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UTILITY OF THE POLYMERASE CHAIN REACTION IN DETECTION OF *TRYPANOSOMA CRUZI* IN GUATEMALAN CHAGAS' DISEASE VECTORS

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Abstract. For effective control programs, accurate assessment of *Trypanosoma cruzi* infection in vectors is essential and has traditionally been performed by microscopic examination. For particular vectors and not others, polymerase chain reaction (PCR) analysis of fecal samples recently has been shown to be an effective means of detection. The sensitivities of the PCR and microscopy for detection of *T. cruzi* in different anatomic sites were compared in the two major vectors of Guatemala, *Triatoma dimidiata* and *Rhodnius prolixus.* Preliminary studies established that *T. cruzi* can be detected by the PCR in the presence of 90% *T. rangeli.* One hundred thirty-five vectors were collected, and samples were obtained from the rectum, intestines, and stomach and analyzed by microscopy and the PCR. For *Triatoma dimidiata* rectal samples, the PCR sensitivity (39.1% *T. cruzi* positive) and the microscopic sensitivity (24.6% positive) was not significantly different. However, in *R. prolixus,* the PCR proved significantly more sensitive than microscopy: 57.6% positive by PCR compared with 22.7% by microscopy. Rectal samples showed the highest rates of infection followed by intestine and stomach samples. However, 10.5% of the *Rhodnius* infections would have been missed if only the rectal sample had been analyzed. Thus, the PCR is significantly more sensitive than microscopy for detection of *T. cruzi* in *R. prolixus.* Analysis of anatomic sites in addition to the rectal sample may be necessary for accurate assessment of infection in particular vectors.

Chagas' disease is caused by the protozoan parasite, *Trypanosoma cruzi,* and is a significant source of morbidity and mortality in Latin America. Although strides have been made in control of this disease in certain countries, an estimated 16–18 million people currently are infected and 25% of the population of Central and South America lives at risk for this disease.[1] The parasite is carried by triatomine vectors and is transmitted to the human host through the vector feces or blood transfusions. A substantial reduction in transmission has occurred in certain countries as a result of an aggressive campaign to eradicate the vectors and to screen blood donors.[2] In Guatemala, the predominant vectors are *Rhodnius prolixus,* which is considered to be largely, if not exclusively, a domestic vector, and *Triatoma dimidiata,* which occupies domestic and sylvatic habitats.[2] Control efforts are beginning in Guatemala and accurate assessment of *T. cruzi* infection in vectors is an essential component of effective control strategies.

Direct microscopic detection of flagellated parasites has been the traditional method for assessment of infection in vectors using either vector feces (usually obtained by abdominal extrusion), or as has been recommended, dissection and analysis of the entire bug.[3] A complication of microscopic assessment of infection in Central America is the presence of the morphologically similar, but apparently nonpathogenic parasite, *T. rangeli,* with overlapping vector and reservoir hosts. Sturm and others introduced a polymerase chain reaction (PCR)–based assay for detection of *T. cruzi* in vectors and patients as an alternative to direct microscopic examination, using primers complementary to the conserved region of the mitochondrial DNA (kinetoplast or kDNA).[4] Several other PCR assays for the detection of *T. cruzi* have also been developed using primer sets complementary to nuclear satellite DNA or other regions of the kDNA.[5–7] The PCR assays based on amplification of the minicircles found in the kDNA are especially sensitive since minicircles are present in 10,000–20,000 copies per parasite. We have recently shown that a PCR assay using primers to the distal ends of the conserved regions of the minicircles of the kDNA can detect approximately 100 minicircles in a 100-μl sample.[8] In addition, other sets of primers have been reported to give PCR products that are different in size for *T. cruzi* and *T. rangeli* when the parasites are present in separate PCRs.[9,10] However, these primers have not been tested in mixed infections.

