%) monkeys were *T. cruzi* positive on the basis of blood smear examination results. Parasitemia was generally low, never exceeding 1 trypanosome/10 hpf (range, 1/10 to 1/200 hpf). Trypanosomes were small C or S curved forms, with free flagella accounting for about a third of the total length. Individual parasites ranged from 18 to 20 μm in length and 0.8 to 1.5 μm in width, with 6- to 7-μm flagella. The nucleus was at, or anterior to the middle part of the body; the kinetoplast was large, round, or oval and was located at the acute end of the body. The undulating membrane was moderately well developed, and the cytoplasm occasionally contained small vacuoles (Figure 1a). Two

Table 2. Oligonucleotides used as primers for polymerase chain reaction (PCR) amplification of DNA from trypanosomes parasites

Trypanosome isolates	Sources	Primer name	Nucleotide sequence (5→3')	Reference
T. cruzi C8 clone 2 *T. cruzi* Vinch	Bristol University UK	TCRUZ	TGCACTCGGCTGATCGTTT ATTCCTCCAAGCAGCGGATA	Modification of TCZ (30)
T. rangeli San Augustin *T. rangeli* RGB	Bristol University UK	TRANG	TATATTGGTACGCGGCGCTT TACCCACTCCTCCCGTTTTCA	
T. evansi Rotat 1.2	Institute of Tropical Medicine Antwerp	ORPHON5J	GATCCCTCTCACCAATCGACCG AACTGCCCCGACCTCCGCAGT	(32)
T. simiae Ken	Bristol University UK	TSM	CCGGTCAAAAACGC AGTCGCCCGGAGTC	(33)
T. minasense	Not available	TMINAS	TGTCCAGCGAATGAATGAAAG ACGCTTTTGGAGCTGGAATT	

Table 3. Primer name, respective annealing temperature (T_A), number of cycle, and length of amplified DNA in base pairs ($amplicon_{length}$)

Primers	Denaturation time (sec)	T_A time (sec)	Polymerization time (sec)	No. of cycles	Final polymerization time (sec)	$Amplicon_{length}$
TCRUZ[1]	30	50°C (90)	72°C (120)	25		168
TRANG[2]	60	52°C (90)	72°C (120)	30	300	464
ORPHON5J[3]	30	68°C (90)	72°C (120)	50	120	246
TSM[4]	60	60°C (120)	74°C (30)	30		437
TMINAS[5]	60	50°C (60)	72°C (120)	30	300	420

[1]*Trypanosoma cruzi*, [2]*T.rangeli*, [3]*T. evansi*, [4]*T. simiae, and* [5]*T. minasense*

Table 4. Comparison of results for 162 monkey blood samples analyzed by blood smear, ELISA and PCR. PCR results are used as "gold standard" to determine the reliability and the predictive value (PV) of blood smear and ELISA tests as well as *T. cruzi* prevalence in the wild caught squirrel monkey population.

		Smear		ELISA		
		-	+	-	+/-	+
PCR						
Negative	119	119	0	116	2	1
Positive	43	33	10	21	6	16
Sensitivity (%)		23		51		
Specificity (%)		100		97		
Positive-PV (%)		100		88		
Negative-PV (%)		78		85		
Prevalence (%)		26		26		

monkeys had *Plasmodium* species identified on blood smears (Figure 1b); one of these animals was also *T. cruzi* positive. On morphologic grounds, the plasmodia were *P. malariae*, which was subsequently confirmed by results of PCR analysis (K. Kain: data not shown) (35). Notice that so-called *P. brasilianum* has been described in NWM. This parasite has the capacity to infect humans and may be identical to *P. malariae* (36–40). One monkey without evidence of trypanosome infection was found to be microfilaremic (probably *Mansonella perstans*) (Figure 1c).

