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Molecular Analysis of *Trypanosoma cruzi* Isolates Obtained from Raccoons (*Procyon lotor*) in Warren and Barren Counties of Kentucky

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MOLECULAR ANALYSIS OF *Trypanosoma cruzi* ISOLATES OBTAINED FROM RACCOONS (*Procyon lotor*) IN WARREN AND BARREN COUNTIES OF KENTUCKY

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Trypanosoma cruzi, the etiologic agent of Chagas disease, infects a variety of wild mammals in the southern United States, but it has only recently been isolated from raccoons trapped in the state of Kentucky. The purpose of the present study was to use a molecular genotyping approach, followed by DNA sequencing to determine the genotypes (type I, or types IIa-IIe) of 15 of the Kentucky isolates. DNA samples were prepared from 15 *T. cruzi*- isolates using a Qiagen mini kit, and PCR amplification was performed using published primers for the 24S α rDNA sequence (D71 and D72), the non-transcribed spacer of the mini-exon genes (TC, TC1, and TC2), the 18S rDNA sequence (V1 and V2), and TCZ1 and TCZ2 primers that amplify a 188-base pair segment of the repetitive 195-bp nuclear DNA sequence of *T. cruzi*. DNA sequencing (ABI 3130 Genetic Analyzer) was performed on all amplification products obtained from the PCR analysis of the RW2 and RB12 isolates (randomly selected to represent both Warren and Barren counties of Kentucky; the number started with an "R" which stood for raccoon, a "W" for Warren County or a "B" for Barren County, followed by a number which represented the order in which animal was trapped). The resulting sequences were edited before analysis using the BLAST database of the National Center for

Biotechnology Information (NCBI) Genbank. All 15 isolates were positively confirmed as *T. cruzi* based upon PCR amplification of a 195 bp repetitive genomic DNA sequence, and all 15 isolates showed identical PCR amplification results with all 4 sets of *T. cruzi*-specific primers. Two positive PCR samples were randomly selected for further DNA sequence analysis, and all samples were positively identified as the type IIa genotype of *T. cruzi* with max identities ranging from 94%-99%. The results of this study confirm that all hemoculture isolates obtained from raccoons trapped in Warren and Barren counties of Kentucky are *T. cruzi.* Furthermore, all BLAST comparisons of amplicon DNA sequences showed high sequence identity to type IIa strains of *T. cruzi*. The type IIa strain of *T. cruzi* is the most commonly reported genotype from raccoons trapped in the U.S.A.

INTRODUCTION

Discovery of Chagas disease

Carlos Chagas, a Brazilian physician and scientist who worked at the Oswaldo Cruz Institute in Rio de Janeiro, discovered the protozoan parasite known as *Trypanosoma cruzi* (Chagas, 1909). At that time, Brazilians were trying to develop the Rio de Janeiro valley, however, malaria was prevalent and was an impediment to railroad construction. Chagas was working on malaria treatment, but he noted that some of his patients displayed unusual symptoms that were not associated with any of the known diseases of the time. These symptoms, including ocular afflictions, glandular enlargement, and edema, are now known to be typical of the disease that came to be known as Chagas disease, or American trypanosomiasis. Chagas investigated the living environment of the patients, and found that blood-sucking bugs (Hemiptera, family Reduviidae, subfamily Triatominae) commonly inhabited the sod walls and thatched roofs of rural homes in Latin America. He examined the Triatomine bugs and found large numbers of flagellated protozoa in their digestive tracts. Chagas allowed infected bugs to feed on marmosets and guinea pigs, and observed that the same flagellated parasites appeared in the blood of the animals within one month. He then investigated the prevalence of the parasite in blood samples obtained from humans and domestic animals. Chagas discovered the parasite in the blood of a domestic cat and in a one-year-old child who also exhibited disease symptoms. By 1916 Chagas described the complete life cycle of the parasite now known as *Trypanosoma cruzi* (Chagas, 1911, 1920, Prata, 1999).

Chagas disease

Chagas disease causes approximately 13,000 deaths per year (WHO, 2002), and more than 28 million people are believed to currently be at risk of infection (Dias et al., 2008). In addition, the World Health Organization has estimated that Chagas disease is responsible for a burden of nearly 670,000 disability adjusted life years (WHO, 2002). The disease is endemic across the vast majority of Latin America, with the exception of the Caribbean nations (Schofield et al., 2006). Chagas disease occurs in three stages: an acute phase, a latent phase, and a chronic phase. The acute phase occurs 4 to 8 weeks after the infection. During the acute phase, the symptoms are generalized (fever, malaise, swollen lymph nodes) and the disease can be difficult to diagnose. The latent (or intermediate) phase can last 10 to 30 years. Patients exhibit few symptoms during the latent period, but remain seropositive. The chronic manifestations of Chagas disease occur in approximately 10-30% of patients (Kirchhoff and Pearson, 2007). Cardiac abnormalities are most common in the chronic phase, and include conduction system abnormalities, ventricular arrhythmias, and congestive heart failure. Chronic gastrointestinal problems may also occur due to megaesophagus or megacolon (Kirchhoff and Pearson, 2007).

Trypanosoma cruzi

Trypanosoma cruzi is a flagellated protozoan parasite in the order Kinetplastida, family, Trypanosomatidae. *Trypanosoma cruzi* displays several developmental stages in its complex life cycle, which occurs in vertebrate and invertebrate hosts. The parasite shows three major stages: amastigote, epimastigote, and trypomastigote (De Souza, 2002).

Rounded amastigotes are the intracellular dividing stages of the parasite found in the tissues of the vertebrate host. Within the cytoplasm of host cells, amastigote stages transform into trypomastigotes, or at the beginning of a new intracellular cycle, trypomastigotes transform into amastigotes. Trypomastigotes are approximately 25 μm long and 2 μm in diameter. The trypomastigote forms are released from infected cells and then enter the blood stream, or re-infect other cells. Moreover, trypomastigotes exist in the liquid phase of mammalian cell cultures and at the stationary phase of growth in axenic cultures. The trypomastigote stage is the infective stage of the parasite for the insect host, and is acquired when the insect takes a blood meal from an infected vertebrate host. Metacyclic trypomastigote stages also accumulate in the rectum of the insect host and are released in the feces of the bug. The metacyclic trypomastigotes become the infective stage for the mammalian host when they cross the mucous membranes of the eyes, nose, or mouth, or enter the wound site created by the bug. Finally, the spindle-shaped epimastigote stages are approximately 20-40 μm long. They are found in the intestine of the invertebrate host, where they undergo rapid division. Epimastigote stages are also observed at the logarithimic phase of growth in axenic cultures (De Souza, 2002).

Cell Biology of *Trypanosoma cruzi*

The cell surface of *Trypanosoma cruzi* is composed of two components, the plasma membrane and an underlying layer of subpellicular microtubules (De Souza, 2002). There is also a single flagellum that is used for general movement of the cell and attachment of the parasites to the surface of vertebrate host cells and to the perimicrovillar membranes that line the intestine of the invertebrate host (Hill, 2003; De