With the limited resources available in endemic countries for critical health care needs, it is important to determine whether or not the labor-intensive, but relatively low cost method of direct microscopic analysis is sufficiently sensitive compared with the PCR for measuring infection rates in the vectors. Only two studies (only one of which used field-collected vectors) have compared the relative sensitivities of the PCR and direct microscopic observation for detection of *T. cruzi* in a large sample of vectors.[11,12] The study on field-collected vectors from Bolivia showed that the PCR was significantly more sensitive than microscopy in the detection of four species of Triatomids.[11] However, for *Triatoma infestans,* the predominant vector in Bolivia, microscopy was actually more sensitive than PCR. In another study using *Triatoma infestans* nymphs allowed to feed on chronically infected patients, the PCR was significantly more sensitive than microscopy.[12] In addition, the relative sensitivities of the PCR and microscopy in dead vectors has not been reported. This present study was undertaken to assess the utility of our primers to distinguish *T. cruzi* from *T. rangeli* in mixed infections and to determine the relative sensitivities of the PCR and microscopy for detection of *T. cruzi* in the two major Guatemalan vectors, *R. prolixus* and *Triatoma dimidiata.* In addition, the PCR and microscopy were compared in dead vectors and the best anatomic site for the detection of *T. cruzi* by the PCR was investigated.

MATERIALS AND METHODS

Collection of vectors. One hundred thirty-five *R. prolixus* and *Triatoma dimidiata* were collected in six rural towns

from five regions of Guatemala as follows (number of vectors): Baja Verapaz (4), Chiquimula (58), Guatemala (4), Santa Rosa (30), and Zacapa (39). Vectors were collected from houses with roofs made of straw or vegetable material and walls made of adobe or mud and stick or vegetable material. All vectors were collected live in houses by the person-hour collection method, using people trained in collecting, usually two people collecting for a half hour per house. Four vectors died in the laboratory while awaiting dissection and 34 vectors died due to overheating in the vehicle during collection (total = 28 *R. prolixus* and 10 *Triatoma dimidiata*). Dead vectors were stored at room temperature and usually dissected within three days of death. Vectors killed by pesticides were not used.

Sample preparation and microscopy. The vectors were transported to the laboratory in plastic vials containing folded filter paper. In the laboratory, a portion of the rectum, stomach, and intestines were removed and samples were placed in separate microfuge tubes for PCR analysis. Dissecting tools were treated with bleach between different anatomic samples. The remainder of the rectum and intestines was dispersed in a drop of phosphate-buffered saline and examined under 400× magnification for approximately 2 min to look for the presence of hemoflagellates. All microscopy was performed by technicians experienced in analyzing samples for hemoflagellate infection. For microscopy, the appearance of any hemoflagellate (*T. cruzi or T. rangeli*) was recorded as a positive result. For the PCR, 200 μl of sterile, deionized water was added to the samples in the microfuge tubes and the tubes were placed in a boiling water bath for 15 min to release the minicircles.[13] Following boiling, the samples were centrifuged 8,000 × *g* at 4°C for 4 min. The supernatant was transferred to a new tube for use in the PCR.

Polymerase chain reaction amplification. The PCR was performed exactly as described[8] using primers for the conserved region of the kinetoplast minicircles (TC1 and TC2). Briefly, 5 μl of DNA template was added to 45 μl of PCR mixture to give final concentrations of 10mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 75 mM KCl, 3.5 mM $MgCl_2$, 200 μM of each dNTP, 20 pmoles of each primer, and two units of *Taq* DNA polymerase (Ampli-Taq®; Perkin-Elmer, Norwalk, CT). An initial denaturation step at 94°C for 3 min was followed by 35 cycles of 94°C, 55°C, and 72°C, each for 1 min, followed by a 10 min extension at 72°C (Programmable Thermal Controller; MJ Research, Watertown, MA). A 10-μl aliquot (20% of the PCR product) was subjected to electrophoresis on a 1.8% agarose gel and visualized on a UV transilluminator following staining with ethidium bromide. A positive control of 10 ng of *T. cruzi* total DNA and a negative control that lacked the DNA template were included with every PCR run. The analysis was repeated on all samples in that run if the controls did not give the expected results.

Strains and DNA isolation. A cloned *T. rangeli* strain, originally from San Agustin, Colombia, was obtained from Dr. A. D'Alessandro (Tulane School of Public Health and Tropical Medicine, New Orleans, LA) and *T. cruzi* strain QRO was obtained from Dr. P. M. Salazar (School of Medicine, Universidad Nacional Autónoma de México, Mexico City, Mexico). Both isolates were grown in liver infusion tryptose media supplemented with 10% fetal calf serum and antibiotics (penicillin and streptomycin).[14] Total DNA was isolated by standard proteinase K/sodium dodecyl sulfate digestion followed by phenol/chloroform extractions and ethanol precipitation.[15] The DNA was quantified on petri plates containing 1% agarose and 1 μg/ml of ethidium bromide and compared with DNA standards of known concentration.