Serologic evaluation: Seventeen of 162 (10.4%) monkey samples were positive by ELISA, using the cut-off value of OD of 0.3 suggested by the manufacturer. The OD range for test-positive animals was 0.315 to 0.986 and for test-negative animals was 0.01 to 0.1 (Figure 2). Another 8 animals had equivocal values, and would have been considered test positive if the arbitrary cut-off value had been lowered to OD = 0.1.

Polymerase chain reaction: Using positive control DNA from various sources (Table 2), we first established the species specificity of the primer pairs chosen. All primer pairs yielded products of the expected size with homospecific DNA, but no products with any of the heterospecific control DNA. A representative experiment using the *T. cruzi* primers is shown in Figures 3 and 4. Forty-three of the 162 monkeys (26.5%) were positive by use of the *T. cruzi* primer pair. Two of the *T. cruzi*-positive animals were also test positive for *T. rangeli*. No animal was test positive for either *T. simiae* or *T. evansi*. All of the 10 smear-positive animals and all but one (16/17) of the ELISA-positive animals were test positive for *T. cruzi* on the basis of results of PCR analysis. Interestingly, the single ELISA-positive, PCR-negative animal was infected with *P. malariae*. In people, the polyclonal B-cell activation associated with chronic malaria infection is known to cause a wide range of false-positive ELISA results (41). A comparison of the three tests is shown in Table 4. The *T. minasense* primer pair yielded identical PCR amplification products from the 10 smear-positive specimens and all trypanosome isolates (Table 2) tested.

Samples from the 19 employees with recent history of monkey-associated injuries had negative results of all tests (data not shown).

Discussion

Squirrel monkeys are widely used in biomedical research. Until recently, most of these animals were captured in the wild rather than bred for research. Squirrel monkeys are often infected with *T cruzi*, *T rangeli* (42, 43), or *Plasmodium brasilianum/malariae* complex (36, 39, 40, 44), parasites with a known capacity to infect humans. These infections can persist for years in monkeys without obvious evidence of disease. Furthermore, they can be transmitted between monkeys by a variety of vector-independent mechanisms resulting in their propagation within monkey colonies over generations. Such naturally acquired, chronic, transmissible infections are a potential source of accidental exposure for animal handlers. As recently pointed out by Sullivan et al. (23), they may also introduce confounding variables into otherwise well-planned and well-performed biomedical studies. Laboratory-acquired infections with *Trypanosoma* and *Plasmodium* species have certainly been reported (45–51). Although the risk of acquiring *Plasmodium* infections appears to be limited to puncture-type blood exposures (45, 46), *T. cruzi* has a documented capacity to infect laboratory personnel by needle puncture (47–49), splash contact with the conjunctiva (50), or abraded skin (51). Such plasticity on the part of the parasite and the asymptomatic nature of most initial infections with *T. cruzi* make this organism of particular concern in settings where squirrel monkeys are used for research purposes. In the study reported here, we document the limitations of routine microscopy and

