Identification of Blood Meal Source and Infection with *Trypanosoma cruzi* of Chagas Disease Vectors Using a Multiplex Cytochrome b Polymerase Chain Reaction Assay

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ABSTRACT

Long-term control of triatomine bugs in Chagas endemic regions will depend on a full understanding of vector–parasite–host interactions. Herein we describe a cytochrome b multiplex polymerase chain reaction (PCR)-based strategy for blood meal source identification in bug foregut contents. This technique discriminates human from animal blood, and has been tested in five *Triatoma* species from México. Host identification has been validated for human, four rodent species, two bat species, dog, rabbit, sheep, and opossum. In addition, *Trypanosoma cruzi* can be identified simultaneously using S34/S67-specific kinetoplast DNA primers. Both host and parasite identification were possible as long as 10 weeks after bug feeding, and in samples stored up to 6 years. The blood meal identification procedure described here represents a powerful tool for large-scale studies identifying the biological, ecological, and environmental variables associated with Chagas disease transmission. Key Words: Triatominae—Blood meal—Chagas disease—PCR.

INTRODUCTION

Chagas disease is caused by the protozoan *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae), present in the feces of triatomine bugs (Hemiptera: Reduviidae), and is usually transmitted to humans via direct fecal contact. The most common secondary transmission routes are blood transfusion, congenital infection, tissue transplant, and food-borne transmission (WHO 2002). This complex anthropozoonosis occurs following establishment of domestic transmission cycles usually within an ecotone area buffering sylvan habitats, where the parasite is harbored in multiple zoonotic hosts from a variety of mammal families (Schofield 1994). Geographic distribution of these sylvan hosts is an important component of each species's ecological niche, and knowledge of triatomine host preferences is therefore essential to study vector capacity, fitness, and domesticity. However, information regarding vector feeding patterns is scarce, due to the limited study of hosts, and limited sen-

sibility and specificity of antibody-based techniques (Quintal and Polanco 1977, Breniere et al. 2004; Freitas et al. 2005). Availability of specific sylvan fauna DNA probes also represents a limitation in the case of a recently described polymerase chain reaction (PCR)-heteroduplex assay (Bosseno et al. 2006).

In contrast to the lack of studies with triatomine bugs, host preferences of other hematophagous arthropods of medical importance have been studied extensively using molecular approaches (Mukabana et al. 2002), as in the case of anophelines (Chow-Shaffer et al. 2000), tsetse flies (Steuber et al. 2005), ticks (Kirstein and Gray 1996), and lice (Lord et al. 1998). Molecular analyses to identify blood meal sources in insects have principally targeted mitochondrial DNA (mtDNA), which is composed of small circular molecules (15–20 kb) having approximately 37 genes coding for 22 tRNAs, 2 rRNAs, and 13 mRNAs (Kocher et al. 1989). As a molecular marker, mtDNA has many advantages since it evolves faster than nuclear DNA (Brown et al. 1982), thereby producing regions with enough variation for population level questions, but sufficiently conserved to establish phylogenetic relationships. Cytochrome b (cyt b) is involved in respiratory chain electron transport in the mitochondrion, and is the only cytochrome coded in mtDNA. It is the most widely used molecular marker gene, and although it evolves slowly in terms of nonsynonymous substitutions, the evolutionary rate for silent positions is relatively rapid (Irwin et al. 1991).

The aim of the present study was to standardize a cyt b PCR-based strategy to identify mammal hosts of triatomines using DNA from bug foregut with the simultaneous identification of *T. cruzi* using specific kinetoplast DNA (kDNA) primers.

MATERIALS AND METHODS

Triatomines, specimen collection, and preservation

Laboratory-bred adult male and female specimens of *Triatoma pallidipennis* were used to standardize DNA isolation and cyt b PCR as-

say. Insects were reared and maintained in an insectary under controlled conditions (38°C, 70% relative humidity). Groups of five insects were starved for a period of 2–3 months, fed on BALB/c mice under controlled conditions, and one group per week dissected at multiple time intervals post-feed (from 1 to 10 weeks). Promesenteric contents (Prm-C) were obtained as previously described (Wisnivesky-Colli et al. 1982). Prm-C were diluted 1:1 in PBS and 100–200 μL of mixture was added to 1 mL of DNAzol (Invitrogen Corporation, San Diego, CA), and genomic DNA was isolated according to the manufacturer's instructions. The final pellet was dissolved in 100 μL of 8 mM NaOH and stored at -20°C until used. The isolated DNA was used as template in PCR experiments. To estimate amplification efficiency following partial vs. complete meals, groups of bugs were interrupted during feeding after 5 minutes, and other groups were left to feed to repletion; the partial meal (5 minutes) was identified by presence of blood, but lack of abdomen distention.

Field-collected bugs were identified taxonomically (Lent and Wygodzinsky 1979), and the presence of *Trypanosoma cruzi* in fecal samples verified using light microscopy. A total of 42 bug specimens (including adults from both sexes and 4th and 5th stage nymphs) were collected from different Chagas endemic regions of México (Table 1) (Ramsey et al. 2000, Enger et al. 2004), preserved in 70% ethanol, and analyzed between 2 months to 6 years later. Approximately 10 mg of solidified Prm-C was extracted and 1 mL of DNAzol added using genomic DNA isolation from ethanol-preserved bugs (EPBs). DNA isolation was performed as described above.