Mixing experiments. Purified DNA from *T. cruzi* and *T. rangeli* was combined in different ratios for a final DNA concentration of 10 ng/μl. One microliter was then used in the PCR and the products were visualized as described above.

Tests for inhibition of the PCR. Vector samples that had tested positive for the rectal sample but negative for a stomach or intestinal sample were identified. Amplification of the negative sample was repeated along with a parallel test in which a second aliquot of the same sample was included in a separate PCR that had been spiked with 0.1 ng of *T. cruzi* DNA.

Data analysis. Chi-square analysis was performed to determine the statistical significance of the data.

RESULTS

Identification of *T. cruzi* and *T. rangeli*. Since the PCR has been shown to be more sensitive than microscopy for detecting *T. cruzi* infection in some vectors but not others, we tested the relative sensitivities of the two methods in the Guatemalan vectors *Triatoma dimidiata* and *R. prolixus.* For microscopy, the appearance of any hemoflagellate (*T. cruzi* or *T. rangeli*) was recorded as a positive result. For the PCR, it was first important to establish that our primers could distinguish between the two parasites in mixed infections. To test the sensitivity of the assay for one parasite in the presence of the other, purified DNA from both parasites in different ratios was mixed and subjected to the PCR. Results showed that the characteristic 235-basepair (bp) *T. cruzi* band was evident in all mixtures of *T. cruzi* and *T. rangeli* (Figure 1), even when *T. cruzi* constituted only 10% of the DNA in the sample (Figure 1, lane 5). *Trypanosoma rangeli* shows a slightly higher band of approximately 350-bp and sometimes an additional band of approximately 700-bp in certain strains. The assay is biased for *T. cruzi* since *T. rangeli* must constitute at least 75% of the sample in the presence of *T. cruzi* for detection by the PCR (Figure 1, lane 4). Based on these results, a positive infection with *T. cruzi* detected by the PCR was recorded if the expected 235-bp band characteristic of *T. cruzi* was obtained in the PCR from any of the three anatomic samples from a vector. A positive *T. rangeli* result was recorded if the 350-bp band was evident.

Sensitivities of the PCR and microscopy. The relative sensitivities of the PCR and microscopy were then tested. The PCR analysis frequently showed the characteristic 235-bp *T. cruzi* band in the rectal and intestinal samples, but no amplification in the stomach sample of a single vector (e.g., Figure 2, lanes 1–3). An unusual pattern was observed in another vector where the *T. cruzi*-specific band was evident in the rectal and stomach samples and the *T. rangeli* band was present in the intestinal sample (Figure 2, lanes 4–6). For all the samples taken together, the PCR was significantly more sensitive than microscopy: 32 of 135 samples were