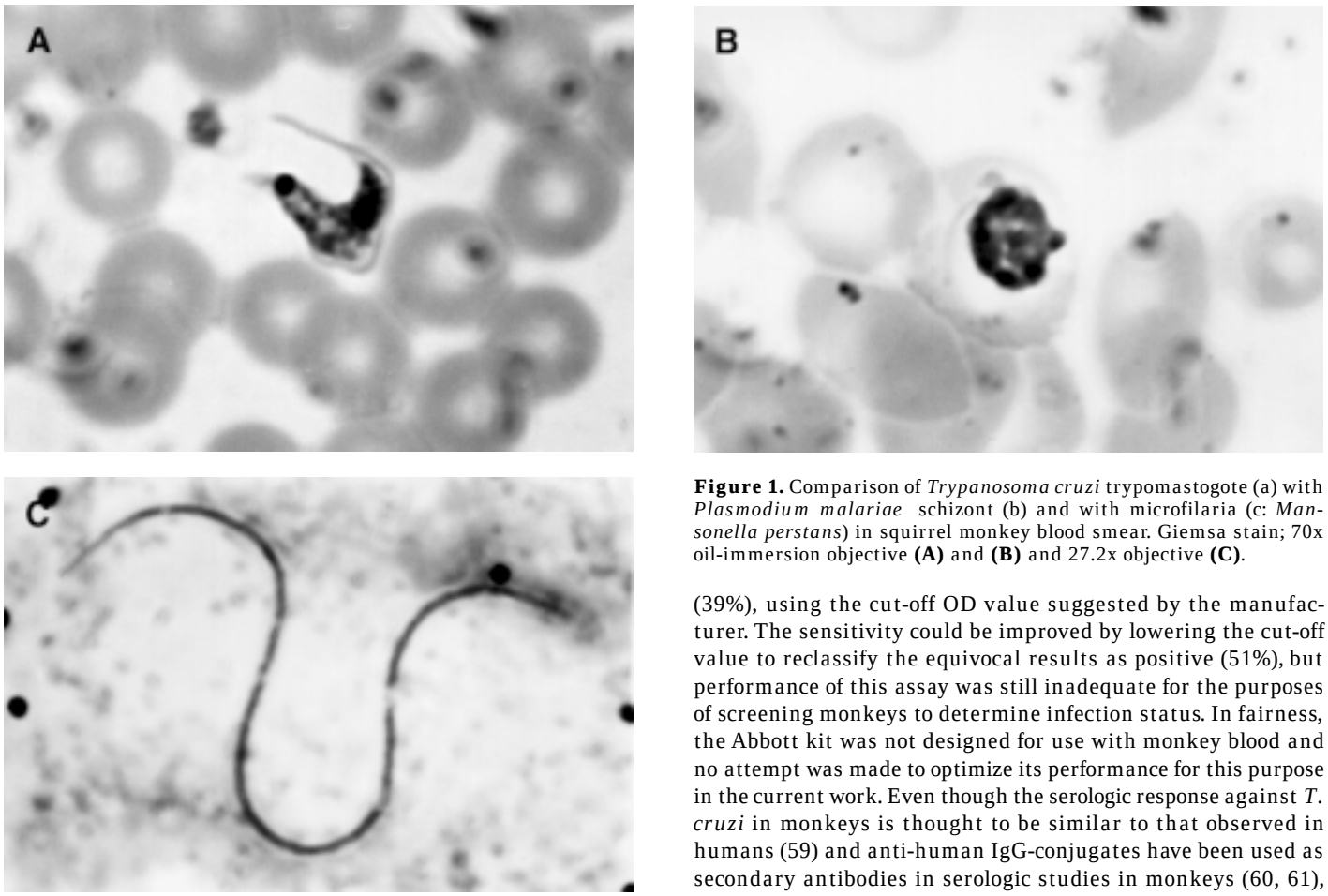

Figure 1. Comparison of *Trypanosoma cruzi* trypomastogote (a) with *Plasmodium malariae* schizont (b) and with microfilaria (c: *Mansonella perstans*) in squirrel monkey blood smear. Giemsa stain; 70x oil-immersion objective **(A)** and **(B)** and 27.2x objective **(C)**.

commercial serologic testing for screening squirrel monkey colonies for trypanosome infections.

Although the precise sensitivity of PCR analysis remains unknown at this time, we documented that this technique has considerable advantages for this purpose despite its technical complexity.

The limitations of microscopy for the detection of *T cruzi* infections in humans and monkeys arise due to the life cycle of these parasites. Although high parasite densities can be readily detected in thin (10^5 organisms/ml) and thick (10^4 organisms/ml) Giemsa-stained slides, such densities are typically present for only a short period after acute infection. Once the infection has become established, most parasites are present as the intracellular amastigote form and the number of trypomastigotes in the blood decreases precipitously to amounts undetectable by microscopy. Studies in the *Cebus apella* monkey model of Chagas' disease and in humans, suggest that the duration and intensity of parasitemia may vary considerably with the strain of *T. cruzi* used (10, 52–56). Although there are fewer data regarding strain variations in human infection, the microscopic evaluation of chronic Chagas' disease in man is also notoriously unreliable (52, 57, 58). Since most of the *T cruzi*-positive monkeys involved in the current study were presumed to have acquired their infection prior to shipment to Canada (arriving between 1985 and 1998), it was not surprising that microscopy performed so poorly, detecting only 10 of the 43 PCR-positive animals (sensitivity, 23%).

