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TRYPANOSOMA CRUZI IN WILD RACCOONS AND OPOSSUMS FROM KENTUCKY

A Thesis
Presented to
The Faculty of the Department of Biology
Western Kentucky University
Bowling Green, Kentucky

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
Brian Chad Groce
August, 2008

TRYPANOSOMA CRUZI IN WILD RACCOONS AND OPOSSUMS FROM KENTUCKY

Date Recommended June 30, 2008

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TRYPANOSOMA CRUZI IN WILD RACCOONS AND OPOSSUMS FROM KENTUCKY

Brian Chad Groce August, 2008 Pages 79

Directed by: Dr.Cheryl D. Davis, Dr. Michael Stokes, and Dr. Kenneth Crawford

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Only 6 autochthonous cases of human Chagas disease have been documented in the U.S.A., however, as many as 5% of immigrants from Latin America may be infected with the etiologic agent, *Trypanosoma cruzi.* The parasite has been isolated from a variety of wild mammals, particularly in the southeastern region of the U.S.A. The goal of our study was to determine if the sylvatic cycle of *T. cruzi* infection occurs in Kentucky, and, if present, to assess the prevalence of infection in Warren and Barren counties. Raccoons and opossums were live-trapped between June and December, 2007. Animals were anesthetized with isoflurane, and blood samples were collected using a vacutainer system. Sera were frozen at -80°C for subsequent analysis, and whole blood samples were inoculated, in duplicate, into liver infusion tryptose (LIT) medium and cultured at 27°C. Seventeen *T. cruzi* isolates from raccoons have been positively identified by hemoculture. A total of 25/44 (57%) raccoon samples were found to be positive by hemoculture or serological analysis. In Warren County 18/25 (72%) of raccoons tested positive for *T. cruzi* and 7/19 raccoons (37%) in Barren County were positive for the parasite. Eighteen of 43 (42%) of the sera collected from opossums in Warren County and 3 of 5 (60%) from Barren County were judged to be positive by either enzyme-linked immunosorbent assay (ELISA) or indirect immunofluorescence

assay tests (IFAT). There were no positive hemoculture results for the opossum samples. The infection rates found in the current study for raccoons were slightly higher than those reported in previous studies in the U.S.A. However, the overall prevalence of *T. cruzi* in opossums (determined by serological analysis) was consistent with previous studies performed in the southeast. To our knowledge, this is the first report of *T. cruzi* from the state of Kentucky.

INTRODUCTION

Procyon lotor

The northern raccoon, *Procyon lotor* Linnaeus, is a member of the Procyonidae family. The fossil record of the Procyonidae is poor compared to other carnivoran families, but the earliest known procyonids in the New World occurred early in the Miocene era (Fulton and Strobeck, 2006). Remains of *P. lotor* have been found in North America dating back to the Pleistocene era and the northern raccoon is currently known to inhabit a wide variety of habitats ranging geographically from Alaska and southern Canada to Panama (Lotze and Anderson, 1979). Raccoons have typically been rare in the Rocky Mountains but a decline in pelt values in the 1990's resulted in population surges and *Procyon spp.* now occur in mountainous areas and deserts where they were formerly rarely seen (Gehrt, 2003).

Management of the raccoon often tends to be very political because of its important roles as a game animal and as a furbearer, with over 3.1 million pelts harvested each year in the United States from 1970-1991 (Gehrt, 2002). Hunters, coon-dog trainers, and animal rights groups all seem to be at odds with lawmakers regarding the management of the raccoon (Chamberlain et al., 1999; Chamberlain et al., 2003; Rogers, 1995). In most states the raccoon is a protected furbearer and seasons are established for hunting (harvest), running (non-harvest), and trapping (Boggess, 1994; Hodges et al., 2000).

The northern raccoon is known to be a nuisance animal in many areas and various states allow for control methods as a means of damage prevention. Raccoons may do extensive damage to gardens and truck crops, particularly sweet corn and watermelons.

They may roll up freshly laid sod to search for food, kill poultry, eat poultry eggs, and destroy the nests of birds in artificial nesting structures, such as bluebirds and wood duck nest boxes (Boggess, 1994).

Raccoons are omnivorous and ecological generalists with opportunistic feeding behaviors; therefore, they are able to exploit a variety of food and habitat sources (Bozek et al., 2007). They typically feed on a wide range of fruits, nuts, grains, acorns, and berries. They also feed on a diverse selection of animal foods including, clams, crayfish, rodents, rabbits, birds and waterfowl, insects, and turtles. Raccoons are considered important predators of bird and turtle eggs (Giles, 1940; Urban 1970; Boggess, 1994; Ratnaswamy et al., 1997).

In their search for food and/or shelter raccoons can cause major damage to buildings. Raccoons naturally den in hollow trees and ground burrows (Berner and Gysel, 1967); however, the availability of den types likely influences their selection of dens (Henner et al., 2004). Therefore, in areas where habitat has been diminished, such as urban or suburban areas, raccoons have found that uncapped chimneys may serve as suitable denning sites in place of hollow trees. Cases have been reported of raccoons pulling shingles or boards off of buildings to gain access to nesting sites in the attic or wall space of a house or outbuilding. Raccoons typically leave evident clues that they are the culprits in such incidents. For example, their “hand-like” paw print and the manner in which they kill poultry, raid nests, and damage crops is characteristically identifiable (Boggess, 1994).

Urbanization heavily influences the biodiversity and density of wildlife through habitat fragmentation and simply by human presence. Some species have been

successful at taking advantage of both anthropogenic and natural resources. These species have been termed "urban adapters." Anthropogenic resources may be abundantly concentrated in smaller areas, leading raccoons to select for smaller home ranges (McKinney, 2002). Raccoons are thought to be one of the most effective of all mesopredators adapted to such urban settings (Bozek et al., 2007; Newbury and Nelson, 2007).