Souza, 1999). The flagellum emerges from an invagination of the plasma membrane known as the flagellar pocket (De Souza, 2002). The flagellar pocket is located posterior to the nucleus in the trypomastigote, however, it is positioned anterior to the nucleus in the epimastigote stage (McConville et al., 2002). As a member of the Trypanosomatidae family, *T. cruzi* possesses only one mitochondrion that extends throughout the cell body (De Souza, 2002). Within the mitochondrial matrix, situated close to the basal body, there is a specialized structure known as the kinetoplast. The kinetoplast contains mini-circle and maxi-circle DNA, and the kinetoplast DNA (K-DNA) represents approximately 30% of the total DNA of *T. cruzi* (De Souza, 2002; 2008). The kinetoplast contains circular DNA, histone-H1-like protein and other basic proteins (Souto-Padron & De Souza 1978, 1979; De Souza et al., 2008). The kinetoplast is a diagnostic characteristic of protozoa in the order, Kinetoplastida (De Souza, 2002). Kinetoplast DNA consists of two types of circular DNA (minicircles and maxicircles). According to Rious and Delain (1969), there are 20,000-30,000 minicircles in the kinetoplast, and each of the minicircles has a length of approximately 0.45μm, corresponding to about 1440 base pairs. In contrast, there are only a few dozen maxicircles, and they range in size from 20-40kb (De Souza, 2009). Maxicircles appear to be similar to the mitochondrial DNA of higher eukaryotes. Maxicircle DNA encodes rRNAs and genes related to the respiratory pathway such as cytochrome-c-oxidase subunit II and III and ATPase subunit 6. However, maxicircle coding DNA sequences contain many frameshifts, and guide RNAs (encoded by the minicircle DNA) must edit RNA transcripts in order for translation to occur (Stuart et al., 1997; De Souza, 2009). This unique molecular process has been termed RNA editing. The morphology of the kinetoplast varies depending upon the stage of the parasite. The

K-DNA is highly condensed and organized (presenting a rod-like shape) in the amastigote and epimastigote stages. The kinetoplast is situated anterior to the nucleus in the epimastigote stage. In the trypomastigote stage, K-DNA is more loosely organized and is situated posterior to the nucleus (De Souza, 1999; 2002). It has been suggested by some investigators that K-DNA is capable of integrating into the host cell DNA of infected vertebrate animals (Teixeira et al., 2006).

The structure of the nucleus of *T. cruzi* is analogous to that of other eukaryotic cells. It is approximately 2.5μm in diameter and consists of 43-50Mb of DNA (De Souza, 2002). Interestingly, the chromosomes do not visibly condense during any stages of the life cycle (De Souza, 2002). The endoplasmic reticulum is visible throughout the cell, and Golgi complexes are positioned at the anterior end of the cell (De Souza, 2002).

Hosts and Life Cycle

The insect vectors of *Trypanosoma cruzi* are blood sucking triatomine bugs in the family Reduviidae (Schofield et al., 1999). They are hemimetabolous and have a life span that ranges from 3 months to 2 years. Triatomines pass 5 instars from birth to adult and the molting process depends upon when they take a blood meal (Stern & Emlen, 1999). The vectors for *T. cruzi* are located in North America, Central America, and South America. The bugs tend to bite people as they sleep during the night (Harder, 2004).

Triatomine bugs ingest trypomastigote stages when taking a blood meal from an infected vertebrate host (see Figure 1). The trypomastigotes transform into epimastigotes and some rounded forms in the midgut of the insect. Then epimastigotes replicate by binary fission in the midgut, and a subpopulation of the dividing epimastigotes transform

into metacyclic trypomastigotes in the hindgut. The infective metacyclic trypomastigote stages are then passed in the feces, frequently during a blood meal. The metacyclic trypomastigotes then enter the bite wound or cross the mucous membranes of the eyes, nose or mouth (De Souza, 2002).

When metacyclic trypomastigotes enter the vertebrate host body, they must quickly enter host cells in order to complete their intracellular cycle of replication. Inside the host cells, they transform into amastigotes and undergo multiple rounds of binary fission. The amastigotes (1 µm in length) then transform into trypomastigotes (20 µm in length) and burst out of the cells. Some trypomastigotes infect nearby tissue cells and others disperse to other tissues and organs by entering the bloodstream. The invertebrate host ingests blood-form trypomastigote stages during a blood meal, completing the parasite's life cycle (De Souza, 2002; CDC, 2010).

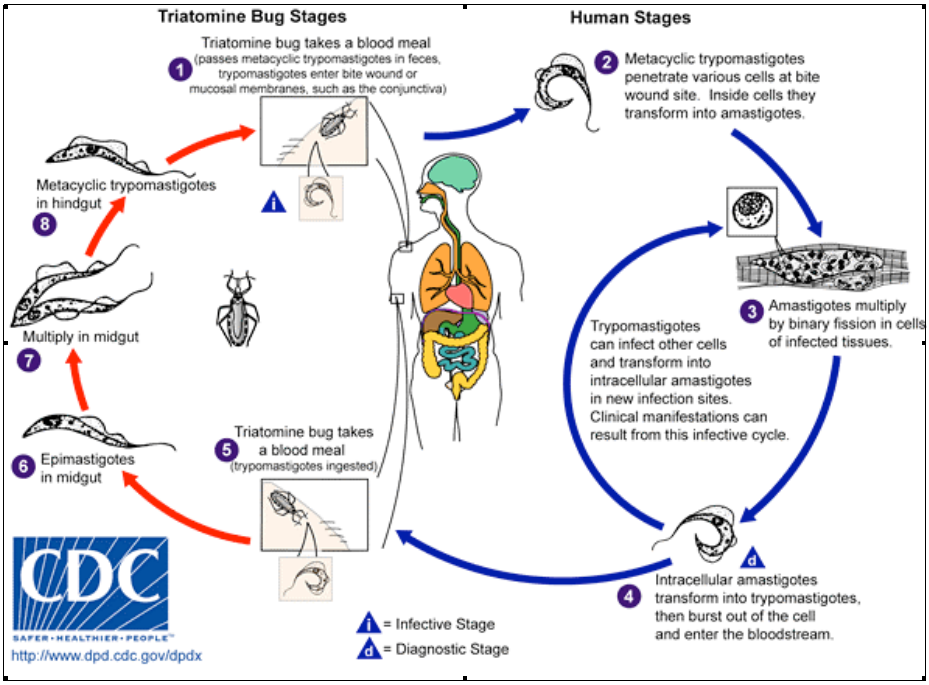

Figure 1. Life cycle of *Trypanosoma cruzi* (CDC, 2010).

Vector facilitated transmission is thought to be the most common mode of transmission of *T. cruzi*, however, blood transfusions, organ transplantation, laboratory accidents, and accidental ingestion of triatomine bugs are all documented routes of infection (WHO, 2002; Woodall, 2007).

Sylvatic cycle of *T. cruzi*

The sylvatic cycle of *T. cruzi* has been reported in many states in the U.S.A., including Alabama, California, Florida, Georgia, Louisiana, Maryland, Oklahoma, North Carolina, South Carolina, Tennessee, Texas and Virginia (McKeever et al., 1958; Olsen et al., 1964; John and Hoppe, 1986; Karsten et al., 1992; Yabsley & Noblet, 2002; Dorn et al, 2007; Hancock et al, 2005). Most recently, the sylvatic cyle of *T. cruzi* was documented in raccoons and opossums from the state of Kentucky (Groce, 2008). *T. cruzi* infection occurs in a wide variety of mammals in the U.S.A. including raccoons *(Procyon lotor*), opossums (*Didelphis virginiana*), gray foxes (*Urocyon cineroargenteus*), striped skunks (*Mephitis mephitis*), macaques (*Macaca silenus*), lemurs (*Lemur catta*), woodrats (*Neatoma magister*), armadillos (*Dasypus novemcincthus*), bats (*Eptiscus fuscus*), moles (*Neurotrichus gibbsii*), and dogs (*Canis familiaris*) (Yabsley et al, 2001; James et al, 2002; Hall et al, 2007). Despite the widespread occurrence of *T. cruzi* in mammals, especially in the southern region of the U.S.A., only 6 cases of autochthonous transmission to humans have been reported (Bern et al., 2007).

Genotypes of *T. cruzi*

T. cruzi has been divided into two highly divergent genotypic groups, designated type I and type II. These strain designations are based upon the results of isoenzyme analysis, riboprinting analysis (restriction fragment length polymorphism analysis of

polymerase chain reaction amplified ribosomal DNA), RNA promoter activity, and analysis of mini-exon gene sequences and microsatellite markers (Tibayrenc, 1995; Souto et al., 1996). Intriguingly, the infectivity of *T. cruzi* strains has been reported to be dependent upon the host from which the strains are isolated, rather than the genotypes of the parasite strains (Bértoli et al., 2006). Strains of *T. cruzi* isolated from sylvatic hosts tend to show significantly higher infectivity in mice than strains isolated from humans and triatomine intermediate hosts (Bértoli et al., 2006).