Mammal tissues

To estimate *T. cruzi* infection rates in small mammals, sylvan, ecotone, and domestic areas were sampled (Oaxaca and Morelos states) (Table 2). Animals were collected live using Sherman traps (Sherman Trap Company, Tallahassee, FL), anesthetized, and killed, and either whole bodies or cardiac tissue were preserved in 70% ethanol. Samples were transported to the INSP laboratory and stored at

TABLE 1. COLLECTION SITES FOR *TRIATOMA* SPECIES USED IN THIS STUDY

Species	n[a]	Year collected	Community	County	State	Latitude	Longitude	Habitat[b]
T. pallidipennis	27	2003	Chalcatzingo	Janteteleco	Morelos	18°41′922″	98°46′30″	I, P
		2004	Temixco	Temixco	Morelos	18°51′16″	99°13′38″	I
		2005	3 de Mayo	Emiliano Zapata	Morelos	18°52′07″	99°12′30″	I
		2005	Chalcatzingo	Janteleco	Morelos	18°41′22″	98°46′30″	S
T. barberi	6	1996	San Pedro Apostol	San Pedro Apostol	Oaxaca	16°44′10″	96°43′35″	I
		1996	La Chilana	San José del Progreso	Oaxaca	16°43′24″	96°43′15″	I, P
		2005	Santa Cruz Papalutla	Santa Cruz Papalutla	Oaxaca	16°57′20″	96°35′02″	I, P
T. dimidiata	6	2000	El Lejem	San Antonio	San Luis Potosí	21°36′09″	98°51′56″	P
		1996	San Juan Comaltepec	San Juan Comaltepec	Oaxaca	17°20′15″	95°58′32″	I, P
		1996	San José Chinantequilla	Totontepec	Oaxaca	17°18′25″	95°59′24″	I
T. phyllosoma	2	1999	Santo Domingo Chihuitán	Santo Domingo Chihuitán	Oaxaca	16°35′20″	95°09′42″	P
T. longipennis	1	2004	Sta. Clara	Zacoalco de Torres	Jalisco	20°13′40″	103°34′10″	S

[a]*n*, number of specimens analyzed.
[b]Habitat: I, intradomestic; P, peridomestic; S, sylan.

TABLE 2. PREVALENCE OF SINGLE AND COMBINED BLOOD MEALS FROM HUMAN, BAT, AND RODENT SOURCES IN DOMESTIC-COLLECTED *TRIATOMA PALLIDIPENNIS*, *T. BARBERI*, *T. DIMIDIATA*, AND *T. PHYLLOSOMA*, AND *SYLVAN/ECOTONE T. LONGIPENNIS*

Blood source	T. pallidipennis		T. barberi		T. dimidiata		T. phyllosoma		T. longipennis	
	Single[a]	Combined[a]	Single	Combined	Single	Combined	Single	Combined	Single	Combined
Myotis sp.	40.0% (2/5)	60.0% (3/5)	—	—	—	—	—	—	—	—
Mus musculus	—	—	100.0% (1/1)	0/1	—	—	—	—	100.0% (1/1)	0/1
Baiomys musculus	0/1	100.0% (1/1)	50.0% (1/2)	50.0% (1/2)	100.0% (5/5)	0/5	100.0% (2/2)	0/2	—	—
Sigmodon mascotensis	—	—	50.0% (1/2)	50.0% (1/2)	—	—	—	—	—	—
Peromyscus levipes	—	—	0/1	100.0% (1/1)	—	—	—	—	—	—
Oryctolagus cuniculus	100.0% (1/1)	0/1	—	—	—	—	—	—	—	—
Didelphis virginiana	100.0% (1/1)	0/1	—	—	—	—	—	—	—	—
Ovis aries	100.0% (1/1)	0/1	—	—	—	—	—	—	—	—
Canis familiaris	100.0% (1/1)	0/1	—	—	—	—	—	—	—	—
Homo sapiens	50.0% (4/8)	50.0% (4/8)	0/3	100.0% (3/3)	—	—	—	—	—	—

[a]Prevalence (%) and number of animals (per type/total).

room temperature until used. A total of 135 mammals from the order Rodentia (Muridae: *Neotoma mexicana*, *Reithrodontomys fulvescens*, *Peromyscus levipes*, *Sigmodon hispidus*, *Mus musculus*, *Baiomys musculus*; Heteromyidae: *Liomys irroratus*, *Liomys pictus*), and from the order Chiroptera (Phyllostomidae: *Glossophaga soriciana*; Mormoopidae: *Mormoops megalophylla*, *Pteronotus parnelli*) were collected. Genomic DNA was isolated from cardiac tissue using DNAzol as described above and used to test the presence of *T. cruzi* using specific kDNA PCR assay (Sturm et al. 1989).

Oligonucleotide design

The cyt b gene sequences from diverse mammal species that are believed to be potential reservoirs, and found in domestic and sylvan habitats, were used to design primers for PCR amplification. A consensus sequence was generated by alignment of available cyt b sequences reported for each expected genus (between 10 to 25 sequences). Alignment of all consensus sequences produces a region with conserved sequences at the 5′ and 3′ ends, including a variable internal region. Primers designed to amplify this region are: DC-cytb-UP, 5′-CRT GAG GMC AAA TAT CHT TYT-3′, and DC-cytb-DW, 5′-ART ATC ATT CWG GTT TAA TRT-3′; the size of the amplified product is 420 bp. Due to broad divergence in the homologous region between the previous and that reported in humans, a third primer (antisense) was designed, to combine with DC-cytb-UP primer; this latter specifically amplifies a shorter region of human cyt b (315 bp): H-cytb-DW, 5′-AGG AGA GAA GGA AA GAA GT-3′. All three primers were used in the multiplex PCR.