The concentration and temporal predictability of anthropogenic food sources is an important determining factor of the home range size and location of both male and female raccoons (Bozek et al., 2007). Raccoons tend to concentrate in areas associated with anthropogenic food sources, such as parks. These high concentrations of animals coupled with the endearing sentiments many people have toward raccoons leads to a willingness to accept food from humans (Smith and Engeman, 2002). It is very difficult to estimate population densities for raccoons because of their movement habits (Hallett et al., 1991). However, Riley et al., (1998) suggested that in areas where humans dominate, raccoon densities may range from 40-120 animals/km^2 or .16-.49 animals/acre. In contrast, Sonenshine and Winslow (1972) estimated densities of one raccoon per 14.3 acre or .07 animals per acre in a natural area in Virginia.

It has been suggested that adult male raccoons may occupy areas of 3-20 square miles while females occupy much smaller territories, 1-6 square miles (Boggess, 1994). Some studies have not observed this type of variation (Gehrt, 2003; Bozek et al., 2007). These differences are likely due to the fact that home ranges are greatly influenced by the availability of resources such as free water, food, and mates (Pedlar et al., 1997; Henner et al., 2004; Newbury and Nelson, 2007). Raccoons are polygynous; therefore, during

the breeding season the home range of the male may expand in order to increase encounters with females, hence increasing his reproductive success (Chamberlain et al., 2003).

The mating season of *P. lotor* varies geographically but usually extends between December and August, with most females mating in May (Lotze and Anderson, 1979) and over half of all births occurring in May (McKeever, 1958). Almost all adult female raccoons breed annually (Fritzell et al, 1985) with an average litter size of 2-5 (Lotze and Anderson, 1979). Larger litter sizes are associated with northern locales (McKeever, 1958).

Raccoon populations are often composed of a high proportion of young animals. In the fall of the year, as much as ¾ of the raccoon population may be less than one year old (Boggess, 1994). Raccoons may live as long as 17 years in captivity, but the average life expectancy of a raccoon in the wild is less than 5 years due to food shortages, hunting by man, disease, and highway mortality (Lotze and Anderson, 1979).

Pathogens of *Procyon lotor*

The raccoon is the definitive host for *Baylisascaris procyonis,* the raccoon roundworm, which is typically found in the small intestine (Page et al., 1999). *B. procyonis* infections are intensely endemic in raccoon populations of the Northeast, Midwest, and mid-Atlantic regions; however, with the exception of mountainous areas, it has been rare in the far-western states, the Southeast, and the southern coastal states (Roussere et al., 2003; Kerr et al., 1997). However, in recent years it has been found in coastal Texas (Kerr, et al., 1997; Long et al., 2006), metropolitan Atlanta (Eberhard et al., 2003), Florida (McCleery et al., 2005) and California (Park et al., 2000; MMWR, 2002).

Baylisascaris procyonis infection rates within raccoon populations can be very high, especially among juveniles. Adult population infection rates have been found to be as high as 70%, while juvenile infection rates may be as high as 90% (Page et al., 2005; Sorvillo, et al., 2002). Parasite intensity tends to be lower in the summer and fall (Smith et al., 1985) but may increase during winter months. In some cases the intensity may increase to the point that intestinal obstruction and death may occur. Kidder et al., (1989) suggested that if the *B. procyonis* burden becomes excessive the raccoon can expel some or all of the worms. Carlson and Nielson (1984) reported finding 1321 worms in the gastrointestinal tract of one raccoon. Snyder and Fitzgerald (1985) found an adult with 328 adult worms that had completely filled portions of the small intestine.

Raccoons are known to preferentially defecate in areas known as latrines (Yeager and Rennels, 1943; Giles 1939). Latrines may be found on flat, raised surfaces including at the base of trees, tree stumps, on rocks, on logs or downed timber, decks, rooftops, chimneys, attics, lofts, patios, and woodpiles (Chris, 2006; Gavin et al., 2005; Rowley, et al., 2000). Multiple latrines will likely exist in a small area. Page et al., (1998) found 5.5 latrines per hectare in an 8.2-hectare area in Indiana. Many raccoons may use one latrine and fresh feces is being deposited to active latrines daily, so eggs of varying developmental stages are present at latrine areas at all times (Page et al., 1999). Roussere et al., (2003) found that 44-53% of 215 latrines tested in northern California contained *B. procyonis* eggs and 16-32% of the latrines contained eggs in the infective stage.

Raccoons infected with *B. procyonis* shed eggs in their feces. An infected adult female will shed an average of 20,000-26,000 eggs per 1 gram of feces (Murray and Kazacos, 1999), and a latrine may contain 370-750 g of feces (Gavin et al. 2005). Once

eggs are shed in the feces they mature into infective larvae, usually in about 2 weeks, depending on the ambient temperature and moisture levels (Murray and Kazacos, 2004). At 22-25° C eggs can mature into infective larvae in 11-14 days; however, it may take weeks or months to become infective at other temperatures (Page et al., 1999). Eggs are very resistant to environmental factors and may remain infective in the soil for years in favorable environmental conditions (Murray and Kazacos, 2004). In cases where the latrine is located on a high log or rooftop, rainwater may wash the eggs onto more accessible areas (Stephenson, 2002). The eggs also possess a sticky proteinaceous covering that allows them to adhere to objects and animals allowing for transport (Stephenson, 2002; Murray and Kazacos, 2004).

Small mammals, especially rodents and birds foraging near latrines, often become infected by *B. procyonis*. Photographic documentation of a latrine in Indiana showed that 28 species of birds and mammals visited the raccoon latrine during the months of September, October, and November. Many of these birds and mammals were foraging on seeds in the raccoon feces (Page et al., 1999). Most of these visitors serve as intermediate hosts for *B. procyonis*. In fact, more than 100 species of birds and mammals in North America can be infected with *B. procyonis*, which is recognized as one of the most common and widespread causes of clinical larva migrans in animals (Gavin et al., 2005). It is hypothesized that *B. procyonis* may play a pivotal role in the drastic decline of some species of wild mammals in recent years, such as the Alleghany woodrat (*Neotoma magister*) and the endangered Key Largo woodrat (*N. floridana smalli*) (McCleery et al., 2005).