We were more surprised by the lack of sensitivity of the ELISA, which detected only 17 of the 43 PCR-positive animals (39%), using the cut-off OD value suggested by the manufacturer. The sensitivity could be improved by lowering the cut-off value to reclassify the equivocal results as positive (51%), but performance of this assay was still inadequate for the purposes of screening monkeys to determine infection status. In fairness, the Abbott kit was not designed for use with monkey blood and no attempt was made to optimize its performance for this purpose in the current work. Even though the serologic response against *T. cruzi* in monkeys is thought to be similar to that observed in humans (59) and anti-human IgG-conjugates have been used as secondary antibodies in serologic studies in monkeys (60, 61), our data suggest that there is no simple (i.e., commercial) solution for the determination of *T. cruzi* status in monkey research colonies. Furthermore, the limitations of serologic testing for the diagnosis of chronic Chagas' disease have long been recognized in the settings of blood donor screening programs (62–65) and the evaluation of patients themselves (21, 66, 67). In selected blood donors in the United States, a small but significant number of seronegative individuals are found to be PCR positive (10 to 20%). In Chagas' disease-endemic areas, the incidence of false-negative ELISA results may be as high as 50% (66). In our study, 49% of the PCR-positive animals were seronegative, including three animals that had positive blood smears. The fact that the single ELISA-positive, PCR-negative animal in our study was found to be infected with *P. brasilianum / malariae* raises the other important limitation of many ELISAs: that of false-positive results.

The PCR test has rapidly become one of the most widely used tools in molecular diagnostics; it is versatile and can use minute quantities of source DNA even when the DNA is of poor quality. The sensitivity of the amplification process is believed to be sufficient to detect a single parasite in 20 ml of blood (30, 68, 69). Furthermore, PCR analysis can distinguish between organisms that are morphologically similar, as is the case for many trypanosome species. Thus PCR has the potential to provide excellent sensitivity and specificity. In the current study, PCR analysis was clearly superior to microscopy and serologic testing as a screening tool for trypanosome infection in monkeys. Interestingly, 10 of 13 (76.9%) of the oldest monkeys shipped from Guyana in the early 1980s were *T. cruzi* positive whereas only 33 of 130 (25%) of