Sequence analysis and oligonucleotide design were performed using the Wisconsin Package, version 10.2 (Genetics Computer Group [GCG], Madison, WI).

T. cruzi

A *T. cruzi* strain isolated from *T. pallidipennis* from Temixco county (Morelos State) was used to obtain DNA for PCR amplifications of positive controls (Flores et al. 2002). Parasites were cultured and maintained *in vitro* in liver infu-

sion tryptose (LIT) medium (Camargo 1964), isolated, and stored at −70°C until used. An estimated 1×10^4 parasites were centrifuged and pellets resuspended in 1 mL of DNAzol and whole DNA isolated as described above; the final DNA pellet was dissolved in an appropriate volume of 8 mM NaOH to obtain a concentration of 10 ng/μL and stored at −20°C until used.

PCR and sequencing

The cyt b PCR assay was carried out using 1 μL (usually 10–70 ng) of DNA obtained from Prm-C, or 10 ng of DNA obtained from cardiac tissue, in a final volume of 12–50 μL of 1× PCR Master Mix (Promega Corporation, Madison, WI) containing *Taq* DNA polymerase with PCR buffer (pH 8.5), 200 mM of each dNTP, 1.5 mM of MgCl$_2$, and 0.5 μM of the primers described above. PCR was performed as follows: initial DNA denaturing step at 94°C, 4 minutes followed by 35 cycles: denaturing (94°C, 30 seconds), annealing (42.5°C, 30 seconds), extension (72°C, 30 seconds), and a final extension step at 72°C for 10 minutes. The human specific primer H-cytb-DW was designed to be used in combination with DC-cytb-UP and DC-cytb-DW (animal primers) in the multiplex PCR approach, to amplify human (315 bp) or animal (420 bp) DNA from blood meals, or to detect both meal sources in the same bug (both bands present). Since the human primer is 100% specific and animal primers are degenerate, concentration and annealing temperatures were defined to obtain optimum conditions for all sources of DNA template. Human DNA used as positive control was isolated from a 10 mL blood sample donated via venous puncture from a donor following informed consent and preserved in DNAzol as described above.

The primers S34 5′-ACA CCA ACC CCA ATC GAA CC-3′ and S67 5′-TGG TTT TGG GAG GGG SSK TC-3′ which amplify the constant region of kDNA were used for *T. cruzi* PCR assay (Sturm et al. 1989). Each primer (0.5 μM) was added to the PCR mix as described above, containing 1 μL of DNA template from Prm-C or 10 ng of the *T. cruzi* DNA control. The amplification reaction was carried out as follows: initial DNA denaturing step at 94°C, 4

minutes followed by 35 cycles: denaturing (94°C, 30 seconds), annealing (60°C, 30 seconds), and extension (72°C, 30 seconds), and a final extension step at 72°C for 10 minutes. All amplified products were separated and visualized in 1% to 1.5% agarose gels, stained with ethidium bromide, and visualized under UV light.

Amplified bands using degenerate primers (420 bp) for sequencing cyt b were separated in a 1% agarose gel, excised, and purified using Wizard SV Gel and PCR Clean-Up System (Promega), and sequenced in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) using the BigDye Terminator v3.1 Cycle Sequencing Kit. Reactions were carried out according to standard protocols described by the manufacturer, using 50 ng of purified PCR product with 20 pM of DC-cytb-UP primer, in a final volume of 20 μL. Sequences were aligned and edited using BioEdit software (Hall 1999), and compared with databases using the BLASTN program (Altschul et al. 1997), to determine homology with potential mammal hosts. To assure quality control of positive human samples (315 bp band) identified in field-collected bugs, 50% of the samples were sequenced as described above.

Statistical analysis

T. cruzi infections analyzed using microscopy and PCR were compared using a χ^2 test. Associations between the identified blood (human or animal) source and the presence of infection and species of triatomine were also evaluated using a χ^2 test. All statistical analyses were performed using SPSS version 10.0 software (SPSS, Inc., Chicago, IL).

RESULTS

Standardization of cyt b PCR for DNA template from animals

To test designed cyt b primers and to standardize PCR conditions, cardiac tissue DNA from three species of rodents collected from
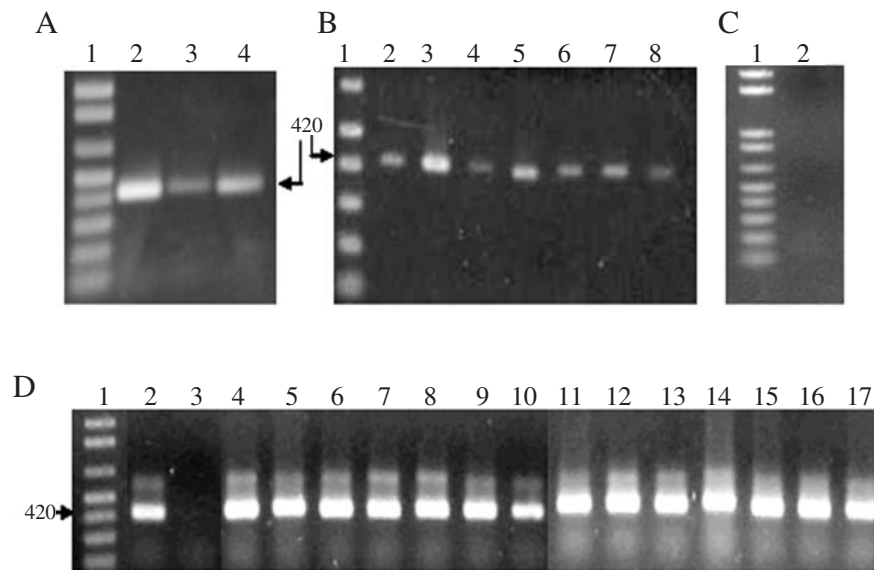

