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FIELD APPLICATION OF POLYMERASE CHAIN REACTION DIAGNOSIS AND STRAIN TYPING OF *TRYPANOSOMA CRUZI* IN BOLIVIAN TRIATOMINES

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Abstract. A new approach for direct identification and characterization of *Trypanosoma cruzi* stocks in biological samples was tested for field applicability on an extensive sample of feces collected from triatomine vectors from four different species found in Bolivia. The first step of the technique is polymerase chain reaction (PCR) amplification of the hypervariable region of kinetoplast DNA minicircles of *T. cruzi* parasites. In this report, 345 fecal samples were analyzed and the PCR results were compared with microscopic examination. For *Triatoma infestans*, the principal Bolivian vector, both techniques were in concordance 85.3% of the time. For the three other species, *Rhodnius pictipes*, *Eratyrus mucronatus*, and *Triatoma sordida*, the fecal samples were all negative by microscopic examination whereas PCR results showed several *T. cruzi*-infected insects in each species. The second step of the procedure is the characterization of the *T. cruzi* clones by means of hybridization of the PCR products with clone-specific probes generated by the PCR. We used two probes corresponding to major clones circulating in high frequency in Bolivia (as shown by previous population genetic studies using isoenzyme characterization). We obtained four primary results: 1) we confirm the importance of two major clones in Bolivia in two distinct regions; 2) we report high rates of mixed infections (multiple clones in a single vector) in *Triatoma infestans*, up to 22% and 35% in Cochabamba and La Paz departments, respectively; 3) the results favor the absence of interaction between different clones; and 4) we find, for the first time, evidence of the major clones circulating in three species of triatomines that are known as mainly sylvatic species. The origin of these clones, sylvatic or domestic, is also discussed.

Trypanosoma cruzi, the causative agent of Chagas' disease, is present as numerous natural clones as evidenced by population genetic studies; these natural clones appear to be evolving with time and dispersing over geographic locations without genetic exchange between organisms, thus maintaining genetically similar populations.¹ Some natural clones are ubiquitous and are the ones most frequently isolated from domestic vectors and from humans; these have been designated as major clones.² Until recently, the characterization of the different clones of *T. cruzi* has been performed by multilocus enzyme electrophoresis (MLEE) after isolation and massive culture of the parasite to obtain sufficient quantities for this analysis. The process of culturing clearly selects for particular clones and may reduce an initial isolate that is composed of several clones to a single clone.³ Even by MLEE analysis, infection of an individual vector with multiple clones is found in approximately 10% of the cases, as has been previously demonstrated by the visualization of double isoenzyme patterns.⁴ This percentage is probably an underestimate because of the necessity of first culturing the parasites.

We and other investigators have recently described a new technique for direct identification of *T. cruzi* stocks in the feces of triatomine vectors and in mammalian blood based on polymerase chain reaction (PCR) amplification of a portion of the minicircle of kinetoplast DNA (kDNA).⁵⁻⁷ Moreover, we have demonstrated the clone specificity of the hypervariable region of the kDNA minicircle (HVRm) based on a population genetics approach.⁶ This result makes possible the development of clone-specific DNA probes that may be used for direct genetic characterization of the natural clones found in the various hosts.⁷ In this paper, we report

the construction of two probes by the PCR that correspond to two major clones of *T. cruzi* circulating in Bolivia.⁶ We hybridized these probes to PCR-amplified kDNA from field samples of triatomine feces to determine the clone infecting each vector, and we assessed the field applicability of this technique by testing a large number of samples from different Bolivian triatomines species, both domestic and sylvatic.

MATERIALS AND METHODS

Vectors. The triatomines tested include four different species found in Bolivia: *Triatoma infestans*, *Rhodnius pictipes*, *Eratyrus mucronatus*, and *Triatoma sordida*. *Triatoma infestans* is a domestic vector whereas the remaining are usually considered sylvatic vectors. The different specimens were captured in the field at different locations (see Table 1 for numbers and collection sites). Moreover, we used as negative controls 28 laboratory-reared, noninfected insects (*Triatoma infestans*).