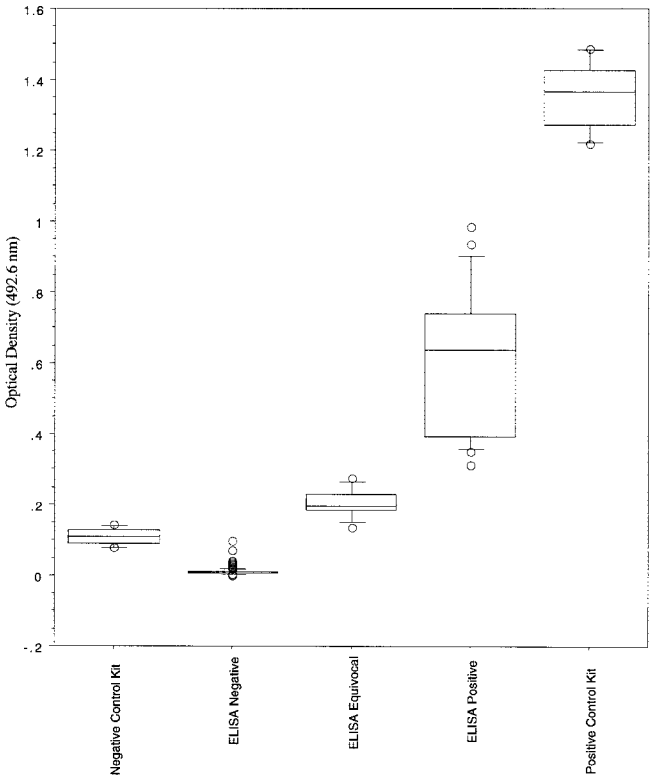

Figure 2. Enzyme-linked immunosorbent assay (ELISA) absorbance values for antibodies to *T. cruzi* in monkey samples. Median values are indicated by horizontal lines within the boxes; the 25th and 75th percentiles are enclosed by the boxes; and the 5th and 95th percentiles are enclosed by the bars outside the boxes. Circles represent outliers.

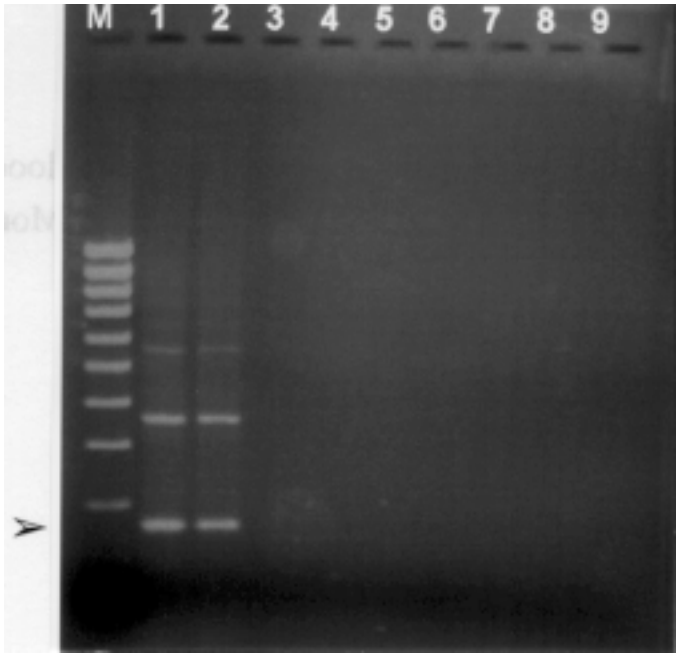

Figure 3. Evaluation of the TCRUZ primers, using known homospecific and heterospecific trypanosome DNA. The polymerase chain reaction (PCR) products were electrophoresed in a 2% agarose gel and stained with ethidium bromide. The 168-bp band (➢) is the expected *T. cruzi*- specific product. The 360- and 550-bp are also specific products resulting from amplification of two or three of the 195-bp repeats found in tandem arrays in the *T. cruzi* genome. Lanes: 1, *T. cruzi* VINCH; 2, *T. cruzi* C8 clone 2; 3, *T. rangeli* San Augustin; 4, *T. rangeli* RGB; 5, *T. evansi* RoTat 1.2; 6, *T. simiae* Ken 2; 7, *T. b. rhodesiense* Etat 1.1-S; 8, *T. b. gambiense* LiTat 1/5; 9, negative control (distilled water); and M, 100-bp ladder.

wild- caught and 0 of 13 of the captive-bred monkeys shipped from Peru between 1990 and 1998 were positive. These findings amply indicate the chronic nature of this infection and variability in prevalence that is likely to be reflected in other NWM colonies throughout the developed world. Only two of the animals included in our study were born in captivity in Canada and neither was found to harbor *T. cruzi* infection. The potential for vector-independent propagation within NWM colonies and the chronicity of this infection strongly suggest that 'once off' PCR screening may not be sufficient to guarantee that any given monkey is free of *T. cruzi*. Indeed, we have recently re-tested a small number of the initially PCR-negative animals (n = 5) and identified two that had become smear and PCR positive. At this time, we cannot determine whether this observation reflects the intermittent nature of the parasitemia in chronic infection, insensitivity of the PCR assay itself, on-going transmission within the colony, or some combination of these factors.