FIG. 1. Standardization of cyt b PCR for sylvan mammals using animal degenerate primers. (**A**) DNA template was obtained from cardiac tissue of *Neotoma mexicana, Peromyscus levipes*, and *Liomys irroratus* (lanes 2, 3, and 4, respectively) and PCR amplified. A single 420 bp band (indicated) was observed in all cases. Molecular weight markers (1 kb Plus DNA Leader, Invitrogen) are shown in lane 1 in A–D. (**B**) Amplification of the specific cyt b band was detected in promesenteric contents of triatomines fed on BALB/c mice 1 week (lane 2), 2 weeks (lane 3), 3 weeks (lane 4), 4 weeks (lane 5), 6 weeks (lane 6), 8 weeks (lane 7), and 10 weeks (lane 8) after the blood meal. (**C**) Negative control (no template) for A and B is shown in lane 2. (**D**) Amplification of cyt b band was possible in eight rodent species: *Neotoma mexicana* (lane 2), *Reithrodontomys fulvescens* (lanes 7, 12), *Peromyscus levipes* (lanes 10, 14), *Sigmodon hispidus* (lanes 4, 17), *Liomys irroratus* (lanes 5, 16), *Liomys pictus* (lanes 8, 15), *Mus musculus* (lanes 6, 13), and *Baiomys musculus* (lanes 9, 11). Negative control (no template) is shown in lane 3.

natural habitats in Morelos and Oaxaca (*N. mexicana*, *P. levipes*, and *L. irroratus*) were used. One specific band of the expected size (420 bp) was amplified from all three species (Fig. 1A). To test if the specific cyt b band could be amplified from Prm-C of triatomines, we evaluated the PCR assay in bugs fed on BALB/c mice. Results showed that the 420 bp band could be amplified at least until 10 weeks after the blood meal (Fig. 1B). The principal limiting factor for cyt b amplification was the amount of blood taken in the meal. Prm-C from bugs fed on small volumes of blood (<5 minutes' feeding) produce less intense bands (Fig. 1B, lane 4) or no amplification at all (data not shown). Elution products from control (unfed) insects were always negative for any bands following PCR (data not shown). Control (unfed) insects were also always negative for any bands following amplification procedures (data not shown). Amplification efficiency and conditions for the cyt b region were identical when other rodent species were used as blood source

(*N. mexicana* and *L. irroratus*). Final standardization methods for this PCR assay were defined according to conditions for eight rodent species (*N. mexicana*, *R. fulvescens*, *P. levipes*, *S. hispidus*, *L. irroratus*, *L. pictus*, *M. musculus*, and *B. musculus*), all of which produce one expected 420 bp band (Fig. 1D).

Standardization of multiplex cyt b PCR assay

Kinetic experiments using DNA from animals or humans, or combinations of both DNA sources, were performed to standardize optimal conditions for the multiplex PCR. The established ratio of degenerate and human primers was $0.5/0.25~\mu$M, and $42.5°$C as the optimal annealing temperature. Results showed that a single 315 bp band was amplified from human DNA, while for all animals, the expected 420 bp band was amplified (Fig. 2A). When DNA extracted from human and rabbit were combined for the multiplex PCR, both bands were amplified (Fig. 2A, lane 6). Both hu-
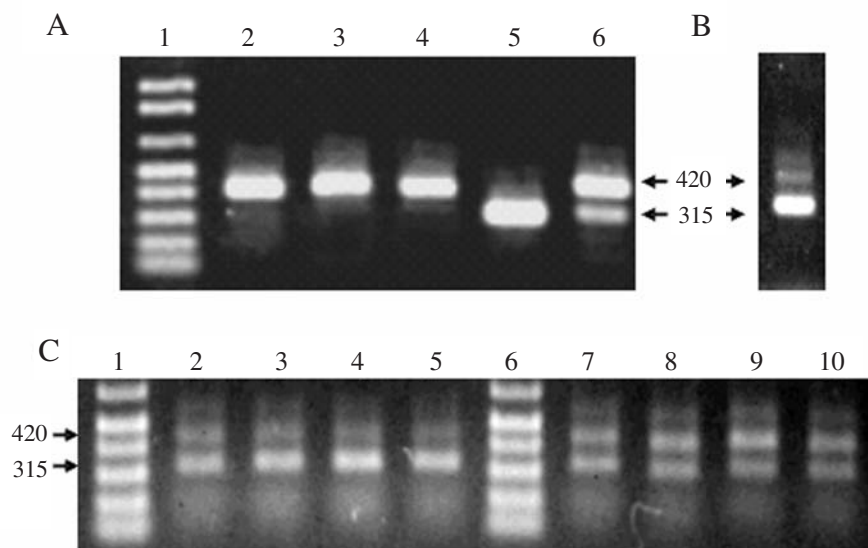
FIG. 2. Standardization of multiplex cyt b PCR for discrimination of animal and human blood sources. (**A**) Multiplex PCR was assessed using forward and reverse animal degenerated primers, and reverse specific human primer. The 420 bp animal band (indicated) was amplified when DNA from BALB/c mice (lane 2), rabbit (lane 3), and dog (lane 4), were used, a 315 bp-specific band (indicated) was amplified when human DNA was used (lane 5). Both bands were amplified from a combined animal (rabbit) and human DNA sample (lane 6). Molecular weight markers (1 kb Plus DNA Leader, Invitrogen) are shown in lane 1 in A and B. (**B**) Animal and human bands were also amplified in DNA obtained from promesenteric contents of triatomines artificially fed with a combined meal of rabbit and human blood. (**C**) Amplification of animal and human bands was possible using a constant concentration (10 ng) of animal DNA (mice) and increasing amounts of human DNA starting with 10 ng (lane 2), 20 ng (lane 3), 30 ng (lane 4), and 40 ng (lane 5). A similar experiment using a constant DNA concentration of human DNA and variable amounts of animal DNA is shown in lanes 7–10. Molecular weight markers are shown in lane 6.