Once the eggs are swallowed they hatch in the small intestine, penetrate the intestinal mucosa, travel through the liver and lungs to the heart where they enter the systemic circulatory system to be distributed to tissues throughout the body. About 5-7% migrate to the central nervous system (CNS) and are encapsulated by eosinophilic granulomas where they remain infective for the duration of the host's life (Moertel et al., 2001; Sorvillo et al., 2002; Gavin et al., 2005).

Baylisascaris procyonis may cause visceral larva migrans, ocular larva migrans, or neural larva migrans in humans (Rowley et al., 2000; MMWR, 2002; Murray and Kazacos, 2004; Gavin et al., 2005). From 1980-2005 there were 13 reported cases of human *B. procyonis* neural larva migrans in the United States. Five of the 14 died and the rest suffered "severe residual deficits" (Gavin et al, 2005). A 4-year old boy from New Orleans is the only known case of full recovery from a *B. procyonis* infection (Pai, et al., 2007).

The median human age of infection is 13 months and children under 4 yrs of age are at greatest risk because of their propensity to place objects in their mouth as they explore the world around them or through pica and geophagia (MMWR, 2002; Pai et al, 2007). There are likely a number of cases of infection with *B. procyonis* that go undiagnosed (Eberhard et al., 2003). Due to the ease of access to large numbers of *B. procyonis* eggs and their ability to survive in the environment for long periods of time, it has been suggested that *B. procyonis* could possibly be used as an agent of bioterrorism (Sorvillo et al., 2002).

As predators, raccoons are also host to a wide range of all types of diseases which may affect other wildlife, domesticated animals and humans. Some common viral

diseases known to be carried by raccoons are canine distemper virus (Rabinowitz and Potgieter, 1984; Roscoe 1993; Paré et al., 1999; Bischof and Rogers, 2005), papillomavirus type I (Rector et al., 2005), rotavirus (Hamir et al., 1990), Venezuelan equine encephalitis virus (VEE) (Bigler, 1971), west nile virus (WNV) (MMWR, 2000), rabies virus (Rabinowitz and Potgieter, 1984; Finnegan et al., 2002; Jones et al., 2003), Canine hepatitis virus (CAV1), Pseudorabies virus (PRV) and canine parvovirus (CPV) (Rabinowitz and Potgieter, 1984). There are also reports indicating that the transmissible spongiform encephalopthies (TSE) known as sheep scrapie and transmissable mink encephalopathy (TME) may be transmitted to raccoons (Hamir et al., 2005). They are also hosts for *Mycobacterium bovis* (Palmer et al., 2002) and *Sarcoptes scabiei* (Fitzgerald et al., 2004). A host of tick-borne diseases and pathogens are associated with raccoons. A few of the more common examples are *Borrelia burgdorferi* (Magnarelli et al., 1995; Magnarelli et al., 2001), *Francisella tularensis, Leptospira interrogans* (Mitchell et al., 1999; Bischof and Rogers, 2005), and *Ehrlichia chaffeensis* (Comer et al., 2000).

Raccoons serve as hosts for a large number of gastrointestinal parasites (Jordon and Hayes, 1959; Harkema and Miller, 1964; Bafundo et al., 1980; Snyder and Fitzgerald, 1985). They also harbor a variety of hemoparasites such as microfilaria (Rabinowitz et al., 1985; Snyder et al., 1989; Telford, Jr. and Forrester, 1991, Pung et al., 1996), *Babesia lotori* (Telford, Jr. and Forrester, 1991), *Toxoplasma gondii* (Mitchell et al., 1999) and *Trypanosoma cruzi* (John and Hoppe, 1986; Karsten et al., 1992; Pung et al., 1995; Pietrzak and Pung, 1998; Herwaldt et al., 2000; Yabsley et al., 2001; James

et al., 2002; Yabsley and Noblet, 2002; Yabsley and Noblet, 2002; Hancock et al., 2005; Dorn et al., 2007; Hall et al., 2007).

Raccoons are thought to serve as an indicator of several zoonotic diseases and various environmental pollutants (Strolle et al., 1978). They carry a wide range of pathogens and their close contact rates at feeding sites allows for intraspecific transmission of pathogens (Totten et al., 2002). These facts coupled with the ability of raccoons to readily adapt to urban areas and their ubiquitous distribution makes them important candidates for further epizootiological investigations.

Didelphis virginiana

The Virginia opossum, *Didelphis virginiana* Kerr, is of the family Didelphidae and is the only native metatherian found north of Mexico (Gardner and Sunquist, 2003). Presently, four subspecies of *Didelphis virginiana* are recognized in North America - *D.v. virginiana*, *D.v. pigra*, *D.v. californica, D.v.yucatanensis* (McManus, 1974). There has been much debate on the taxonomy of the Virginia opossum and confusion plagues the current literature. This work will follow the nomenclature of Gardner (1973, 1993).

The evolutionary origin of *Didelphis* is poorly understood and the literature is full of inconsistencies. *Didelphis* has often been referred to as a “living fossil” left over from the age of dinosaurs (Clemens, 1968). However, chromosomal studies (Reig et al., 1977), nuclear DNA evidence (Kirsch et al., 1993), and mitochondrial DNA sequences (Patton et al., 1996) suggest that *D. virginiana* most likely evolved from the South American white-eared opossum, *Didelphis albiventris* (Gardner and Sunquist, 2003). Some authors claim that *Didelphis* may have entered North America by crossing the Panamanian Land Bridge during the Pliocene epoch (Shaw and McDonald, 1987;

Gilmore, 1977). However, some fossil remains seem to suggest that they inhabited North America during the Upper Cretaceous period (Clemens, 1967; Gardner and Sunquist, 2003).

Virginia opossums are nocturnal and omnivorous. They are also ecological generalists; therefore, they are adaptive to a wide range of habitats and climates, including areas of urbanization. They are ubiquitous in much of the southeastern United States and their distributional range extends from northwestern Costa Rica and Mexico to southern Ontario and British Columbia, Canada (Gardner and Sunquist, 2003). The range of the opossum has been estimated to be expanding northward at a rate of 6.4 km per year. The diet of the opossum commonly consists of rabbit, squirrel, mice, birds, eggs, worms, snakes, frogs, skunk, opossum, grapes, apples, berries, grains and insects (Taube, 1947).