Kinetoplast DNA has been a frequent target for PCR-based detection and diagnosis of *T. cruzi*, due to its unique molecular characteristics. The PCR amplification of conserved regions of mini-circle K-DNA has been shown by a number of investigators to be a more efficient and sensitive method to detect *T. cruzi* than microscopic analysis (Shikanai et al., 1996; Avila et al., 1993; Dorn, 1997; Kirchhoff et al., 1996). In addition, the primers designated TCZ1 and TCZ2, which are specific for an 188bp segment of a repetitive 195bp nuclear DNA sequence, have been shown to be highly sensitive and specific for the detection of *T. cruzi* (Lanar et al., 1981; Moser et al., 1989). PCR amplification with TCZ1 and TCZ2 allows for the detection of *T.cruzi* isolates from widely separated geographic regions, but importantly, does not detect mice or human DNA, or the DNA of closely related species such as *Leishmania* spp. or African trypanosomes (Moser et al., 1989).

To better understand the complexity of pathogenic behavior, genetic diversity, and epidemiology of *T. cruzi*, many investigators have attempted to devise a straightforward scheme for genotyping parasite strains. Miles and coworkers (1978) analyzed a variety of isolates of *T cruzi* using multilocus enzyme electrophoresis (MLEE)

and described the presence of three zymodemes (Z1, Z2, Z3). The Z1 and Z3 zymodemes consisted of parasites in the sylvatic cycle and a few isolates from human acute cases. The Z2 zymodeme consisted only of isolates from domestic cycles. Romanha (1982) and Carneiro and coworkers (1990) characterized *T. cruzi* strains isolated from chronic chagasic patients, and described four zymodemes (designated ZA, ZB, ZC and ZD). The zymodeme ZA was shown to be the same as the previously described zymodeme, Z2. Thus, a total of six different *T.cruzi* isoenzyme groups (Z1, Z2 or ZA, Z3, ZB, ZC and ZD) were designated.

A variety of investigators proposed independent and unrelated genotyping schemes subsequent to the study of Carneiro and co-workers in 1990. However, Tibayrenc (1995) proposed a widely accepted genotyping system that divided *T. cruzi* into two primary phylogenetic lineages (type I and type II) based upon MLEE and random amplified polymorphic DNA analyses (RAPD). Similar conclusions were also drawn by other investigators based upon their analysis of the 24Sα rRNA genes and the non-transcribed spacer of mini-exon genes (Souto et al., 1996), the promoter region of rRNA and mini-exon genes (Nunes et al., 1997), and the 195bp repeated satellite DNA sequence of *T. cruzi* (Bastrenta et al., 1999). After MLEE and RAPD analysis of a larger set of *T. cruzi* isolates, Brisse and coworkers (2000) also found two primary lineages (type I and type II) with type II further subdivided into five lower subdivisions that were designated IIa, IIb, IIc, IId and IIe. The rRNA and mini-exon sequences of *T. cruzi* have been widely used in epidemiological and evolutionary studies, however, the correspondence between MLEE and RAPD classification and rRNA and mini-exon PCR analyses has not yet been determined.

Brisse and co-workers (2001) confirmed the existence of six type II lineages based upon a molecular typing scheme employing PCR analysis of a 24Sα rRNA gene sequence, an 18S rRNA gene sequence, and a non-transcribed spacer sequence of a mini-exon gene from *T. cruzi*. Recently, Roellig and co-workers (2008) used Brisse's molecular typing approach to genotype over 100 isolates of *T. cruzi* from the U.S.A. Only type I and type IIa strains of *T. cruzi* were observed. The type I strain was the genotype that occurred most often in opossums, and to date, it is the only genotype that has been isolated from autochthonous human infections in the U.S.A. The type IIa genotype is the most common genotype that has been reported from domestic dogs, raccoons, ring-tailed lemurs, and skunks in the U.S.A. Both type I and type IIa genotypes of *T. cruzi* have been isolated from triatomine bugs collected in the U.S.A. (Roellig et al., 2008).

Previous investigations from our laboratory (Groce, 2008) documented the existence of the sylvatic cycle of *T. cruzi* in Kentucky (Groce, 2008). A total of 17 hemoculture isolates of *T. cruzi* were obtained from raccoon blood samples collected from Warren and Barren counties of Kentucky during the study. The purpose of the present study was to use the molecular typing approach described by Brisse (2001) and Roellig et al. (2008) followed by DNA sequencing and BLAST analysis to determine the genotypes (type I, or types IIa-IIe) of 15 of the 17 raccoon isolates obtained from south-central Kentucky.

MATERIALS AND METHODS

Parasite Cultures

Hemocultures of *T. cruzi* were initially established by inoculating one ml of whole blood from each raccoon into sterile liver infusion tryptose (LIT) medium containing 10% newborn calf serum (heat inactivated at 56°C for 30 minutes), penicillin G (100U/ml) and streptomycin (100µg/mL; complete LIT; Groce, 2008). Flasks (T-25) were incubated at 28° C for up to three months and were checked weekly under an inverted light microscope at 200X magnification (Groce, 2008). Positive cultures were expanded into 75cm^2 tissue culture flasks, and culture forms in the log phase of growth were frozen in cryovials at -70C in a sterile freezing solution containing 10% DMSO and 90% newborn calf serum (v/v). Fifteen of the frozen stocks were thawed and inoculated immediately into complete LIT medium and maintained at 28ºC to establish log phase cultures for use in the present study.

CV-1 Cell Cultures

African green monkey kidney cells (CV-1) were grown under sterile conditions in RPMI-1640 medium supplemented with 10% newborn calf serum, penicillin G (100U/ml) and streptomycin (100µg/mL; complete RPMI). Cells were maintained in 75 cm^2 culture flasks and were incubated at 37°C and 5% CO_2 under high humidity.

Infection of CV-1 Cells with *Trypanosoma cruzi* isolates

Four raccoon isolates of *T. cruzi*, two from Barren County and two from Warren County (RB14, RB12, RW15, and RW10) were randomly selected for CV-1 cell infectivity assays. A 5ml volume of LIT culture medium containing live epimastigote stage of *T. cruzi* in exponential growth phase was subjected to centrifugation at 1000 xg

for 10 min. After centrifugation, the supernatant was discarded and the pellet was resuspended in 1ml of complete RPMI medium. A 0.5mL aliquot of each parasite suspension was inoculated into two separate T-75 flasks containing confluent CV-1 cells. Cells were cultured at 37°C in a 5% CO_2 incubator under high humidity, and supernatants were removed and replaced with fresh complete RPMI medium after 48 hr. Flasks were examined daily for evidence of motile trypomastigote stages under an inverted light microscope at 200X magnification.

DNA Isolation

Genomic DNA was extracted from each of the 15 culture isolates while they were in the logarithmic phase of growth in complete LIT culture medium. A 10ml volume of LIT culture medium containing live epimastigote stages of *T. cruzi* was subjected to centrifugation at 1000 xg for 15 min. After centrifugation, the supernatant was discarded and the pellet was resuspended in 200μl PBS. DNA was then isolated from each parasite isolate using a QIAamp DNA mini kit (Qiagen, Valencia, CA) according to manufacturer's instruction. DNA yield was determined using a Thermo Nanodrop ND-100 spectrophotometer. DNA samples were stored at -20 °C until use.