man and animal bands were also amplified from Prm-C obtained from *T. pallidipennis* adults artificially fed on a mixture of human and rabbit blood (Fig. 2B). Amplification of the cyt b region from both animal and human DNA was always possible, even when the concentration of one template varied as much as fourfold with respect to the other (Fig. 2C).
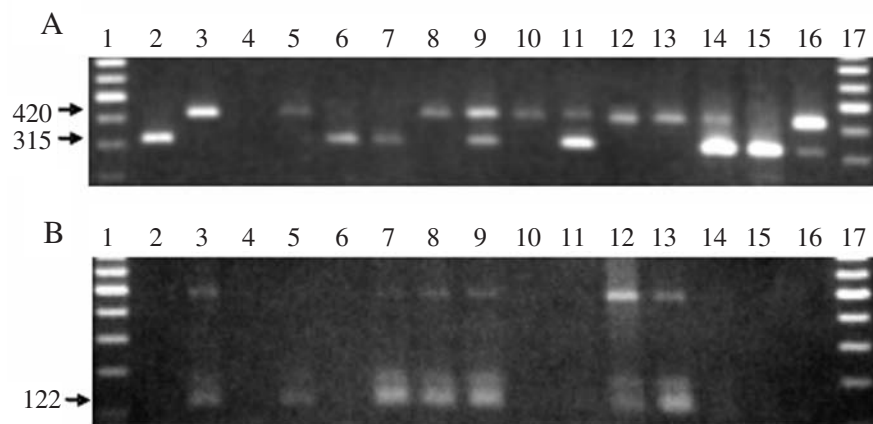
Identification of bug hosts

Bugs collected in endemic areas were analyzed for host identification using the standardized cyt b PCR as described above. DNA was amplified from all samples visibly containing blood in promesenteron, while DNA could not be amplified from 16.7% (7 of 42) of samples, all of which had no visually identifiable blood meal. Blood meal sources from *T. pallidipennis, T. barberi, T. dimidiata, T. phyllosoma,* and *T. longipennis* specimens collected from domestic and sylvan/ecotone habitats were identified (Table 2). DNA obtained from insects collected and preserved for as long as 6 years in 70% ethanol could all be amplified.

Positive samples for only animal bands in the multiplex PCR assay were reamplified using only animal primers (DC-cytb-UP and DC-

cytb-DW), and products were purified, sequenced, and compared with reported sequences using BLASTN search. In all cases, sequences having greater than 85% homology were considered significant and crossed with lists of known Mexican fauna from the collection area (Comisión Nacional de Biodiversidad, CONABIO, www.conabio.gob.mx, last accessed August 2006) or from lists of mammals collected over the course of studies. A representative experiment is shown in Figure 3, in which samples represent PCR amplifications from DNA obtained from different species of triatomines, collected in different years and villages, and includes samples with different blood sources, having a single (animal or human) or combined blood meal source (animal and human). Frequencies of blood meal sources for these test samples are summarized in Table 2. We identified blood from ten species of mammals in field-collected bugs, including six species of rodents, one species of bat, opossum, sheep, dog, and human.

Despite the bias of low sample numbers, a higher proportion of *T. pallidipennis* had single blood meals (55.6%) than *T. barberi* (33.3%) (Table 2), although this difference was not significant ($p = 0.274$). Domestic *T. pallidipennis*

FIG. 3. Identification of blood meal sources and *Trypanosoma cruzi* infection in field-collected triatomines. (**A**) Human (315 bp band) and animal (420 bp band) DNA were amplified by multiplex cyt b PCR in bugs collected in distinct regions of study (see Table 1); a representative experiment is shown. Results showed samples positive for animal blood only (lanes 5, 8, 10, 12, and 13), samples positive for human blood only (lanes 6, 7, and 15), and samples positive for both blood sources (lanes 9, 11, 14, and 16). Positive human control is shown in lane 2, positive animal control in lane 3, and negative control (no template) in lane 4. (**B**) Identification of *T. cruzi* in the same DNA samples was performed by PCR using S67/S34-specific kDNA primers that amplified a 122 bp sequence (indicated) of the constant minicircle region. A negative control consisting of DNA obtained from triatomines fed on uninfected mice is shown in lane 2, a positive control (*T. cruzi* DNA) in lane 3, and a no template control in lane 4. Molecular weight markers (1 kb Plus DNA Leader, Invitrogen) are shown in lanes 1 and 17 in A and B.