If threatened, *D. virginiana* often assumes a defensive posture and tries to "bluff" potential predators by hissing, growling, and baring its fifty teeth (McManus, 1974). If the threat continues, the opossum resorts to two mechanisms of passive defense. Initially, it may secrete a foul smelling greenish substance from papillae located near the anus. It has been reported that opossums may also spray this substance from the perianal region during times of threat (Weidorn, 1954). It is of particular relevance to the present study that the developmental cycle of *Trypanosoma cruzi,* which usually occurs in the gut of triatomine insects, has been demonstrated to occur in the anal glands of opossums. Urdaneta-Morales and Nironi (1996) found all stages of *T. cruzi* in the anal secretions of opossums. Many of the epimastigotes observed were undergoing binary fission, giving

rise to infective trypomastigotes. Schofield (2000) hypothesized that marsupials may have acted as one of the original vectors of *T. cruzi.*

If the threat is strong enough, the opossum may resort to its most well-known behavioral advantage - the ability to feign death. During this event the animal enters a catatonic state that may last from a few short minutes up to 6 hours. The animal becomes immobile and refractory in many aspects (McManus, 1970). It is unclear whether the purpose of this response is to cause a predator to loose visual contact with the opossum or if this response is coupled with the malodorous perianal secretion to deter predatory attack (McManus, 1970). The latter seems most likely because it has been repeatedly demonstrated that the stimuli must be fairly strong in order to produce the catatonic death feigning state (McManus, 1970; Ladine and Kissell, Jr., 1994). There is also evidence suggesting this response was developed mainly in response to agonistic intraspecific encounters (Francq, 1969; McManus, 1970).

There is a great deal of uncertainty concerning the population dynamics and movement patterns of opossums, despite the considerable amount of attention given to the subject by researchers. Some researchers have suggested that opossums are highly nomadic and do not establish well-defined home ranges (Reynolds, 1945). While Lay (1942) reported home ranges of 11.5-38.4 acres, Fitch and Sandidge (1953) described home ranges of 42-70 acres and Verts (1963) estimated a home range of 96 acres.

Population estimates are highly varied as well. Holmes and Sanderson (1964) reported an average population at midsummer to be approximately 300 animals per square mile (2 per acre), Lay (1942) reported 160 animals per square mile (1 per 4 acres), Fitch and Sandidge (1953) estimated 32 animals per square mile (1 per 20 acres), Verts

(1963) reported 10 opossums per square mile (1 per 64 acres), and Hamilton (1958) reported 20 per square mile (1 per 30 acres). The average life expectancy of an opossum in the wild is less than 1.3 - 7 years, and populations may turnover in as little as 4.8 years (McManus, 1974).

Many factors, including landscape, food abundance, temperature, mate availability, predator influence, population density, and urbanization influence the home range, population and movement pattern of opossums. Therefore, it is impossible to estimate a value for the home range or determine a method of calculating the population size that can be applied universally to local populations without considering these variables (Holmes and Sanderson, 1965; Gillete 1980).

The mating season of the polygynous opossum depends upon geographic location (McKeever, 1958; McManus, 1970; Hardy, 1997). There are usually two mating seasons per year ranging from December to August (McKeever, 1958; McManus 1970; Grote and Dalby, 1973; Winegarner, 1982). Both the onset and duration of the mating seasons are reportedly affected by latitude (Tyndale-Biscoe and Mackenzie, 1976). Adult females may give birth to as many as 16 young but are limited by number of teats to raising 13 with an average litter size of 7-9 (McManus, 1970; McManus, 1974). The sex ratios of pouch young and of live-trapped juveniles tend to average 50:50 (Sanderson, 1961; Holmes and Sanderson, 1964) and are not affected by maternal diet (Hardy, 1997).

The opossum is an ideal animal model for many types of biomedical research because of its very short gestation period and the fact that marsupial pouch young give researchers access to what are essentially “extrauterine embryos” (Marx, Jr. et al., 1970). Due to the altricial state of birth, newborn opossums are equivalent to a 4-6 week old

human embryo (Miller, 1983). Studies have shown newborn opossum tissues to have regenerative properties (Mizelle, 1968). Wintzer (2004) showed that newborn opossums are actually able to heal complete transections of the spinal cord. The opossum has also been used in neuro-psychiatric investigations (Wierdon, 1954), and extensive work has been done in the field of immuno-genetics using the opossum (Hayes, 1968; Rowlands, Jr. 1968; Taylor and Burrell, 1968; Rowlands, Jr. 1969; Rowlands, Jr. et al., 1974; Miller et al., 1998; Belov et al., 2007).

Immune development and immune competence in marsupials are areas of intense research interest, and both may influence the animals' role in the transmission of zoonotic diseases. *Didelphis virginiana* has an internal gestation period of only 13 days at which point the young migrate from the uterus into the marsupium (Jurgelski, Jr. et al., 1976). Neonatal organogenesis is in a very immature state and the young are born without organized lymphoid tissues or organs. Consequently, the immune system develops in the presence of pathogens (Rowlands, Jr. et al., 1971; Belov et al., 2007). Metatherian neonates are; therefore, born with no adaptive immune system and must, for the first few days of life, depend on passive immunity acquired through lactation for protection during development outside the sterile intrauterine environment (Belov et al., 2007). The thymus begins to appear at day two of life, and the spleen by day 17-20. Embryonic antibody production has been recorded as early as seven days post-partum, with peak antibody production occurring as early as 14 days (Rowlands, Jr. et al., 1971). The young permanently suckle until day 16 and then suckle intermittently thereafter, which builds immuno-competence through exposure to new pathogens. The cessation of maternal

antibodies across the intestinal epithelium occurs at 60 days of age and weaning generally coincides with this phenomenon (Belov et al., 2007).