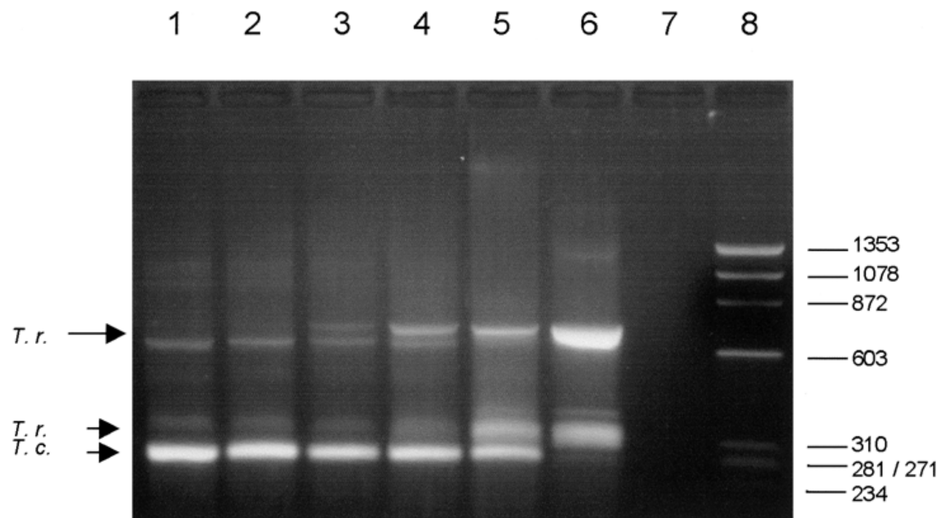

FIGURE 1. Amplification of *Trypanosoma cruzi* (*T.c.*) in the presence of *T. rangeli* (*T.r.*) shown by agarose gel electrophoresis of polymerase chain reaction (PCR) products. Different ratios of *T. cruzi* and *T. rangeli* DNA were combined to give a final concentration of 10 ng of DNA in the PCR. The percentage of *T. cruzi* in the PCR shown in each lane is as follows: lane 1, 100% (*T. cruzi* alone); lane 2, 90%; lane 3, 50%; lane 4, 25%; lane 5, 10%; lane 6, 0% (*T. rangeli* alone). Lane 7, negative control (no DNA); lane 8, molecular weight markers (ϕX174 DNA digested with *Hae* III. Values on the right are in basepairs.

positive by microscopy and 65 of 135 were positive by the PCR ($\chi^2 = 8.76$, $P < 0.01$; Table 1). This significant difference was largely due to detection in *R. prolixus*: 22.7% positive by microscopy (15 of 66) compared with 57.6% positive by the PCR (38 of 66) ($\chi^2 = 8.34$, $P < 0.01$: Table 1). Infection with *T. cruzi* was detected in 10 additional samples in *Triatoma dimidiata* by the PCR (27 of 69, 39.1%) compared with microscopy (17 of 69, 24.6%); however, this was not a statistically significant difference ($\chi^2 = 1.69$, $P > 0.05$; Table 1).

Within the limits of detection of this assay, no *T. rangeli* infection was observed in any of the *Triatoma dimidiata* samples. *Trypanosoma rangeli* was present in 12 (18%) of 66 *R. prolixus* samples tested by the PCR, by itself in 1.5% of the samples, and found along with *T. cru*zi in 16.7% of the samples. Of the 12 samples identified as *T. rangeli* pos-

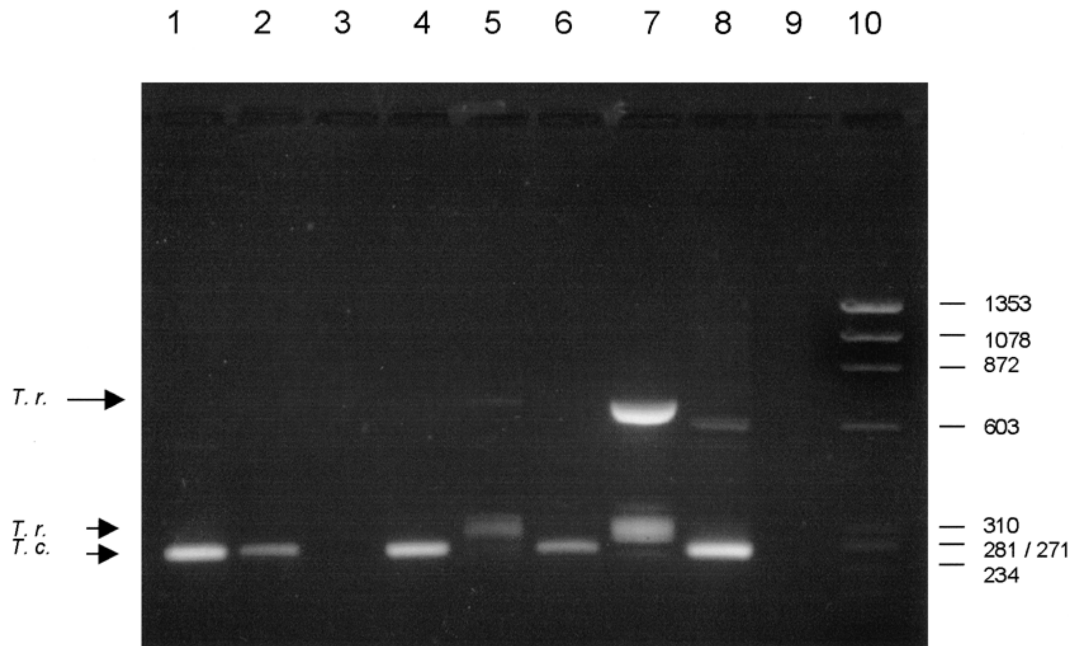

FIGURE 2. Typical (lanes 1–3) and atypical (4–6) results of polymerase chain reaction (PCR) amplification of vector samples shown by agarose gel electrophoresis of PCR products. Amplification of anatomic parts from two individual vectors (lanes 1–3 and 4–6) is as follows: rectum (lanes 1 and 4), intestine (lanes 2 and 5), and stomach (3 and 6). Lane 7, amplification of 10 ng of *Trypanosoma rangeli* (*T.r.*) DNA; lane 8, 10 ng of *T. cruzi* (*T.c.*) DNA; lane 9, negative control (no DNA template); lane 10, molecular weight markers (ϕX174 DNA digested with *Hae* III). Values on the right are in basepairs.