***Trypanosoma cruzi* primers for PCR**

Four different primer sets were used to amplify DNA samples. The first primer set was designed to amplify 188bp of a 195-bp repetitive nuclear sequence of *T. cruzi* (Moser et al., 1989) and consisted of the following primer sequences:

TCZ1 (5'-CGA GCT CTT GCC CAC ACG GGT GCT 3')

TCZ2 (5'-CCT CCA AGC AGC GGA TAG TTC AGG 3')

The second primer set was designed to target the D7 divergent domain of the 24Sα rDNA of *T. cruzi* (Brisse, 2001) and consisted of the following sequences:

D71 (5'-AAG GTG CGT CGA CAG TGT GG 3')

D72 (5'-TTT TCA GAA TGG CCG AAC AGT 3')

The third primer set was designed to amplify the non-transcribed spacer of the mini-exon genes of *T. cruzi* (Brisse, 2001). All three of the following primer sequences were used for PCR amplification:

TC (5'-CCC CCC TCC CAG GCC ACA CTG 3')

TC1 (5'-GTG TCC GCC ACC TCC TTC GGG CC 3')

TC2 (5'-CCT GCA GGC ACA CGT GTG TGT G 3')

The fourth primer set was designed to amplify the size-variable domain of the 18S rDNA sequence of *T. cruzi* (Brisse et al., 2001) and consisted of the following primer sequences:

V1 (5'-CAA GCG GCT GGG TGG TTA TTC CA 3')

V2 (5'-TTG AGG GAA GGC ATG ACA CAT GT 3')

PCR Setup

DNA samples were analyzed by PCR for the presence of *T. cruzi* DNA using each of the primer sets described above. For each analysis, a 50μl reaction was set up containing 20μl 2.5X Master Mix (5 Prime, Gaithersburg, MD) (including 0.06 U/μl *Taq* DNA polymerase, 2.5x *Taq* reaction buffer with 125 mM KCl, 75 Tris-HCl pH 8.4, 4 mM Mg^{2+}, 0.25% Nondet-P40, 500 μM each of dNTP, stabilizers), 1 μl (4 μM) forward; 1 μl (4 μM) reverse primer ; 50 ng template DNA; and sterile nanopure water to bring the total volume to 50 μl. A negative control containing sterile nanopure water in place of template DNA was included with each reaction set. Two positive controls using template

DNA prepared from *T. cruzi* type I and type IIa reference strains from Dr. Yabsley lab were also included with each reaction set. Reaction mixtures were loaded into an automated DNA thermal cycler to undergo amplification according to the protocols outlined in Tables 1-4.

Table 1. Thermocycler program for PCR amplification using the TCZ1 and TCZ2 primers.

	Temperature (ºC)	Time	# of cycles
Denature	94	2 min	1
Denature	94	1 min	30
Annealing	64.5	30 sec	
Extension	72	15 sec	
Final extension	72	5 min	1
Hold	4	Indefinite	1

PCR products were subjected to electrophoresis at 80V for 2 hr in 3% agarose gels with 0.5 X TBE buffer (adapted from Moser, 1989).

Table 2. Thermocycler program for PCR amplification using D71 and D72 primers.

	Temperature (°C)	Time	# of cycles
Denature	94	2 min	1
Denature	94	1 min	30
Annealing	55	1 min	
Extension	72	1 min	
Final extension	72	5 min	1
Hold	4	Indefinite	1

PCR products were subjected to electrophoresis at 80V for 2 hr in 3% agarose gels with 0.5 X TBE buffer (adapted from Brisse et al., 2001).

Table 3. Thermocycler program for PCR amplification using the TC series primers.

	Temperature (°C)	Time	# of cycles
Denature	94	2 min	1
Denature	94	1 min	30
Annealing	55	1 min	
Extension	72	1 min	
Final extension	72	5 min	1
Hold	4	Indefinite	1

PCR products were subjected to electrophoresis at 90V for 90 min in 1.5% agarose gels with 0.5 X TBE buffer (adapted from Brisse et al., 2001).

Table 4. Thermocycler program for PCR amplification using the V1 and V2 primers.

	Temperature (ºC)	Time	# of cycles
Denature	94	2 min	1
Denature	94	1 min	30
Annealing	50	1 min	
Extension	72	1 min	
Final extension	72	5 min	1
Hold	4	Indefinite	1

PCR products were analyzed by electrophoresis at 80V for 2 hr in 3% agarose gels with 0.5 X TBE buffer (adapted from Brisse et al., 2001).

In addition, to allow direct comparison of amplification products from control *T. cruzi* type I and type IIa DNA samples to representative isolates from Warren and Barren counties (RW2 and RB12), PCR products were analyzed side by side in the same gel by electrophoresis at 80V for 3h in 3% agarose gels with 0.5 X TBE buffer. Following electrophoresis, agarose gels were stained with ethidium bromide and visualized under ultraviolet light.

DNA sequencing of PCR amplicons

DNA sequencing was performed on all amplification products obtained from the PCR analysis of the RW2 and RB12 isolates (randomly selected to represent both Warren and Barren counties of Kentucky). Each PCR sample was purified using an Ultraclean PCR purification kit (MOBIO). A 10 μl Big Dye Terminator v3.1 (Applied Biosystems

Inc., Foster City, CA) reaction was set up as follows: 2 μl 5X sequencing buffer, 2 μl ready reaction mix, 5 pmol of forward or reverse primer, 30 ng DNA template, and sterile nanopure water to bring the total volume to 10 μl. The sequencing reaction conditions are shown in Table 5 below.

Table 5. DNA sequence reaction conditions.

	Temperature (°C)	Time	# of cycles
Initial Denature	94	2 min	1
Denature	94	1 min	25
Annealing	55	1 min	
Extension	72	1 min	
Hold	4	Indefinite	1

Each sequencing reaction product was purified using a DyeEx 2.0 Spin kit (Qiagen), dried in a speedvac for15 min with no heat, and rehydrated with 20 μl of HiDi formamide buffer. Samples were then loaded onto an ABI 3130 Genetic Analyzer for sequencing. The sequences were edited before searching the BLAST database of the National Center for Biotechnology Information (NCBI) Genbank for sequence matches.

RESULTS

Infection of CV-1 Cells with *Trypanosoma cruzi* isolates

T. cruzi is an obligate intracellular parasite that must invade mammalian host cells in order to reproduce and complete its life cycle. Four raccoon isolates of *T. cruzi*, two from Barren County and two from Warren County (RB14, RB12, RW5, RW10), were randomly selected from 15 isolates for testing in a CV-1 cell infectivity assay. All four isolates were highly infective for the CV-1 cells. The length of incubation time, between the inoculation of the cultures and the first appearance of trypomastigote stages in culture supernatants, ranged from four to five weeks. Figure 2 shows the results of a representative assay in which CV-1 cells were heavily infected by the RB14 isolate of *T. cruzi*.

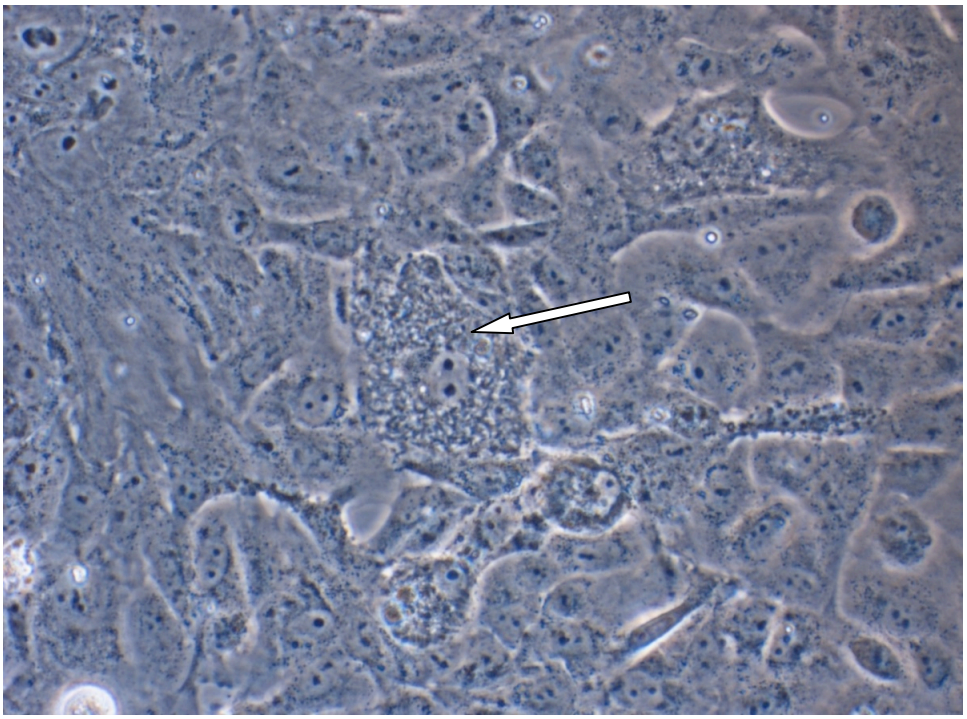

Figure 2. CV-1 cell monolayer heavily infected by the RB14 raccoon isolate of *T. cruzi*. The arrow indicates a CV-1 cell packed with trypomastigote stages that are ready to burst out of the cell.