The completed genome of the grey, short-tailed opossum, *Monodelphis domestica*, (Mikkelsen et al., 2007) suggests that the marsupial immnogenome is very similar to eutherians, although it may work at a slower pace (Rowlands, Jr., 1970). Miller et al. (1998) characterized the V_H repertoire of the opossum and Belov et al. (2007) showed that marsupials posses highly conserved genes which code for immunoglobulins G, A, E and M, α β, and γ δ T cell receptors, and major histocompatability complex (MHC) proteins.

The opossum has been identified as a host to a wide variety of endo- and ecto-parasites (Barr, 1963; Alden, 1995). It is also a carrier for diseases that commonly affect livestock (Bigler, 1970), and a known reservoir host of trypanosomes that affect humans (Barr, 1963; Woo and Soltys, 1970). These reasons coupled with the fact that the opossum makes such a good animal model for laboratory and field investigations, as well as the ecologically adaptive nature of the opossum, make it an important subject of epizootiological studies.

Trypanosoma cruzi

Trypanosoma cruzi, originally known as *Schizotrypanum cruzi*, was discovered in 1909 by Carlos Chagas (Chagas, 1909). Chagas, a Brazilian scientist employed at the Oswaldo Cruz Institute in Rio de Janeiro, was working on the prophylaxis of malaria in the Rio das Velhas Valley when he observed a group of patients who displayed symptoms inconsistent with those of any known disease in the region at that time. However, many of these patients exhibited symptoms that are now known to be

associated with trypanosomiasis, such as glandular enlargement, ocular afflictions and edema. Chagas also noted a large insect of the order Hemiptera that was known to live and proliferate in the walls of the rural homes in the region and then take blood meals from the inhabitants at night. Knowing the important role hematophagous vectors could play in the transmission of infectious diseases, Chagas decided to examine the intestinal contents of the insects and discovered a flagellated parasite. Because the monkeys in the Rio das Velhas Valley were all naturally infected with *Trypanosoma minasense*, a trypanosome described by Chagas in 1908, the role of the hematophagous bug in the transmission of the parasite could not be determined in the field. Therefore, numerous Hemiptera were collected and sent to Oswaldo Cruz in Manguinhos where they were allowed to feed on uninfected monkeys. Once the role of the hematophagous insect in the transmission of the parasite was confirmed, Chagas set out to survey the humans and domestic animals that resided in homes known to be infested with the insect, *Triatoma megista*. A blood sample from a cat was found to be heavily infected with *T. cruzi*; however, no signs of the trypanosome were found in the human samples. Finally, blood from a small child who was exhibiting signs such as glandular enlargement, spleenomegaly and a swollen face was examined and *T. cruzi* was found. Thus, a new disease, Chagas disease, was described (Chagas, 1920; Prata, 1999). Since Chagas first described the parasite it has been found, due to paleoparasitological efforts, in four million year old mummies from Chile (Guhl et al., 2000) and 9000 year old mummies in northern Chile and southern Peru (Aufderheide, 2003).

Chagas disease is endemic in many parts of Latin America with the exception,

historically, of the Caribbean nations (Morel, 1999; Kirchoff, 2003). In 1991 the World Health Organization (WHO) estimated, based on serological data, that there were approximately 16-18 million people infected with *T. cruzi* (Umezawa and Silveira, 1999). More recent estimates have projected that about 11 million people are currently infected (MMRW, 2007) with an additional 28 million people at risk of infection and 12,500 deaths occurring each year due to Chagas disease (Dias, et al., 2008). It is the third most common parasitic disease in Latin America behind malaria and schistosomiasis (WHO, 1997). However, the social and economic impact associated with Chagas disease far outweighs the combined effects of other parasitic diseases. In 1993, The World Bank estimated an annual loss of 2,740,000 disability-adjusted life years (DALYs) (Dias and Schofield, 1999). That same year the WHO estimated the economic loss in endemic countries of Latin America to be approximately $6.5 billion (U.S.) (WHO, 1997).

The biology of *Trypanosoma cruzi* has been studied extensively since its discovery almost a century ago (Araújo-Jorge, 1999; Souza, 1999). In his 1909 report, Chagas included schematic drawings of Giemsa-stained *T. cruzi* found in blood, cultures, and from the gut of the insect. In 1950, Meyer and Porter published the first electron micrographs of *T. cruzi* (Meyer and Porter, 1954). Electron micrographs from 1978 revealed new information on the kinetoplast, the flagellum, and other cytoplasmic structures, such as the one mitochondrion per cell. More recently, technological advances have made it possible to identify other aspects of the fine structure of *T. cruzi*, such as the organelles known as acidocalcisomes and reservosomes (De Souza, 1999).

During the life cycle of *Trypanosoma cruzi* it undergoes transformations into three different forms; the epimastigote, the trypomastigote, and the amastigote (Chagas,

1920; Williams, 1984). These body forms are most likely adaptations that allow the parasite to utilize the different environments present in the invertebrate and mammalian hosts (Colli and Alves, 1999). Spindle-shaped epimastigotes are about 20-40 µm long and are found primarily in the midgut of the insect where they divide by binary fission. Non-dividing, infective trypomastigotes are found in the hindgut, feces, and urine of the insect and in the bloodstream of the vertebrate host. Slender trypomastigotes are about 25 µm long and 2 µm in diameter. When found in the gut, feces or urine of the insect these are referred to as metacyclic trypomastigotes, although the morphology is the same as those found in the bloodstream of the vertebrate host. Finally, the rounded intracellular stage of *T. cruzi* is known as the amastigote. Amastigotes are approximately 3-5µm in diameter and are found within infected vertebrate cells where they divide via binary fission (Walter et al., 2000). Studies have found this stage to also be infective for mammalian cells (De Souza, 2002).