Microscopic observation. The microscopic observation was considered positive if flagellated parasites were observed in the feces of a triatome specimen during a 5-min examination of a drop of feces mixed with phosphate-buffered saline at a 400 × magnification.

Processing of triatomine feces for the PCR. Feces samples were prepared by the addition of distilled water, followed by boiling and centrifugation. Briefly, 10-20 µl of triatomine feces were individually collected in sterile Eppendorf (Hamburg, Germany) tubes using forceps and gloves rinsed in bleach between handling of each sample, and the samples were stored -20°C. The samples were collected in the entomologic room that was separate from the other room where the PCR procedure was undertaken. They



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TABLE 1
Geographic origin and collection site of the vectors

Department	Province	Triatomine species*	No. of communities sampled	Collection sites	No. of triatomines analyzed
Cochabamba	Campero	<i>T. infestans</i>	1	Houses/chicken coop	6
	Capinota	<i>T. infestans</i>	6	Houses/chicken coop	34
La Paz	Nor Yungas	<i>T. infestans</i>	12	Houses/chicken coop	124
	Caranavi	<i>T. infestans</i>	1	Houses/chicken coop	53
	Sud Yungas	<i>R. pictipes</i>	1	Chicken coop	36
	Franz Tamayo	<i>E. mucronatus</i>	1	Houses/chicken coop	68
		<i>T. sordida</i>	1	Houses/chicken coop	24

* *T.* = *Triatoma*; *R.* = *Rhodnius*; *E.* = *Eratyrus*.

were diluted by the addition of 200 μ l of distilled water. The parasites were lysed by boiling for 10 min followed by two 10-min centrifugation at 8,000 \times *g*. As a control, one tube with water was subjected to the same treatment as the fecal samples (boiled and centrifuged). We believe that the water template is a good negative control because it is free of the possible presence of PCR inhibitors. Ten microliters of the supernatant was used as a template in each of the PCR assays. One-tenth of the PCR product was analyzed by electrophoresis on a 0.8% agarose gel and visualized by staining with ethidium bromide.

Polymerase chain reaction. The PCR was performed according to a previously described method.⁷ Briefly, the sequences of the oligonucleotide primers used were CV1: 5'-GATTGGGGTTGGAGTACTACTAT-3' and CV2: 5'-TTGAACGGCCCTCCGAAAAC-3' (chosen to amplify all *T. cruzi* isolates). They were obtained from the Genset Laboratory (Paris, France).⁶ Two restriction sites (*Sca* I and *Sau* 96 I) were artificially introduced at the 3' end of each oligonucleotide and used for purification of the HVRm-amplified sequence away from the oligonucleotide primers that contain part of the conserved region of the minicircle. Samples were amplified in 67 mM Tris-HCl (pH 8.8), 16.6 mM (NH₄)₂SO₄, 6.7 mM MgCl₂, 10 mM mercaptoethanol, 0.01 mg/ml of bovine serum albumin, 75 pM of each deoxynucleotide triphosphate, and 75 pM of each oligonucleotide in a total reaction volume of 50 μ l. For the amplification, which was performed using a Trio thermoblock PCR device (Biometra, Gottingen, Germany), 2.5 U of *Thermus aquaticus* DNA polymerase (Promega, Madison, WI) was used. The amplification involved three distinct steps: 1) an initiation step with DNA denaturation (95°C for 5 min), oligonucleotide primer annealing (48°C for 2 min), and elongation (72°C for 2 min); 2) an amplification step with 30 cycles (95°C for 5 sec, 48°C for 30 sec, and 72°C for 1 min), and 3) a cooling step (4°C for variable times). Each run include 1) one positive control of total DNA template and 2) two negative controls with water instead of DNA template, with one of them subjected to the same treatment as each feces sample as described above. A systematic aliquoting of the PCR components (buffer, primers, oligonucleotides, and water) was done in 30–40 sterile microtubes. Each aliquot was covered with 50 μ l of paraffin to prevent evaporation and was stored at –20°C until use. The absence of contamination in the aliquots was previously checked by a test using a water template.

Sensitivity of detection. The sensitivity was evaluated by

the addition of 10 fg to 20 pg of *T. cruzi* total DNA (reference strain) in the feces samples from different laboratory-reared *Triatoma infestans*.