DNA Isolation and PCR

Genomic DNA was successfully isolated from all 15 *T. cruzi* isolates using a QIAamp DNA mini kit (Qiagen). DNA yields ranged from 17.2- 82.9 ng/µl. Four different *T. cruzi*-specific primer sets were then used to amplify each of the 15 genomic DNA samples. The first primer set, TCZ1 and TCZ2, was used to first verify that the isolates were *T. cruzi,* and not some other species of trypanosome. The TCZ primers, which amplify a 188-bp segment of a 195-bp repetitive nuclear sequence, have been shown to be highly sensitive and specific for *T. cruzi* (Moser et al., 1989). Optimization of the PCR reaction with the TCZ primer set and control *T. cruzi* genomic DNA resulted in a PCR product of 188bp. The 188 bp product was specifically amplified in all 15 of the raccoon isolates, confirming the presence of the *T. cruzi* parasite (see Figure 3). An unexpected low intensity amplicon of approximately 400 bp also showed in lanes 1-15. The 400bp band is not a contaminant since it is not present in the negative control lane, nor is it present in subsequent gels showing the results of PCR amplification with the three additional *T. cruzi*-specific primer sets.

All 15 raccoon isolates showed identical PCR amplification results with the other three sets of *T. cruzi*-specific primers (see Figures 4-6). PCR amplification of the D7 divergent domain of the 24Sa rDNA sequence of *T. cruzi* with the D71 and D72 primer pair resulted in an amplicon of 130 bp (see Figure 4, lanes 1-15). Amplification of the non-transcribed spacer of the mini-exon genes of *T. cruzi* (using TC1, TC2, and TC3 primers) resulted in a PCR product of 400 bp (see Figure 5, lanes 1-15). PCR amplification of the size-variable domain of the 18S rDNA sequence of *T. cruzi* with the

V1 and V2 primer pair resulted in a PCR product of 155bp in all 15 isolates (see lanes 1-15, Figure 6).

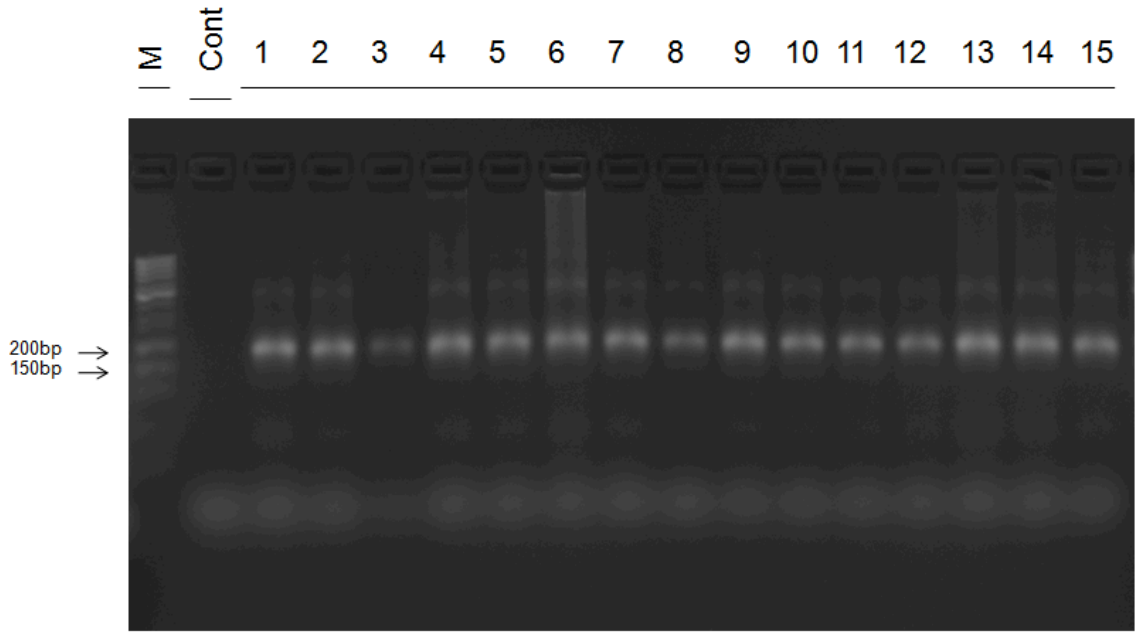

Figure 3. PCR products obtained after amplification of a 188bp fragment from a 195bp repetitive nuclear sequence from *T. cruzi* with primers TCZ1 and TCZ2 followed by agarose gel electrophoresis (3% gel). M indicates the 50 bp DNA ladder (exACTGene, Fisher Scientific, Pittsburg, PA) lane. Cont indicates the no template control lane. Lanes 1-15 show the PCR products amplified from genomic DNA from each of the 15 isolates.

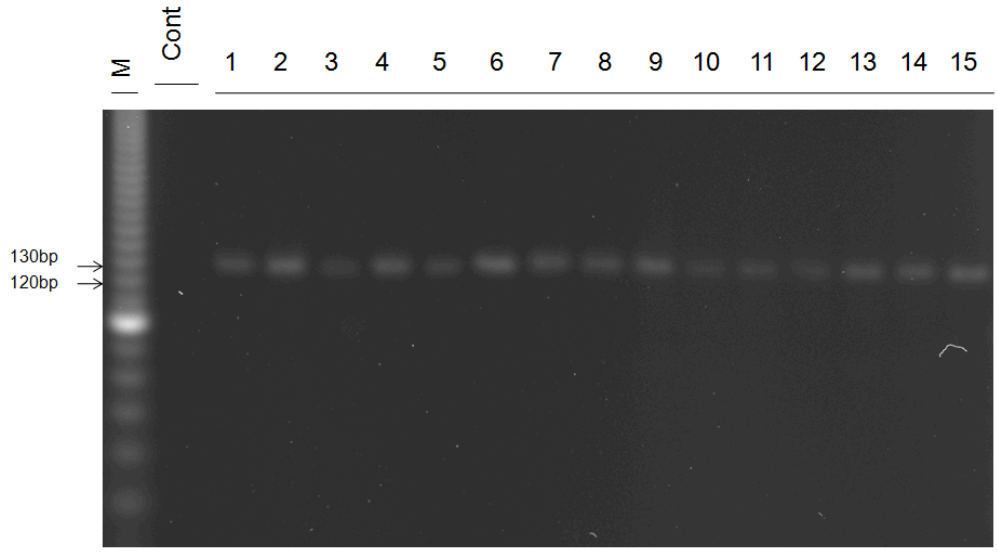

Figure 4. Agarose gel electrophoresis (3%) of PCR products (130bp) amplified using D71 and D72 primers, which recognize the D7 domain of the 24Sα rDNA of *T. cruzi*. M indicates the 10 bp DNA ladder (Invitrogen Corporation, Carlsbad, CA) lane. Cont indicates the no template control lane. Lanes 1-15 show the PCR products amplified from genomic DNA from each of the 15 isolates.