There are a number of important and diagnostic anatomical characteristics of trypanosomatids. The surface of the trypanosome is composed of two main parts, the plasma membrane and the subpellicular microtubules. The plasma membrane is covered by a glycocalyx, which is a thin outer coat composed of polysaccharides. Below the plasma membrane is a series of hollow microtubules equally spaced 44 nm apart and connected to each other and to the plasma membrane. The presence of this layer is diagnostic of members of the family *Trypanosomatidae*. The intricate association of the subpellicular layer and the plasma membrane likely contributes to cell rigidity and makes physical disruption of the cell very difficult (De Souza, 2002). In addition, all trypanosomes possess a single flagellum which plays a pivotal role in the survival of and

potential pathogenicity of the parasite. The flagellum is used for general movement of the parasite and wave propagation can originate from the base or the tip, allowing the cell to change directions (Hill, 2003). It is also used to attach to the cell surfaces of vertebrate hosts and the perimicrovillar membranes that line the intestines of the invertebrate host. The length of the flagellum depends on the developmental stage of the parasite. In amastigotes it may be as short as 1 μm, and in trypomastigotes it may be as long as 20 μm (De Souza, 2002). The flagellum emerges from an invagination in the cell surface known as the flagellar pocket - a specialized invagination of the plasma membrane that contains receptors and plasma membrane transporters and serves to deliver glycoproteins, glycolipids, and numerous other molecules to the cell surface (De Souza, 1999; McConville et al., 2002; De Souza, 2002). In the epimastigote stage, the flagellar pocket is located anterior to the nucleus, but in the trypomastigote stage it is located posterior to the nucleus (McConville et al., 2002). The flagellum of trypanosomes is not only attached to the cell at the basal body but also along the length of the flagellum. This unique attachment couples cell movement with wave movement of the flagellum, giving the appearance of an undulating membrane on one side of live parasites when viewed microscopically (Hill, 2003).

Members of the family *Trypanosomatidae*, possess a single mitochondrion which stretches the length of the cell body (De Souza, 2002). In the mitochondrion, near the basal body, is a highly specialized region that contains a condensation of extranuclear DNA (K-DNA) that accounts for about 30% of total cellular DNA. This K-DNA is collectively referred to as the kinetoplast, which forms a rounded structure at the base of the flagellum and is a diagnostic characteristic of the family *Trypanosomatidae* (De

Souza, 1999; De Souza, 2002). The kinetoplast is composed of 20,000-30,000 minicircles which are approximately .045 µm, corresponding to about 1440 base pairs (De Souza, 1999). These minicircles account for about 95% of the K-DNA (McGhee and Cosgrove, 1980). The other 5% is comprised of a number of highly conserved maxicircles of 6-11 µm. The morphological characteristics of K-DNA are dependent upon the developmental stage of the parasite. During the epimastigote and amastigote stages the K-DNA is compactly organized, giving a rod-like appearance. The kinetoplast is located anterior to the nucleus during the epimastigote stage. During the trypomastigote stage the K-DNA is located posterior to the nucleus, and is more loosely organized, giving a more rounded or basket-like appearance (De Souza, 1999; De Souza, 2002).

The nucleus of *T. cruzi* is typical of that found in other eukaryotic cells, possessing a nuclear membrane with pores. In the trypomastigote stage, the nucleus is elongated and centrally located within the cell, while the nucleus found in epimastigotes and amastigotes is rounded and measures approximately 2.5 µm. The endoplasmic reticulum is seen throughout the cell, while the cisternae of the Golgi complex are always located at the anterior end of the cell, near the flagellar pocket and the kinetoplast. Membrane-bound vesicles serve in trafficking from the Golgi body to the flagellar pocket to release contents to the extracellular space, rather than utilizing the endosomal-lysosomal pathway (De Souza, 2002). Other organelles of interest are the cytosome, acidocalcisome, the glycosome and the reservosome all of which will only be mentioned here (De Souza, 1999; Soares, 1999; De Souza, 2002).

The life cycle of *T. cruzi* is complex (see Figure 1), involving both vertebrate and

invertebrate hosts and a wide range of enzyme-driven physiological and morphological changes (Almeida et al, 1999; Travassos and Camargo, 1999). Hematophagous insects from the family Reduviidae ingest blood-form trypomastigote stages of *T. cruzi* as they feed on mammalian hosts. In the stomach of the insect, the trypomastigotes transform into epimastigotes and some rounded trypomastigote forms. The rounded trypomastigotes possess a free flagellum and may give rise to either short replicative epimastgotes or to long non-dividing forms. The epimastigotes replicate repeatedly via binary fission in the midgut portion of the intestine and attach to the perimicrovillar membranes. In the rectum, the epimastigotes transform into infective metacyclic trypomastigotes that are subsequently passed in the feces, oftentimes at the time of a blood meal. Transmission to the vertebrate host occurs when the metacyclic trypomastigotes enter a break in the skin, mucous membrane or conjuctiva (Krieger et al., 1999; De Souza, 2002; Kirchoff et al., 2003).

Once the parasite has entered the bloodstream of the mammalian host it uses the flagellum to assist in the invasion of various host cells. During the several hours following the invasion of a cell by a trypomastigote, the parasite goes through an intermediate form as it progressively assumes the rounded shape associated with the amastigote. In the amastigote form, the flagellum shortens to approximately 1 μm. For the following 35 hours the amastigote increases in size and then begins replicating by binary fission. The kinetoplast and nucleus divide and a new flagellum is formed, then anterior and posterior clefts divide the cell. The cytokinetic process is completed in approximately 25 minutes, and the doubling time for the parasite is about 14 hours. Subsequent replications occur for a few days and then, still in the host cell, the

amastigotes transform into trypomastigotes. This transformation from amastigote to trypomastigote takes several hours during which time the flagellum grows to about 20 µm and the kinetoplast is reorganized within the cell. At the end of the transformation process the movement of the long flagella of the newly differentiated trypomastigotes helps to rupture the cell releasing the trypomastigotes into the bloodstream. Some of the released trypomastigotes invade nearby tissues while some use the lymphatic system and bloodstream to travel to distal sites where they invade cells and tissues. This asynchronous cycling maintains trypomastigotes in the bloodstream that are infective for invertebrate vectors which feed on the host, completing the life cycle (De Souza, 2002; Kirchoff et al., 2003).