Southern blot. One-tenth of each PCR-positive sample was subjected to electrophoresis on a 0.8% agarose gel and transferred after alkali denaturation (0.5 N NaOH, 1.5 M NaCl, twice for 15 min) onto charged nylon membranes (Hybond N+; Amersham, Buckinghamshire, UK) by vacuum blotting. For the vacuum transfer, the gel was placed on a prewetted membrane, which consisted of filter paper backed with a piece of diaper. This sandwich was covered with plastic wrap and placed on a gel dryer (Bio-Rad, Richmond, CA) and vacuum was applied for 10 min without heat.

Probes. The two clone-specific probes (20 and 39) were purified from their respective HVRm DNA fragments produced by the PCR from TPk1 (clone 39) and So34 c14 (clone 20) *T. cruzi* stocks.^{1,6,8} Briefly, the PCR-amplified 270-basepair HVRm fragments from 10 runs were purified by electrophoresis on 0.8% preparative agarose gel (Sigma, St Louis, MO). The fragments were eluted by electroelution using a 422 electro-eluter devise (Bio-Rad) according to the manufacturer's instructions, precipitated with 0.2 M NaCl in 2.5 volume/volume (v/v) of pure ethanol, and resuspended in 100 μ l of Tris-EDTA buffer. The DNA was digested with the restriction endonucleases *Sau* 96 I and *Sca* I (Promega) to eliminate part of the oligonucleotide primers selected in the conserved region of the minicircle. After digestion, the DNA was precipitated with ethanol and resuspended in a volume of 100 μ l, as described above. The amount of DNA was quantitated by electrophoresis of sequenced dilutions. These probes have previously been shown to be highly specific for *T. cruzi* clones 20 and 39 and genetically closely related clones.^{6,7}

Labeling and hybridization conditions. The probes were labeled and the filters were hybridized using the enhanced chemiluminescence gene detection system according to the manufacturer's recommendations (Amersham). Briefly, the membranes were incubated at 42°C in hybridization buffer (0.12 ml/cm²) for 15 min. At the same time, each of the purified probes was labeled for 10 min at 37°C. A total of 10 ng of labeled probe was added to the membranes per milliliter of hybridization buffer. Hybridization was performed at 42°C overnight in a rotary oven (Appligen, Illkirch, France). To remove nonspecific hybridization products, the membranes were washed twice under highly stringent conditions (6 M urea, 0.1 \times SSC [1 \times SSC = 0.15 M NaCl,

TABLE 2
Detection of parasites in feces: comparison of microscopic observation and the polymerase chain reaction (PCR) technique

Triatomine species ¹	No.	Microscopic results				Comparison of both techniques				
		+		-		%		%		
		+	-	+	-	MOB	PCR	concordance [‡]	A	B
<i>T. infestans</i>	217	95	22	10	90	92.1	82.7	85.3	10.1	4.6
<i>E. mucronatus</i>	68	0	0	13	55	—	—	80.9	—	19.1
<i>T. sordida</i>	24	0	0	3	21	—	—	87.5	—	12.5
<i>R. pictipes</i>	36	0	0	19	17	—	—	47.2	—	52.8

¹ T = *Triatoma*, U = *Utravirus*, R = *Rhodnius*.

² The sensitivity of each technique was calculated by the following formula: ni/N with ni = positive by a particular technique and N = positive by either technique. MOB = microscopic observation.

³ % concordance = % of samples positive or negative by both techniques.

⁴ % discrepancy A = % of samples positive by microscopy and negative by PCR; B = % of samples positive by PCR and negative by microscopy.

0.015 M sodium citrate] in 0.4% sodium dodecyl sulfate at 42°C for 20 min), and then twice in 2× SSC at room temperature for 10 min. Two exposures were performed (1 min and 30 min) on autoradiography film (Hyperfilm[®]-MP; Amersham).

RESULTS

To test the field applicability and sensitivity of PCR-based diagnosis of *T. cruzi*, we analyzed 345 fecal samples from four triatomine species obtained from domestic and peri-domestic habitats (Table 1) for the presence of parasites by direct microscopic observation and by PCR. The results are summarized in Table 2.