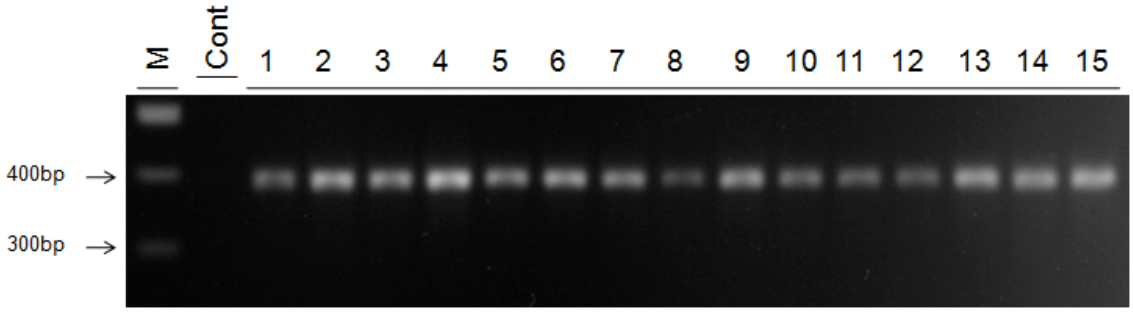

Figure 5. Agarose gel electrophoresis (1.5%) of PCR products (400bp) amplified using TC, TC1, and TC2 primers that recognize a sequence from the non-transcribed spacer of the mini-exon gene of *T. cruzi*. M indicates 100 bp DNA ladder (New England Biolabs, Ipswich, MA) lane. Cont indicates the no template control lane. Lanes 1-15 show the PCR products amplified from genomic DNA from each of the 15 isolates.

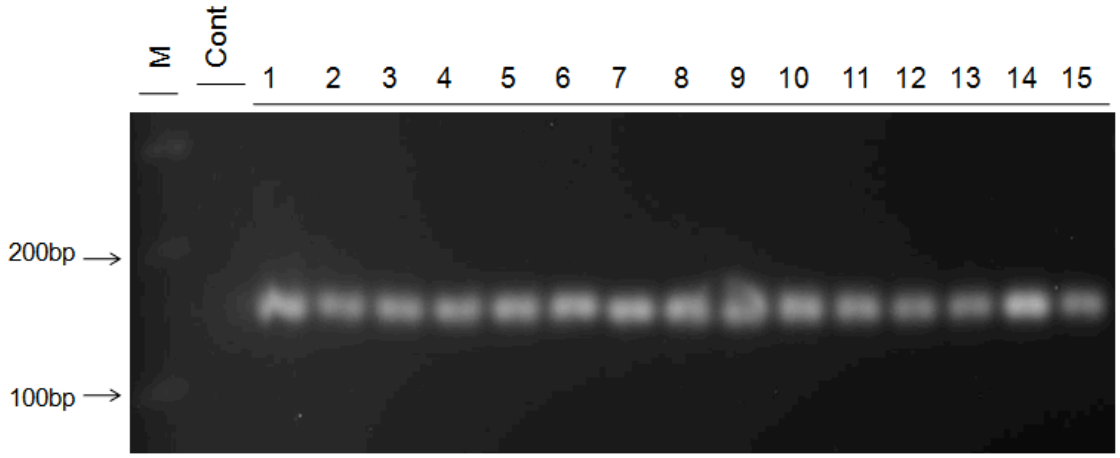

Figure 6. Agarose gel electrophoresis (3%) of PCR products (155bp) amplified using V1 and V2 primers that recognize a sequence from the 18S rDNA gene of *T. cruzi*. M indicates 100 bp DNA ladder (New England Biolabs) lane. Cont indicates the no template control lane. Lanes 1-15 show the PCR products amplified from genomic DNA from each of the 15 isolates.

To allow a direct comparison of amplification products from control type I and type IIa *T. cruzi* genomic DNA samples to representative raccoon isolates from Warren and Barren counties (RW2 and RB12), PCR products were analyzed side by side in the same agarose gel. Figure 7 depicts the results of this electrophoretic analysis. With the D71/D72 primer set, PCR amplification of type I genomic DNA showed a 110 bp product, the type IIa genomic DNA sample showed a 130bp product and a 100 bp product, and all 15 raccoon isolates showed an amplicon size of 130bp. With the TC series primers, the type I genomic DNA sample showed a 350bp product, the type IIa sample showed no amplification, but all 15 raccoon isolates showed a prominent

amplicon of approximately 400 bp**.** With the TCZ1 and TCZ2 primer set, all DNA samples showed an amplicon of 188bp, indicating that they are all *T. cruzi.* The type I genomic DNA samples and all 15 isolates also showed a faint band of 400bp which was not evident in the control lane. With the V1/V2 primer set, the type I genomic DNA sample showed a 170bp product and the type IIa DNA sample and all 15 raccoon isolates showed a 155bp product.

DNA Sequencing Results

Positive PCR samples (RW2 and RB12) were randomly selected for sequencing analysis. All samples, from all 4 PCR analyses, showed highest identity to type IIa strains of *T. cruzi* (9212210r, Can III, and STC10R), with identities ranging from 94%-99%. Figures 8 through 11 show representative sequence alignments following BLAST analysis of positive PCR samples from both Warren and Barren Counties.