TABLE 1

Comparison of *Trypanosoma cruzi* detection by polymerase chain reaction (PCR) and microscopy in *Triatoma dimidiata* and *Rhodnius prolixus*

All vectors	*T. dimidiata* (n = 69) No. of PCR results		*R. prolixus* (n = 66) No. of PCR results		All vectors (n = 135) No. of PCR results	
	Positive	Negative	Positive	Negative	Positive	Negative
Microscopy positive	17	0	14	1	31	1
Microscopy negative	10	42	24	27	34	69

itive by the PCR, only six were positive for hemoflagellates by microscopy. There was no obvious selection against coinfection with both parasites: of the 12 vectors showing infection with *T. rangeli,* 10 also showed infection with *T. cruzi.*

Comparison of living and dead vectors. Because some of the vectors had died (38 of 135, 28.1%) before being tested for infection with *T. cruzi,* this allowed analysis of whether the survival status of the bug effected the sensitivities of the two assays. Results of analysis of the vectors that were dissected while still alive showed that the PCR was significantly more sensitive than microscopy in living vectors ($\chi^2 = 4.22$, $P < 0.05$; Table 2). The significance was even greater when the results of the PCR and microscopy in vectors that had died before dissection were compared. Of 10 dead *Triatoma dimidiata,* the PCR and microscopy results were in agreement: one vector was positive for *T. cruzi* infection and nine were negative. For 28 dead *R. prolixus,* 13 were positive by the PCR and all 28 were negative by microscopy. Therefore, for both species together, infection was detected by microscopy in only one of the 14 dead vectors identified as positive by the PCR ($\chi^2 = 7.01$, $P < 0.01$; Table 2).

Optimal anatomic site. An analysis to determine the anatomic site best suited for detecting *T. cruzi* infection by the PCR was performed. Dissected rectal, intestine, and stomach samples had been processed separately and amplified by the PCR. Rectal samples showed the highest rates of infection, 45.7% (59 of 129), followed by intestine, 33.6% (41 of 122) and stomach samples, 20.3% (16 of 79). Due to the lower levels of PCR-positive samples from the stomach and intestine, samples from these two anatomic sites were tested for inhibition of the PCR. Vectors with a positive rectal but negative stomach or intestinal sample were identified. Negative samples were then spiked with *T. cruzi* DNA and reamplified. Results of this analysis showed that five of six stomach samples failed to amplify even when exogenous *T. cruzi* DNA had been added. In contrast, six of seven intestinal samples did show an amplification product with the added DNA. Following these studies, stomach samples were excluded from the analysis.

Rates of infection in different regions. A comparison of *T. cruzi* vector infection in the different regions of Guatemala showed the highest rates of infection in Zacapa (almost exclusively infested with *R. prolixus*) and Santa Rosa (infested only with *Triatoma dimidiata,* Table 3). Since there were only four vectors each in the regions of Baja Verapaz and Guatemala, these regions were excluded from this comparison.

TABLE 2

Comparison of *Trypanosoma cruzi* detection by polymerase chain reaction (PCR) and microscopy in living and dead vectors

	Living vectors (n = 97) No. of PCR results		Dead vectors (n = 38) No. of PCR results	
	Positive	Negative	Positive	Negative
Microscopy positive	30	1	1	0
Microscopy negative	21	45	13	24

DISCUSSION

Although the PCR has been shown to be an exquisitely sensitive technique for the detection of *T. cruzi* in vectors and patients in laboratory studies, its utility in large-scale monitoring of specific vector populations has not been adequately assessed. Only one large-scale study using field-collected samples has been published, and results of that study indicated that microscopy was actually more sensitive than PCR for the most predominant vector in Bolivia, *Triatoma infestans.*[11] Conflicting results were obtained by Shikanai-Yasuda and others, who showed that the PCR was a more sensitive method of detection for *Triatoma infestans* than microscopy.[12] These different results may be due to the use of different primer sets and sample processing techniques. Vector control efforts are currently underway in Guatemala and accurate means of assessing infection in vectors is critical to the success of these efforts.