Among the 217 fecal samples recovered from *Triatoma infestans*, 117 showed flagellated parasites by microscopic observation, whereas only 105 showed an amplified band by PCR. The two techniques were in agreement 85.3% of the time. Discrepancy between the techniques occurred with two different patterns. The first showed a negative PCR result when the microscopic observation was positive; this pattern occurred in 10% of the insects examined. This rate of false-negative results was higher than expected. The second showed a positive PCR result when the microscopic observation was negative; this pattern occurred in 5% of the samples examined. Considering the sum of positive samples (positive by microscopic observation and/or positive by PCR), we compared the sensitivity of both techniques. Microscopic observation appears to be significantly more sensitive than the PCR technique (Yates' $\chi^2 = 5.18$, $P < 0.05$). We did not find significant differences in the sensitivity of the PCR technique between locales (La Paz and Cochabamba Departments). The 28 fecal samples from laboratory-reared *Triatoma infestans* were all PCR-negative as expected. Likewise, the sensitivity evaluated by the addition of *T. cruzi* total DNA to fecal samples of laboratory-reared *Triatoma infestans* insects always gave a positive amplification with 0.2 µg of DNA. On the other hand, the PCR was positive in only 30% of fecal samples artificially infected with either 10 or 100 µg of DNA. If one considers that a cell contains approximately 150–200 fg of DNA, the PCR method permitted the detection of one parasite in a feces sample. A discrepancy in the sensitivity of the PCR-based diagnosis was observed when comparing the results of studies of the 217 fecal samples recovered from the field with the fecal samples from artificially infected, laboratory-reared insects.

The relative sensitivities of the PCR and microscopic observations were reversed (higher) for the three sylvatic species compared with the results obtained with *Triatoma infestans*. All of the fecal samples collected from *R. pictipes*, *E. mucronatus*, and *Triatoma sordida* species were negative by microscopic observation. However, we obtained 52.8%, 19.1%, and 12.5% positive results, respectively, by the PCR.

We then proceeded to identify the particular clones present in the infected triatomines (Figure 1). A total of 136 PCR-positive samples were hybridized with probes specific for the two *T. cruzi* clones previously identified by isoenzyme analysis as major clones circulating in the domestic cycle in Bolivia (clones 20 and 39, see Materials and Methods). A large majority of the samples tested (77.9%) were recognized by one, or in the case of mixed infections, both probes (Table 3). Clones 20 and 39 were present in both domestic and sylvatic vectors. However, 30 samples (22.1%) were not recognized by either probe and the percentage of unrecognized samples was higher among sylvatic species of vectors (51.4%) than in domestic ones (11.9%) (Table 3).

We detected a much higher percentage of mixed infections in *Triatoma infestans*, up to 22.7% and 35.4% in Cochabamba and La Paz samples, respectively, (Table 4) than had been previously reported; 10% of the mixed infections were identified by isoenzyme analysis.^{4,9} Mixed infections were also observed in *E. mucronatus* and *Triatoma sordida*. To determine whether or not infection with one clone affected the likelihood of infection with the other (inhibition or facilitation), we compared the observed rates of mixed infections with theoretical rates based on observed rates of infection of each clone in the population. The calculated numbers ($N \times (\% 20 \times \% 39)$, with N = total population number, % 20 = observed % of triatomines infected by clone 20 and % 39 = observed % of triatomines infected by clone 39) did not differ significantly from the observed rates when they were compared by Yates' modified chi-square test (Table 4).