The risk of transmission of *T. cruzi* from a NWM to a human will depend on the status of the monkey (infected/uninfected) and the nature of the injury, as well as the actual parasite density at the time of the injury/exposure. It is likely that a hierarchy of risk exists so that injuries/exposures associated with smear-positive animals represent a substantially higher risk of transmission than those associated with smear-negative, but PCR-positive animals. However, there is every reason to believe that a single trypomastigote can give rise to Chagas' disease in a susceptible individual (70). Hence, workers who suffer injuries/exposures with even smear-negative, PCR-positive animals should be monitored carefully.

Although the data generated in the current study only permitted us to evaluate the status of 19 workers with exposures/injuries involving *T. cruzi*-positive animals that were smear and/or PCR positive, it is certainly reassuring that none of these workers appears to have acquired Chagas' disease.

In addition to the issue of laboratory worker safety, accurate diagnosis of chronic parasitic infections may have important implications for the research being conducted with these animals. Although the short- and long-term pathologic changes associated with several of these infections are not fully understood in squirrel monkeys, closely related NWM are widely used as animal models for a variety of human illnesses/conditions (9, 10, 15, 71–73). The judicious application of sensitive and specific tests for chronic *Plasmodium* and trypanosome infections should permit investigators to make informed decisions regarding these possible confounding influences.

In this study, we detected a striking prevalence of *T. cruzi* and other blood parasites in a well-controlled, well-maintained colony of mixed wild-caught and captive-bred squirrel monkeys. The wild-caught animals implicated had been imported from two widely separated geographic areas in South America. These findings strongly suggest that laboratories working with NWM need to consider screening these animals for chronic parasitoses. Such precautions may be particularly relevant in older, wild-caught animals. In screening for monkey trypanosome in-

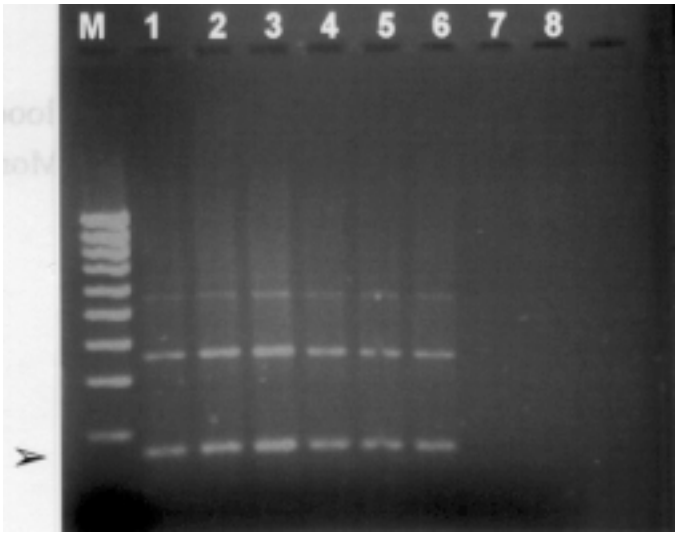

Figure 4. Example of PCR results obtained for seven monkey samples, using the TCRUZ primers. Blood samples were processed as described in Materials and Methods. The PCR products were electrophoresed in a 2% agarose gel and stained with ethidium bromide. The 168-bp band (➢) is the expected *T. cruzi*-specific product. The 360- and 550-bp are also specific products resulting from amplification of two or three of the 195-bp repeats found in tandem arrays in the *T. cruzi* genome. Lanes: 1 to 6 contain the amplification products of DNA from *T. cruzi*-infected monkeys (5 were also test positive by blood smear); 7, blood from non-infected monkey; 8, negative control (distilled water); and M, 100-bp ladder.

fections, PCR analysis had a clear advantage over microscopy and commercial ELISA. Although there was no evidence of monkey-to-human transmission in the current study, the combination of high prevalence and high parasite burden prompted reevaluation of animal use and purchasing practices as well as the monitoring of monkey-associated injuries in the institution where this work was conducted.

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