By varying the ratio of purified *T. cruzi* and *T. rangeli* DNA in artificial mixed infections, we showed that *T. cruzi* can be detected even in the presence of a large excess of *T. rangeli.* Thus, coinfection with *T. rangeli* does not interfere with detection of *T. cruzi* by the PCR with these primers. In contrast, *T. rangeli* must be present at high levels in mixed infections to be detected. Therefore, the level of mixed infections is likely underestimated in this study. In addition, results suggest that microscopy may not be very useful for the detection of *T. rangeli.* Only half of the samples identified as *T. rangeli* positive by PCR were detected by microscopy. In addition, infection with one parasite does not appear to preclude infection with the other since several coinfections were observed.

We have compared microscopy to the PCR for the two main Chagas' disease vectors in Guatemala and the results show that the PCR is significantly more sensitive than direct microscopy for detection of *R. prolixus,* but not *Triatoma dimidiata.* It may be that *R. prolixus* supports a lower parasitemia than does *Triatoma dimidiata,* so that infections are more likely to be missed by microscopy. Based on these

TABLE 3
Comparison of rates of *Trypanosoma cruzi* infection in *Triatoma dimidiata* and *Rhodnius prolixus* from different regions of Guatemala*

Department	*T. dimidiata*		*R. prolixus*	
	+ microscopy	+ PCR	+ microscopy	+ PCR
Santa Rosa	13/30 (43.3%)	18/30 (60.0%)	No samples	No samples
Chiquimula	4/30 (13.3%)	7/30 (23.3%)	0/28 (0%)	12/28 (42.9%)
Zacapa	0/1 (0%)	1/1 (100%)	15/38 (39.5%)	26/38 (68.4%)

* PCR = polymerase chain reaction.

results, care must be taken when interpreting infection rates in *R. prolixus* based on microscopy. The one vector in which *T. cruzi* was identified by microscopy but missed by the PCR lacked a rectal sample for the PCR. It may be that the intestines and stomach were not infected with *T. cruzi* or that there was inhibition of the PCR in those particular samples. In addition, because the analysis of infection often takes place long distances from where the vectors are collected, vectors may have died by the time of analysis. Results shown here clearly demonstrate that with dead vectors, microscopy is inadequate for detecting *T. cruzi* in *R. prolixus* and the PCR should be used to assess rates of infection. More samples of *Triatoma dimidiata* need to be studied to determine the relative effectiveness of the PCR and microscopy in dead vectors.

Rhodnius prolixus showed higher rates of infection (57.6%) than *Triatoma dimidiata* (39%). This may explain why high levels of seropositivity are found in houses infested with *R. prolixus* even though, by previous microscopic analysis, *R. prolixus* appeared to have a lower infection rate than *T. dimidiata* in Guatemala (Monroy C, Mejia M, Rodas A, Tabaru Y, 1995, unpublished data). The results shown here stress the importance of *R. prolixus* as a vector for *T. cruzi* in Guatemala. Analysis of three regions of Guatemala showed very high rates of *T. cruzi* infection in vectors from Santa Rosa and Zacapa. To efficiently target control efforts, more samples from other regions need to be tested by the PCR.

To reduce the cost and risk of cross-contamination in the PCR analysis, it is preferable to use just one sample per vector. Therefore, we analyzed which of the three anatomic parts would be best for the detection of *T. cruzi* by the PCR. Results showed that the stomach sample inhibited the PCR; thus, the stomach is not a good sample for the PCR and the estimate of infection of the stomach is likely an underestimate. Previous work in our laboratory has shown that the PCR is not inhibited by 5% blood, which suggests that the inhibition is not due to blood remaining from a blood meal but to some other component found in the insect stomach. There was much less inhibition seen in the intestinal samples, and the rectal samples showed the highest rates of infection with the parasite. However, not all the infections would have been detected by testing only the rectal sample. Five of the positive samples would have been missed: one *Triatoma dimidiata* (3.7% of the positive samples) and four *R. prolixus* (10.5% of the positive samples). We are currently testing a combined rectum plus intestinal sample as a single sample from each vector for maximal detection of *T. cruzi.*

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