DISCUSSION

In this study, we have demonstrated the applicability of PCR-based diagnosis and strain typing for epidemiologic studies of *T. cruzi*. The first effort was to evaluate the sensitivity of the PCR technique. The level of sensitivity of the PCR reached 82.6% with samples from *Triatoma infestans*, a rate lower than expected considering the high theoretical sensitivity of the PCR. This may be explained by the lack

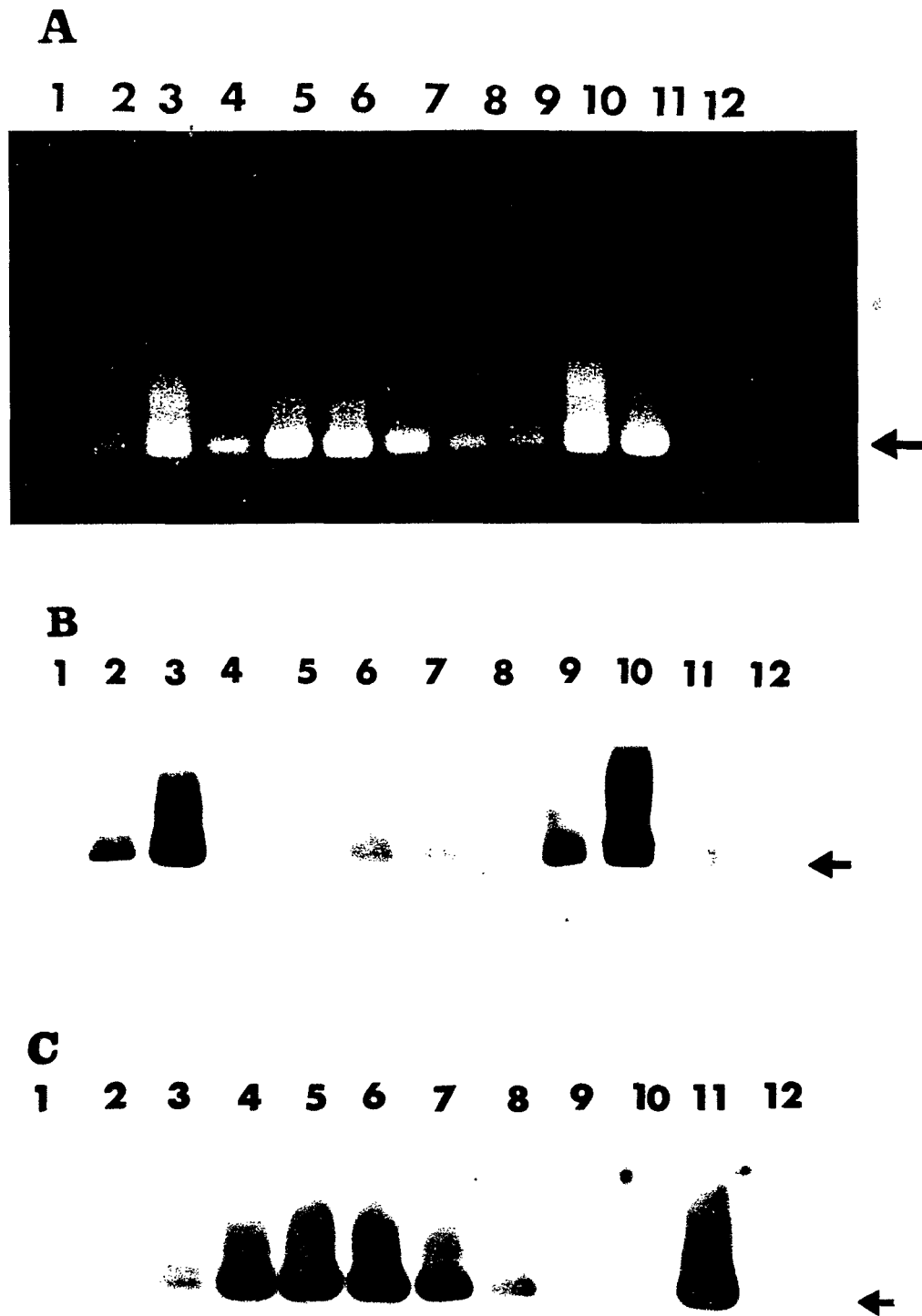


FIGURE 1. A, ethidium bromide-stained 0.8% agarose gel containing polymerase chain reaction products from *Trypanosoma cruzi*-infected *Triatoma infestans* feces. B and C, hybridization patterns of these products with clone-specific probes corresponding to natural clones 20 and 39, respectively. Lanes 1 and 12, control samples (using distilled water as the template); lanes 2-10, positive *Triatoma infestans* feces from Cochabamba Department; lane 11, positive control (using 10 ng of total DNA from the reference *T. cruzi* stock pertaining to natural clone 39 as the template). The arrows indicate the major amplified bands (270 basepairs).