feeding uniquely on one species did so on domestic/livestock hosts (*Oryctolagus cuniculus*, *Ovis aries*, and *Canis familiaris*) or the ubiquitous *Didelphis virginiana*, while blood of sylvan species (*Baiomys*) was only found in combined meals. Both human and bat blood were found in equivalent proportions in single and combined meals of this bug species. Sylvan/ecotone-collected *T. pallidipennis* had low human blood prevalence rates (14.3%, 1/7), and meals were combined with sylvan species (*Myotis* sp., *M. musculus, B. musculus,* or *S. mascotensis*) (data not shown). Domestic-collected *T. barberi* (Table 2) was not found to feed on domestic host species (i.e., dog or cat), or solely on humans (always in combined meals), while the frequency of single and combined meals from sylvan rodents was similar. Single blood meals from *B. musculus* were identified in domestic-collected *T. dimidiata* and *T. phyllosoma*, while a single *M. musculus* blood meal was identified from *T. longipennis*.

T. cruzi *infection in field-collected bugs*

Using the same DNA sample obtained from Prm-C of field-captured bugs, *T. cruzi* infection was determined by PCR and results compared with light microscopic observation of fecal samples. Due to bug mortality, microscopic identification of *T. cruzi* in feces was only pos-

sible in 30 of 42 (71.4%) samples, while presence/absence was possible for only 41 samples using PCR. Using the PCR assay as the gold standard and comparing the 30 samples analyzed with both techniques, microscopic examination had a sensitivity of 46.6% and a specificity of 73.3%. The positive and negative predictive values for this methodology were 63.6% and 57.9%, respectively.

The prevalence of *T. cruzi* infection in domestic-collected *T. pallidipennis*, *T. barberi*, *T. dimidiata*, and *T. phyllosoma* using PCR according to blood meal type is summarized in Table 3. *T. barberi* containing animal blood had the highest infection rate with *T. cruzi* among all species (66.7%); 50.0% of *T. pallidipennis*, 50.0% of *T. phyllosoma*, and 20.0% of *T. dimidiata* were also infected with animal blood. In contrast, *T. pallidipennis* had the highest infection rate (62.5%) for bugs containing human blood, while *T. barberi* was significantly lower (33.3%). Eighty percent of *T. pallidipennis* and one *T. dimidiata* which had no amplifiable blood meal were also infected with *T. cruzi*.

It is noteworthy that the highest infections of *T. cruzi* in domestic *T. pallidipennis* were found associated with bats (*Myotis* sp.), domestic and ecotone rodents (*Baiomys* and *Sigmodon*), and humans. Bugs with blood from stationary penned livestock (*O. cuniculus*) had higher infection rates than those with blood from small

TABLE 3. INFECTION[a] OF DOMESTIC *TRIATOMA PALLIDIPENNIS*, *T. BARBERI*, *T. DIMIDIATA*, AND *T. PHYLLOSOMA*, WITH *T. PHYLLOSOMA* WITH TRYPANOSOMA CRUZI, ACCORDING TO BLOOD MEAL SOURCE

Blood source	T. pallidipennis		T. barberi		T. dimidiata		T. phyllosoma	
	%	(+/total)	%	(+/total)	%	(+/total)	%	(+/total)
Myotis sp.	60.0	(3/5)	—	—	—	—	—	—
Mus musculus	—	—	100.0	(1/1)	—	—	—	—
Baiomys musculus	100.0	(1/1)	100.0	(2/2)	20.0	(1/5)	50.0	(1/2)
Sigmodon mascotensis	—	—	50.0	(1/2)	—	—	—	—
Peromyscus levipes	—	—	0.0	(0/1)	—	—	—	—
Didelphis virginiana	0.0	(0/1)	—	—	—	—	—	—
Ovis aries	0.0	(0/1)	—	—	—	—	—	—
Canis familiaris	0.0	(0/1)	—	—	—	—	—	—
Oryctolagus cuniculus	100.0	(1/1)	—	—	—	—	—	—
Total animal	50.0	(5/10)	66.7	(4/6)	20.0	(1/5)	50.0	(1/2)
Human	62.5	(5/8)	33.3	(1/3)	—	—	—	—
No amplification cyt b[b]	80.0	(4/5)	—	—	100.0	(1/1)	—	—

[a]*Trypanosoma cruzi* infection was determined by PCR using specific kinetoplast DNA primers S67/S34.
[b]Control amplification functional.

mammals or dogs. The highest infection rates in *T. barberi* occurred in domestic and ecotone rodent species.

T. cruzi *infection in potential sylvan hosts*

Rodent specimens collected from Valles Centrales, Oaxaca, where *T. barberi* is the only domestic vector species, have high *T. cruzi* infection rates (Table 4). Infections in *M. musculus*, *B. musculus*, *Sigmodon* spp., *R. fulvescens*, and *L. irroratus* confirm bug infections. Bug species from ecotone and domestic habitats positive for *T. cruzi* from Morelos also correlated with blood meal identification in bugs (Table 4).