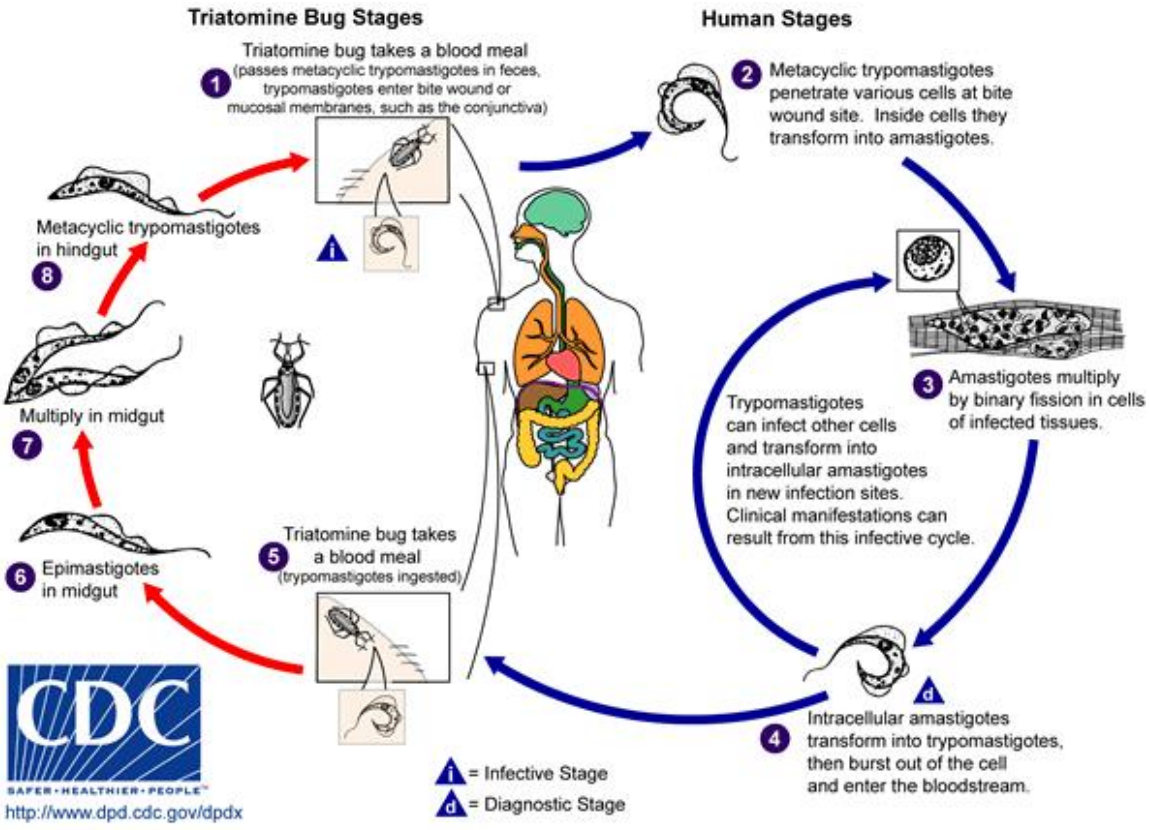

Figure 1. The life cycle of *Trypanosoma cruzi.* (CDC, 2008)

The vector-facilitated method of transmission is considered to be the most common mode of transmission of the parasite, but there are a number of other ways to become infected - congenital transmission (WHO, 2002; Gűrtler et al., 2002), organ transplantation, laboratory incident, and ingestion of triatomine insects (WHO, 2002; Woodall, 2007). Since *T. cruzi* may be found in the blood of as many as 50% of infected persons for years after symptoms have subsided, there is a risk of infection by blood transfusion and organ transplantation (Schmunis, 1999). Also, about 50% of persons infected are in the latent phase and may not realize they are even infected (Moraes-Souza, 1999). The risk of transfusional infection is considered to be approximately 20%, the second most common mode of infection (WHO, 2002; MMWR, 2007). Blood bank infections in Argentina, Brazil, and Chile have been shown to range from 1.4-18% and reach levels as high as 48% in Bolivia (Schmunis, 1999). Ten South American countries and three Central American countries now screen 100% of all blood donations (WHO, 2002). The dramatic increase in the number of Hispanic immigrants has led to an elevated risk of transfusional infection in the U.S. It is estimated that as many as 50/1000 legal immigrants and 59/1000 undocumented immigrants in the U.S. may be infected with *T. cruzi* (Schmunis, 2007). A study of blood donors in Los Angeles County, California showed 1/9,850 donors were seropositive in 1996 and 1/5,400 donors positive in 1998. A 2005 study conducted at 3 sites - Los Angeles, CA, Oakland, CA., and Tucson, AZ, tested 148,969 blood-donation specimens and found 1/2,365 samples contained antibodies against *T. cruzi*. On January 29, 2007, the American Red Cross and Blood Systems, Inc., who are responsible for approximately 65% of the U.S. blood supply, started to screen all donations for *T. cruzi* while providing testing services for

smaller blood-collection centers and hospitals upon request (MMWR, 2007).

Chagas disease is considered to occur in 3 stages - the acute phase, latent phase, and chronic phase. The acute phase usually lasts 4-8 weeks after the time of infection. An indurated erythematous lesion often occurs at the point of entry of the parasite within 10-14 days post-infection. Alternatively, if the parasite enters through the conjunctiva, a painless unilateral periorbital edema may occur, known a Romaña's sign. These occurrences are the first signs of the acute phase of Chagas disease. As the parasite disseminates and multiplies, the patient may experience nonspecific symptoms that are easily confused with a host of other diseases. The symptoms may include fever, malaise, edema of the face and lower extremities, generalized lymphoadenopathy, a morbilliform rash known as shizotrypanides, and hepatosplenomegaly. In some cases heavy parasitism of the muscles, myocarditis (leading to fatal congestive heart failure), meningoencephalitis, and invasion of the central nervous system may occur, although these occurrences are rare in the acute phase. Within 4-8 weeks, circulating parasite numbers diminish and the symptoms of the acute phase spontaneously disappear as the patient enters the intermediate phase of the disease. The often asymptomatic intermediate phase may last from 10-30 years and is associated with subpatent parasite levels. During this time one would expect to see a low parasitemia and elevated levels of anti-*T. cruzi* antibodies in the bloodstream of the patient. (Kirchoff et al, 2003).