TABLE 3

Identification of major clones 20 and 39 of *Trypanosoma cruzi* by specific DNA probes in polymerase chain reaction-amplified feces of vectors

Triatomine species*	Department	No.	Clone identification, no. (%)†			
			20 only	39 only	20 + 39	neither 20 nor 39
<i>T. infestans</i>	Cochabamba	22	15 (68.2)	0 (0)	5 (22.7)	2 (9.1)
	La Paz	79	24 (30.4)	17 (21.5)	28 (35.4)	10 (12.7)
Total		101	39 (38.6)	17 (16.8)	33 (32.7)	12 (11.9)
<i>E. mucronatus</i>	La Paz	13	1 (7.7)	2 (15.4)	2 (15.4)	8 (61.5)
<i>T. sordida</i>	La Paz	3	1 (-)	0 (-)	1 (-)	1 (-)
<i>R. pictipes</i>	La Paz	19	8 (42.1)	2 (10.5)	0 (0)	9 (47.4)
Total		35	10 (28.6)	4 (11.4)	3 (8.6)	18 (51.4)

* *T.* = *Triatoma*; *E.* = *Eratyrus*; *R.* = *Rhodnius*.

† - = not calculated (sample too small).

of DNA purification. In fact, we have chosen a very simple method of template preparation, involving just boiling the feces in water, to facilitate processing large numbers of samples and to avoid cross-contamination. This crude lysate may contain various factors that are inhibitory in the PCR. Some of these inhibitors include blood breakdown products, such as heme,¹⁰ which may vary in quantity and quality, depending on the digestive status of blood present in the gut of individual bugs. It is worth noting that the relationship between the percentage positive by microscopic observation and the percentage negative by PCR-based diagnosis varies between localities and in some, the false-negative PCR responses are absent. However, we have observed 35.7% and 58.3% false-negative PCR samples in two localities. The estimated sensitivity of the PCR technique is approximately one parasite per sample. The presence of PCR inhibitors in bug feces may explain the failure to detect a single parasite in some samples. The PCR technique was able to identify *T. cruzi* in some samples that could not be identified by microscopic examination (4.6% for *Triatoma infestans*, 19.1% for *E. mucronatus*, 12.5% for *Triatoma sordida*, and 52.8% for *R. pictipes*). Most notably, the PCR detected *T. cruzi* in three sylvatic species that were not detected by microscopic examination. We believe that the discrepancy in the techniques is due to the low parasite burden in these sylvatic species, which is missed by microscopic examination but detected by the PCR. These results bring to light a potential vector role for these sylvatic species in Bolivia.

Contamination with previous amplification products is the

main problem with the PCR technique, but we do not believe it is an explanation for the discrepancy between negative microscopic examination results and positive PCR results in this study because of the following arguments. Among positive samples that showed a negative result by microscope observation, some give weak PCR amplification but others give strong amplification (generally contamination gives a weak band), and the negative and positive controls of each protocol always gave appropriate results. The hybridization patterns (see below) indicate the presence of several clones of *T. cruzi* in a group of triatomine samples tested in each experiment, and false-positive PCR results (contamination) occurred mostly due to the same source (the same clone of *T. cruzi*). It is worth noting that some samples were not recognized by either of the two probes that were used.

The PCR has the advantage of providing a more specific diagnosis than one that relies only on the detection of flagellated parasites in areas where other parasites, such as *T. rangeli* or *T. cruzi marenkellei*, could be present as well.