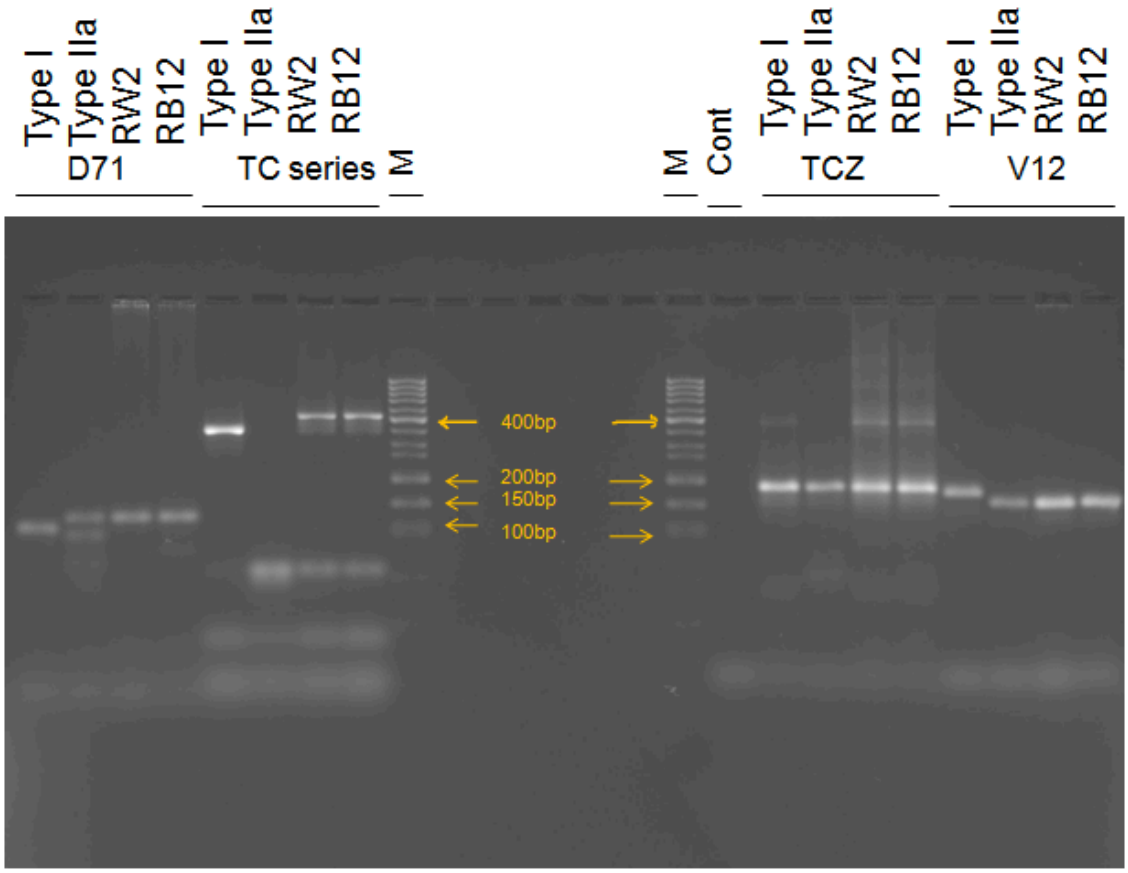

Figure 7. Agarose gel electrophoresis (3%) showing representative PCR products obtained from PCR amplification using all 4 sets of *T. cruzi* -specific primers (D71 and D712; TC series; TCZ1 and TCZ2; V1 and V2). PCR analyses were performed with one representative genomic DNA sample obtained from Warren County (RW2), one representative genomic DNA sample obtained from Barren County (RB12), and 2 positive control (type I and type II) *T. cruzi* genomic DNA samples. M indicates the 50 bp DNA ladder (Fisher Scientific) lane. Cont indicates the no template control lane.

```
   gb|EU178923.1|  Trypanosoma cruzi strain CanIII satellite sequence
Length=195

 Score =  242 bits (268),  Expect = 2e-61
 Identities = 137/139 (98%), Gaps = 0/139 (0%)
 Strand=Plus/Plus

Query  1    GGCTGCTGCATCACACGTTGTGGTCCAGATTTTTGTTGCGAATTGTGAATGGTGGGAGTC  60
            ||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  48   GGCTGCTGCATCACACGTTGTGGTCCAGATTTTTGTTGCGAATTATGAATGGTGGGAGTC  107

Query  61   AGAGGCACACTCTGTCACTACGTGTCTGCGTGTTCACACACTGGACACCAAACAACCCTG  120
            |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  108  AGAGGCACACTCTGTCACTACGTGTTTGCGTGTTCACACACTGGACACCAAACAACCCTG  167

Query  121  AACTATCCGCTGCTTGGAG  139
            ||||||||||||||||||
Sbjct  168  AACTATCCGCTGCTTGGAG  186
```

Figure 8. Representative sequence alignment for a TCZ PCR product amplified from RB12. Lowercase letters within the query sequence represent a low complexity sequence that was filtered by BLAST to prevent any artificial hits between sequences that were not truly related.

```
   gb|AY367114.1|  Trypanosoma cruzi 92122102r 24S-alpha ribosomal RNA gene, partial
sequence
Length=126

 Score = 80.6 bits (88),  Expect = 1e-12
 Identities = 53/56 (94%), Gaps = 2/56 (3%)
 Strand=Plus/Minus

Query  5    CAccccccccGTAA-GAGAAGGGAAAGAGAGCaaaaaaaaaaaaGAAACTCCCCC  59
            ||||||||||||||| |||||||||||||||| |||||||||||| |||||||||
Sbjct  73   CACCCCCCCCCCGTAAAGAGAAGGGAAAGAGAGC-AAAAAAAAAAAGAGACTCCCCC  19
```

Figure 9. Representative sequence alignment for a D71/D72 PCR product amplified from RB12. Lower case letters within the query sequence represent a low complexity sequence that was filtered by BLAST to prevent any artificial hits between sequences that were not truly related.

```
   gb|AY367124.1|  Trypanosoma cruzi 92122102r spliced leader mini-exon repeat region,
partial sequence
Length=711

 Score =  596 bits (660),  Expect = 3e-167
 Identities = 334/335 (99%), Gaps = 1/335 (0%)
 Strand=Plus/Minus

Query  1    CGTCACGGACGGCGTCGGGGGCCGGAATGGACGCAACCGGCGAGCGTGGAGCGGCAAATG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  518  CGTCACGGACGGCGTCGGGGGCCGGAATGGACGCAACCGGCGAGCGTGGAGCGGCAAATG  459

Query  61   CAGGCGAGCGCGTGCGCaaagcaaaaaagagggaaaaaacacaaaagcaccggaaaatgc  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  458  CAGGCGAGCGCGTGCGCAAAGCAAAAAAGAGGGAAAAAACACAAAAGCACCGGAAAATGC  399

Query  121  aggaaaagccgtaaaaCACGTGGAGGAGGCGGAGAGCAAAAGGAAACGAGACGGGGAAAA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  398  AGGAAAAGCCGTAAAACACGTGGAGGAGGCGGAGAGCAAAAGGAAACGAGACGGGGAAAA  339

Query  181  CACGAAACAACAACGGCGCAAACAAAGGC-AGCGGCGAAACAAAAATGAAACAGCTGGGG  239
            |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  338  CACGAAACAACAACGGCGCAAACAAAGGCAAGCGGCGAAACAAAAATGAAACAGCTGGGG  279

Query  240  GGTGGTGGAGGACACGGAACGTGGGGGGACAGTGCTGCACTGGAGGGGAAGCTGCAGGAC  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  278  GGTGGTGGAGGACACGGAACGTGGGGGGACAGTGCTGCACTGGAGGGGAAGCTGCAGGAC  219

Query  300  AGCCGCACGAGGGTGCCGGGCACACACACACGTGT  334
            ||||||||||||||||||||||||||||||||||
Sbjct  218  AGCCGCACGAGGGTGCCGGGCACACACACACGTGT  184
```

Figure 10. Representative sequence alignment for a TC series PCR product amplified from RW2. Lowercase letters within the query sequence represent a low complexity sequence that was filtered by BLAST to prevent any artificial hits between sequences that were not truly related.

```
   gb|FJ555630.1|  Trypanosoma cruzi isolate STC10R 18S ribosomal RNA gene, partial
sequence
Length=764

 Score =  192 bits (212),  Expect = 3e-46
 Identities = 108/109 (99%), Gaps = 0/109 (0%)
 Strand=Plus/Plus

Query  1    TTGTGTGGCACTCGTCGCCTTTGTGGGAAATCCGTGTGGCACTGTTTGTGTTGTTGGCGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  327  TTGTGTGGCACTCGTCGCCTTTGTGGGAAATCCGTGTGGCACTGTTTGTGTTGTTGGCGG  386

Query  61   ACTTCGGTCTTGCCTTCGCATATTTCACATGTGTCATGCCTTCCCTCAA  109
            ||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  387  ACTTCGGTCTTGCCTTCGCATATTTCGCATGTGTCATGCCTTCCCTCAA  435
```

Figure 11. Representative sequence alignment for a V12 PCR product amplified from RB12. Lowercase letters within the query sequence represent a low complexity sequence that was filtered by BLAST to prevent any artificial hits between sequences that were not truly related.

DISCUSSION

Trypanosoma cruzi, the etiologic agent of Chagas disease, has been found in triatomine bugs, wild mammals, and domestic mammals throughout the southern United States. Although confirmed reports of autochthonous human cases have been rare, sylvatic infection has been documented in Alabama, California, Florida, Georgia, Louisiana, Maryland, Oklahoma, North Carolina, South Carolina, Tennessee, Texas, Virginia, and Kentucky (McKeever et al., 1958; Olsen et al., 1964; John and Hoppe, 1986; Karsten et al., 1992; Yabsley and Noblet, 2002; Dorn et al, 2007; Hancock et al, 2005; Groce, 2008). In addition, the number of human cases in the United States, Canada, and Europe among immigrants or tourists from Latin American countries has increased steadily in recent years (CDC, 2007). In response to the growing risk to our blood supply (Leiby et al., 2008), the US Food and Drug Administration began recommending a screening assay for blood donors to US blood banks in January 2007. Despite public health concerns about the potential for vector borne transmission of *T. cruzi* to humans and domestic animals in the United States, a systematic study of *T. cruzi*-infection in wild mammals, domestic animals, and triatomine bugs in the United States has not been conducted.