DISCUSSION

A first step to understanding triatomine ecological niches is the analysis of host preference and specificity in the habitats they occupy (sylvan, ecotone, domestic). In México, most triatomine species belonging to the *protracta* and *phyllosoma* complexes are implicated in Chagas disease transmission, including the species studied herein. Data regarding host feeding preferences of these species are very scarce or nonexistent for most regions in México, and no information is available regarding host substitution for microhabitat variability. Immunodiffusion assays have been used for the identification of feeding preferences of *phyllosoma* complex species in peridomestic habitats in Jalisco State (Breniere et al. 2004), although

these results are severely biased due to the number of antisera available, and their lack of species specificity. Recently, the identification of triatomine feeding preferences was approached by using a heteroduplex DNA migration pattern assay (Bosseno et al. 2006). Although this molecular method is more sensitive than immunologically-based approaches, data obtained for feeding preferences had similar limitations: blood source identification is limited by the number of available specific probes (hybridization drivers), and host identification taxonomically only attains the order level. This methodological restriction impedes urgently needed studies on vector population dynamics.

Specificity and sensitivity of immunologically based techniques are influenced by the quality of collected bugs, the time since last feeding, and the specificity/diversity of host-specific antibodies used. In the present study, standardization of preservation procedures in 70% ethanol permitted a uniform and efficient preservation of DNA (host and parasite). We were able to amplify the cyt b gene in samples stored for periods up to 6 years, and the amplification was possible at least until 10 weeks after the blood meal, thereby providing a sensitive approach for vectors that often go through months of fasting. The use of degenerate animal primers provides broad host identification, and combined with the human primer, the assay immediately identifies vector preferences. Together with *T. cruzi* identifica-

TABLE 4. INFECTION OF MAMMAL SPECIES[a] WITH *TRYPANOSOMA CRUZI* USING PCR WITH S67/S34 PRIMERS

Mammal species	Collection site	n	T. cruzi positive (%)
Baiomys musculus	VC Oaxaca	26	46.2
	Morelos	30	26.6
Liomys irroratus	VC Oaxaca	24	25.0
Mus musculus	VC Oaxaca	3	0.0
Neotoma mexicana	VC Oaxaca	4	0.0
Peromyscus levipes	VC Oaxaca	3	0.0
	Morelos	7	28.6
Reithrodontomys fulvescens	VC Oaxaca	31	54.8
Sigmodon hispidus	VC Oaxaca	7	42.9
	Morelos	91	8.8

[a]Representing rodents captured in (Valles Centrales [VC] Oaxaca), ecotone, and domestic (Morelos) habitats, in dry and rainy reasons.

tion, these assays standardize methods across collection habitats and therefore reduce bias for population dynamic studies, as demonstrated for other vector-borne diseases (Mukabana et al. 2002).

Despite the low numbers of field-collected bugs analyzed in this pilot host study, human blood was the most frequent source for most triatomine species in domestic habitats, in a single or mixed blood meal. Prevailing anecdotal citations in the literature refer to *T. barberi* as a strictly domestic species specialized on human blood sources; this was not substantiated from the specimens analyzed herein. In fact, *T. barberi* appeared to be less anthropophilic than *T. pallidipennis,* as human blood was only identified in combined meals in the former species. High *T. cruzi* prevalence was found in bugs having all types of blood meals in the domestic habitat (livestock, sylvan rodents, and humans) suggesting broad opportunism once the bugs are located in this "novel" environment. Interestingly, we expected a higher prevalence of canine blood meals in domestic-collected bugs, but this was neither the case in *T. pallidipennis* nor *T. barberi*. Future studies analyzing representative numbers of bugs and reservoirs from all environmental compartments will be required to understand host substitutions across habitats.

T. cruzi infection in cardiac tissue of sylvan bats and rodents correlated with bug blood meal data. Cross-reactivity of specific *T. cruzi* kDNA primers with bat trypanosomes such as *T. cruzi marinkellei* is unlikely since kDNA sequences (Telleria et al. 2006) and random amplified polymorphic DNA (Brisse et al. 2000) from *T. cruzi* I, predominant in all areas where present bug samples were collected (Bosseno et al. 2002), and *T. cruzi marinkellei,* are unrelated.

One of the two most prevalent rodent species in ecotone areas, *B. musculus*, also had high *T. cruzi* infection rates in at least three of the bug species. *Myotis* sp., an insectivorous bat species not frequently found near human housing, was not collected using conventional trapping methods; rather it was identified from ultrasound recordings of peridomestic habitats (Anabat detection system, Fenton et al. 2001). It is important to note that bugs collected in this study containing *Myotis* blood had high *T. cruzi*

infection rates, equivalent to those containing human blood.

Despite the efficacy of this PCR technique, its usefulness and cost in microhabitat studies or in large-scale ecological niche studies may be limited. However, few tools are available for dynamic population studies of triatomines and *T. cruzi*, and precise identification of vector–host associations as well as vector–parasite–host associations is essential to identify vector dispersion mechanisms, bug domestication processes, and the epidemiological importance of vector populations.

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REFERENCES

Altschul, SF, Madden, TL, Schaffer, AA, Zhang, J, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997; 25:3389–3402.