The second part of the investigation was to characterize the clones circulating in the vectors. We used two probes made with the PCR that encompass the HVRm of kDNA and hybridized these to PCR products from each sample of feces. We found high levels of two major clones that were previously reported from the same area using nuclear DNA markers (isoenzymes). Clones 20 and 39 (or closely related clones) were identified from the domestic vector (*Triatoma infestans*). The PCR-based analysis is therefore a powerful tool for determining the distribution of clones because of the

TABLE 4

Prevalence of mixed *Trypanosoma cruzi* clone infections: comparison of the observed and theoretical rates

Triatomine species*	Department	No.	No. (%) recognized‡		Mixed infections‡§		P§
			By probe 20	By probe 39	Observed	Expected	
					No. (%)		
<i>T. infestans</i>	Cochabamba	22	20 (90.9)	5 (22.7)	5 (22.7)	5 (22.7)	>0.05
	La Paz	79	52 (65.8)	45 (57.0)	28 (35.4)	30 (38.0)	>0.05
<i>E. mucronatus</i>	La Paz	13	3 (23.1)	4 (30.8)	2 (15.4)	1 (7.7)	>0.05
<i>T. sordida</i>	La Paz	3	1 (-)	0 (-)	1 (-)	-	
<i>R. pictipes</i>	La Paz	19	8 (42.1)	2 (10.5)	0 (0)	1 (5.3)	>0.05

* *T.* = *Triatoma*; *E.* = *Eratyrus*; *R.* = *Rhodnius*.

† - = not calculated (sample too small).

‡ Mixed infections observed = number and % of fecal samples hybridizing with both probes 20 and 39. Mixed infections expected = number of fecal samples calculated by the formula $N \cdot (\%20 \cdot \%39)$ with N = number of stored samples and %20 and %39 = observed % of fecal samples hybridized by probes 20 and 39, respectively. The expected % are deducted dividing the expected numbers by the total number of samples.§ By Yates' chi-square test, $ddl = 1$.

facility of the method in dealing with large numbers of samples without the time, expense, and loss of clones during culturing, which is required for isoenzyme analysis.

The results of the PCR with *Triatoma infestans* confirm earlier work with isoenzymes indicating the importance of major clones 20 and 39 in the domestic cycle in Bolivia.¹ Although the sample sizes are as yet too small to allow epidemiologic conclusions, this technique is clearly able to identify the microdistribution of particular clones: e.g., in our sample, the Department of Cochabamba showed a significantly higher percentage of clone 20 (90.9%) than the Department of La Paz (65.8%, Table 3, Yates' $\chi^2 = 5.65$, $P < 0.05$).

Of particular interest is finding clones 20 and 39 in three nondomestic species. This is the first report of the presence of these major clones, known from the domestic cycle, in sylvatic species in Bolivia. Since these normally sylvatic triatomines were collected in domestic and peridomestic areas, it is not clear whether these vectors are tending towards domesticity and acquiring domestic clones or whether clones 20 and 39 exist in the sylvatic situation as well. The origin of these major clones, sylvatic or domestic, remains to be determined. Application of this PCR-based technique to sylvatic mammals and triatomines should resolve this issue. Contrary to what was observed in *Triatoma infestans*, the majority of the clones present in *E. mucronatus* and *R. pictipes* are neither clone 20 nor clone 39 and thus likely represent infections by additional clones whose taxonomic status remains to be determined.

Amplification with the PCR followed by hybridization was also a much more powerful technique than MLEE for detecting mixed infections. The probes used here do not cross-hybridize, as previously verified on a large range of reference strains (characterized by isoenzymes) pertaining to natural clones 20, 39, and others.⁶ This result strongly suggests the detection of mixed infections (clone 20 and clone 39).⁶ As more specific *T. cruzi* clone probes are developed and more strains are identified, we expect to find even higher rates of mixed infections. Since the expected rates of mixed infections with clones 20 and 39 (Table 4) did not differ significantly from the observed rates, we have no evidence for interaction between the clones in which the presence of one would affect the presence or development of the other.

The approach developed here should allow characterization of many clones from different regions. When the PCR products from an infected host (vector or mammal) do not hybridize with the probes that are used, it will be possible to develop a new probe from these PCR products. This new probe will be hybridized first with strains that have been genetically characterized (by MLEE) to determine its specificity and then used to test biological samples for epidemiologic studies if the specificity results are satisfactory. If we consider the clonal nature of *T. cruzi* populations as well as the linkage between HVRm DNA and nuclear DNA, it should be possible to develop specific probes from HVRm kDNA. Moreover, this approach, when applied to human infection, should allow investigation of the medical conse-

quences of infection with a particular clone or multiple clones.

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