Our research group documented the sylvatic cycle of *T. cruzi*-infection in Kentucky in 2008 (Groce, 2008). In this study, Groce trapped 44 raccoons in Warren and Barren counties of Kentucky between June 2007 and December 2008. A total of 17 isolates of *T. cruzi* were successfully established by the in vitro culture of whole blood in complete liver infusion tryptose (LIT) medium. The purpose of the present study was to use the molecular typing approach described by Brisse et al. (2001) and Roellig et al.

(2008) followed by DNA sequencing and BLAST analysis to determine the genotypes (type I, or types IIa-IIe) of 15 of these 17 raccoon isolates.

Many different strains of *T. cruzi* have been subjected to extensive genotypic analysis, and it is evident that it is a highly heterogeneous species. However, only type I and type IIa genotypes of *T. cruzi* have been reported from triatomine and mammalian hosts in the United States. Both type I and type IIa genotypes of *T. cruzi* have been isolated from triatomine bugs (CDC, 2008), and the type IIa genotype is the most common genotype that has been reported from domestic dogs, raccoons, ring-tailed lemurs, and skunks. In contrast, all of the characterized autochthonous human strains and the majority of opossum strains from the United States have been type I (Roellig et al., 2008).

In the present study, all 15 parasite isolates were first positively identified as *T. cruzi* based upon successful PCR amplification of a 195 bp repetitive nuclear DNA sequence with the primers designated TCZ1 and TCZ2. This sequence has been shown to be particularly abundant in the genome of all strains of *T. cruzi* that have been analyzed, and it has been estimated that it represents approximately 9% of the total nuclear DNA (Moser et al. 1989). The TCZ1 and TCZ2 primers are highly specific for *T. cruzi* and do not amplify DNA from related trypanosome species (Moser et al., 1989). A 188 bp fragment was amplified in DNA samples from all 15 isolates from Kentucky (see Figure 3), and this amplicon size corresponds well to the published results of Moser et al (1989), the first group to develop and utilize the TCZ1 and TCZ2 primer pair for PCR. An additional low intensity amplicon of approximately 400 bp was also visible in all samples. The 400 bp band is not a contaminant since it is not present in the control lane,

nor is it present in subsequent gels showing the results of PCR amplification with the three additional *T. cruzi*-specific primer sets.

DNA samples from all 15 raccoon isolates also showed identical PCR amplification results with the other three sets of *T. cruzi*-specific primers (see Figures 4-6). The primers designated D71 and D72 recognize the D7 domain of the 24Sα rDNA of *T. cruzi*. A 130 bp fragment was amplified from all 15 parasite DNA samples, a result consistent with Brisse et al. (2001), who also reported a 130 bp PCR product for a North American type IIa strain of *T. cruzi*. In contrast, Roellig et al (2008) reported a 120 bp or 110 bp product when DNA from type IIa strains were subjected to PCR using the D71 and D72 primer pair.

Amplification of the non-transcribed spacer of the mini-exon genes of *T. cruzi* (using TC, TC1, and TC2 primers) resulted in a PCR product of 400 bp (see Figure 5). Whereas Brisse et al. (2001) reported that type IIa and type IIc DNA samples show no amplification with the TC, TC1, TC2 primer series, other investigators have reported products of 350 bp or 400 bp with both type I and type IIa strains of *T. cruzi* (Yeo et al. 2005; Roellig et al., 2008).

Amplification of the 18S rDNA sequence with V1 and V2 primers reportedly results in consistent differences in PCR product size between genotypes of *T. cruzi*. Brisse et al. (2001) reported that PCR amplification of this sequence resulted in amplification products of 155 bp in all type IIa strains analyzed. In contrast, *T. cruzi* lineages type IIb, IIc, and IId consistently showed an amplification product of 165 bp. Roellig et al. (2008) reported a 155 bp amplification product for North American type IIa strains, and a 175 bp amplification product for North American type I strains.

To allow a direct comparison of amplification products from control type I and type IIa *T. cruzi* genomic DNA samples to representative raccoon isolates from Warren and Barren counties (RW2 and RB12), PCR products were analyzed side by side in the same agarose gel (see Figure 7). The 15 raccoon isolates of Kentucky analyzed in the present study showed similar, but not identical PCR amplification results as the control type IIa *T. cruzi* genomic DNA sample generously donated to us by the Yabsley lab at the University of Georgia. When amplified with the TC series primers, the type I genomic DNA sample showed a 350bp product, all 15 raccoon isolates showed a prominent amplicon of approximately 400 bp**,** however, the control type IIa sample showed no evidence of amplification.

The type IIa strain of *T. cruzi* is the most commonly reported genotype reported from raccoons trapped in the United States. In the present study, the combined results of PCR analysis using 4 different sets of *T. cruzi*-specfic primers also strongly suggested that the 15 raccoon isolates from Kentucky were also the type IIa genotype. However, due to the lack of complete agreement between PCR results using the control type IIa genomic DNA sample and DNA from our 15 parasite isolates, we decided to sequence and perform a BLAST analysis on representative samples of PCR products from Warren and Barren Counties of Kentucky (RW2 and RB12). All samples, from all 4 DNA sequence from 4 PCR amplified DNA analyses, showed highest identities to type IIa strains of *T. cruzi* (9212210r, Can III, and STC10R), with max identities ranging from 94%-99%. The 9212210r is the Type IIa *T. cruzi* isolated from Raccoons in Georgia, USA (Lewis et al., 2009).

Although DNA sequencing followed by BLAST analysis allowed us to confirm the genotype of our 15 isolates of *T. cruzi*, this approach would most likely not be practical for routine genotyping of new isolates. One limitation of this approach is that matching sequences have to exist in the Genebank database. If the appropriate sequences have not been entered into Genebank, the BLAST will not yield useful results. In addition, sequencing analysis is not practical for a large cohort of isolates due to the amount of time necessary for the analysis.

One other limitation of the genotyping technique employed in the present study is that DNA ladders from different companies tend to yield different estimates of PCR fragment size in agarose gels. Therefore, DNA estimates based only on comparisons to DNA markers should be regarded as only approximate values. Genomic DNA samples from confirmed genotypes of *T. cruzi* should be analyzed along with DNA samples from new isolates whenever possible.

Other approaches that have been used by investigators for the genotypic analysis of *T. cruzi* isolates include restriction fragment length polymorphism (RFLP), multilocus sequence typing (MLST), and multilocus microsatellite typing (MLMT). RFLP is a practical method and is relatively inexpensive. The techniques of MLST and MLMT allow for the discrimination of *T. cruzi* genotype, but they are complex and time-consuming techniques and are not practical for analysis of a small number of samples (Lewis et al., 2009). Lewis et al. (2009) has recommended using a combination of PCR product size polymorphism of the 24Sα rDNA sequence, combined with a PCR-RFLP assay for the HSP60 and GPI proteins of *T. cruzi* (Lewis et al., 2009). According to Lewis et al. (2009), this approach would involve minimal cost, minimal time, and low

quantities of sample material. Lewis et al. (2009) further suggests that this approach would allow for reproducible results with high resolution.

T. cruzi is an obligate intracellular parasite that must invade mammalian host cells in order to reproduce and complete its life cycle. In addition to the genotypic analyses performed in this study, cell infectivity assays were performed on four of the raccoon isolates, two from Barren County and two from Warren County (RB14, RB12, RW5, and RW10). All four isolates were highly infective for the CV-1 mammalian cell line, providing further evidence that the parasite isolates are definitely *T. cruzi*, and not a related trypanosome such as *T. rangeli* that does not have an intracellular cycle of replication.

The 15 isolates of *T. cruzi* analyzed in the present study are the first strains to be reported from the state of Kentucky. Future studies are urgently needed to determine if the sylvatic cycle of *T. cruzi* is endemic in the neighboring states of Missouri, Illinois, Indiana, Ohio, and West Virginia. It will also be important to determine the prevalence of *T. cruzi* in triatomine vectors in the state of Kentucky, as well as in other wild mammals in the state such as skunks, opossums, wood rats, and coyotes.

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