Bosseno, MF, Barnabe, C, Gastelum, EM, Lozano, KF, et al. Predominance of *Trypanosoma cruzi* lineage I in Mexico. J Clin Microbiol 2002; 40:627–632.

Bosseno, MF, Garcia, LS, Baunaure, F, Gastelum, EM, et al. Identification in triatomine vectors of feeding sources and *Trypanosoma cruzi* variants by heteroduplex assay and a multiplex miniexon polymerase chain reaction. Am J Trop Med Hyg 2006; 74:303–305.

Breniere, SF, Pietrokovsky, S, Gastelum, EM, Bosseno, MF, et al. Feeding patterns of *Triatoma longipennis* Usinger (Hemiptera, Reduviidae) in peridomestic habitats of a rural community in Jalisco State, Mexico. J Med Entomol 2004; 41:1015–1020.

Brisse, S, Barnabé, C, Tibayrenc, M. Identification of six *Trypanosoma cruzi* lineages by random amplified poly-

morphic DNA and multilocus enzyme electrophoresis. Int J Parasitol 2000; 30:35–44.

Brown, WM, Prager, EM, Wang, A, Wilson, AC. Mitochondrial DNA sequences of primates: tempo and mode of evolution. J Mol Evol 1982; 18:225–239.

Camargo, EP. Growth and differentiation in *Trypanosoma cruzi*. I. origin of metacyclic trypanosomes in liquid media. Rev Inst Med Trop Sao Paulo 1964; 12:93–100.

Chow-Shaffer, E, Sina, B, Hawley, WA, De Benedictis, J, et al. Laboratory and field evaluation of polymerase chain reaction-based forensic DNA profiling for use in identification of human blood meal sources of *Aedes aegypti* (Diptera: Culicidae). J Med Entomol 2000; 37:492–502.

Enger, KS, Ordonez, R, Wilson, ML, Ramsey, JM. Evaluation of risk factors for rural infestation by *Triatoma pallidipennis* (Hemiptera: Triatominae), a Mexican vector of Chagas disease. J Med Entomol 2004; 41:760–767.

Fenton, MB, Bernard, E, Bouchard, S, Hollis L, et al. Choosing the 'correct' bat detector. J Trop Ecol 2001; 17:511–524.

Freitas, SP, Lorosa, ES, Rodrigues, DC, Freitas, AL, et al. Feeding patterns of *Triatoma pseudomaculata* in the state of Ceara, Brazil. Rev Saude Publica 2005; 9:27–32.

Hall, TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 1999; 41:95–98.

Irwin, DM, Kocher, TD, Wilson, AC. Evolution of the cytochrome b gene of mammals. J Mol Evol 1991; 32: 128–144.

Kirstein, F, Gray, JS. A molecular marker for the identification of the zoonotic reservoirs of Lyme borreliosis by analysis of the blood meal in its European vector *Ixodes ricinus*. Appl Environ Microbiol 1996; 62:4060–4065.

Kocher, TD, Thomas, WK, Meyer, A, Edwards, SV, et al. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc Natl Acad Sci USA 1989; 86:6196–6200.

Lent, H, Wygodzinsky, P. Revision of the Triatominae (Hemiptera, Reduviidae) and their significance as vectors of Chagas' disease. Bull Am Mus Nat Hist 1979; 163:123–520.

Lord, WD, DiZinno, JA, Wilson, MR, Budowle, B, et al. Isolation, amplification, and sequencing of human mitochondrial DNA obtained from human crab louse, *Pthirus pubis* (L.), blood meals. J Forensic Sci 1998; 43:1097–1100.

Mukabana, WR, Takken, W, Knols, BG. Analysis of arthropod bloodmeals using molecular genetic markers. Trends Parasitol 2002; 18:505–509.

Quintal, RE, Polanco, GG. Feeding preferences of *Triatoma dimidiata maculipennis* in Yucatan, Mexico. Am J Trop Med Hyg 1977; 26:176–178.

Ramsey, JM, Ordonez, R, Cruz-Celis, A, Alvear, AL, et al. Distribution of domestic triatominae and stratification of Chagas disease transmission in Oaxaca, Mexico. Med Vet Entomol 2000; 14:19–30.

Schofield, CJ. *Triatominae: Biologia y Control.* West Sussex: Eurocommunica Publications; 1994.

Steuber, S, Abdel-Rady, A, Clausen, PH. PCR-RFLP analysis: a promising technique for host species identification of blood meals from tsetse flies (Diptera: Glossinidae). Parasitol Res 2005; 97:247–254.

Sturm, NR, Degrave, W, Morel, C, Simpson, L. Sensitive detection and schizodeme classification of *Trypanosoma cruzi* cells by amplification of kinetoplast minicircle DNA sequences: use in diagnosis of Chagas' disease. Mol Biochem Parasitol 1989; 33:205–214.

Telleria, J., Lafay, B., Virreira, M., Barnabé, C., et al. *Trypanosoma cruzi*: sequence analysis of the variable region of kinetoplast minicircles. Exp Parasitol 2006; 114:279–288.

WHO. *Control of Chagas Disease*. 2nd Ed. Technical Report Series 905. Geneva: WHO; 2002.

Wisnivesky-Colli, C, Gurtler, RE, Solarz, N, Salomon, D, et al. Feeding patterns of *Triatoma infestans* (Hemiptera: Reduviidae) in relation to transmission of American trypanosomiasis in Argentina. J Med Entomol 1982; 19:645–654.

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