As many as 30% of patients with long-term *T. cruzi* infections will eventually go on to develop the symptomatic chronic phase of Chagas disease. The persistence of the parasites within cardiac muscle stimulates an inflammatory response that may cause

dysrythmias, bundle branch blocks, and premature ventricular contractions resulting in dizziness, syncope, and death. Fibrosis and cardiomyopathy may also occur which may lead to congestive failure, clot formation with thromboembolization and ventricular apical aneurysm. Many patients who develop chronic Chagas disease also experience megaesophagus and/or megacolon (Kirchoff et al, 2003).

The sylvatic cycle of *T. cruzi* infection has been reported in several states, 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 and Noblet, 2002; Dorn et al, 2007; Hancock et al, 2005;). There has been at least one previous attempt to isolate *T. cruzi* from opossums (*Didelphis virginiana*) in Kentucky but no positive results were obtained (Aliff, 1970). A wide range of mammals, many of which are either indigenous to or well established in the southeastern United States, have been shown to serve as reservoir hosts for the parasite. In the United States, *T. cruzi* has been found in 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).

While *T. cruzi* infection is common among many domestic and sylvatic mammals, autochthonous human cases in the United States are very uncommon. There have been only 6 published reports of infections originating in the U.S. The documented cases include 3 infants from Texas, 1 infant from Murfreesboro, TN., a 56-year-old woman

from California, and a 74-year-old Louisiana woman (Dorn et al, 2007). The reasons for the low prevalence of *T. cruzi* infection in the U.S are not entirely clear. It is likely to be due to a number of factors including, lower virulence of North American strains of *T. cruzi,* lack of suitable dwellings for the triatomine vectors, decreased zoophilicity of the vector, and delayed defecation by vectors after blood meal (Pung, et al. 1995).

There are various methods of detecting *T. cruzi* infections but the most common methods are by hemoculture and serological analysis, such as enzyme-linked immunosorbent assay (ELISA) or immunofluorescent assay test (IFAT). Serological analysis is more sensitive than hemoculture (Yabsley et al., 2001; Yabsley and Noblett, 2002); therefore, serological surveys typically show a higher percentage of positive samples than hemoculture. Hemoculture results from South Carolina indicated that 14% of raccoons were infected with *T. cruzi* (Yabsley and Noblet, 2002). Lemurs from St. Catherine's Island, Georgia, were shown to be 0-6.5% infected based on culture of blood samples, and 20-51.2% infected based on serological analysis using ELISA (Hall et al., 2007). Surveys for *T. cruzi* infections in raccoons (*Procyon* lotor) from Georgia have shown from 15-60% infection rates based on hemoculture results (Pung et al., 1995; Pietrzak and Pung, 1998). Cultured blood samples from raccoons in Oklahoma showed an infection rate of 62% (John and Hoppe, 1986).

Trypanosoma cruzi is considered to be a heterogenous species, and parasite populations are typically classified as either- *T. cruzi* I or *T. cruzi* II based upon genotypic analyses. In Latin America *T.* cruzi I is thought to be associated with marsupial animals and the sylvatic cycle of transmission. *T. cruzi* II, which is further subdivided into 5 subgroups, IIa, IIb, IIc, IId and IIe is associated with

placental mammals and the domestic cycle of transmission (Jansen et al., 1999; El-Sayed et al, 2005). However, results from molecular characterization of strains taken from various animals in Latin America using polymerase chain reaction (PCR) have shown randomized distribution of strains among marsupials and placental mammals (Lisboa et al., 2007). In North America most of the isolates characterized from raccoons and lemurs have been type IIa, while human and opossum strains have been identified as type I (Clark and Pung, 1994; Barnabe et al, 2001; Hall et al, 2007; Rolllig et al, unpublished). Both *T. cruzi* I and *T. cruzi* IIa have been detected in triatomid insects in the U.S. (Barnabe et al, 2001; Roellig et al, unpublished).

The invertebrate vectors for *T. cruzi* are insects from the order Hemiptera, belonging to the family Reduviidae, subfamily triatominae (Schofiled et al., 1999). Triatomines are hemimetabolous and live an average of 3 months to two years. Immature triatomines molt approximately 5 times (Kobylinsky and Connelly, 2006) and the molting process is driven by the abdominal stretching which occurs when the bug feeds (Stern and Emlen, 1999). Both male and female triatomines feed on humans (Kobylinsky and Connelly, 2006). Feeding usually occurs at night, and triatomines have a tendency to bite people on the face which lends the nickname, “kissing bug” (Harder, 2004). The vector for *T. cruzi* varies among geographical locations throughout North and South America. This variation may have an impact on the transmission of the parasite from the insect to the vertebrate host. There are over 100 identified species of triatomid insects, and about half of them are known to be naturally infected with *T. cruzi* or a *T. cruzi*-like organism (Ryan et al., 1985). In South America the most common vectors are *Triatoma infestans, T. dimidiata, T. brasiliensis, Panstrongylus megistus, P. geniculatus, Rhodnius*

prolixus, R. robustus, R. picitipes and R. ecuadoriensis (Coimbra, Jr. et al., 1988; Aguilar et al., 1999; Azambuja et al., 1999; Costa, 1999; Garcia, 1999; Sherlock, 1999; Steindel, 1999; Valente, 1999; Azambuja et al., 2005). *Rhodnius prolixus* and *T. dimidiata* are known to be the most domesticated species, living in and around houses in endemic areas. Others such as *P. geniculatus* are considered to be important in the sylvatic cycle of *T. cruzi* because they dwell in rural and peridomestic habitats, such as nests of birds and mammals, chicken coops, and swine and goat facilities (Schofield et al., 1999). In Mexico the most important vector is *Meccus longipennis* (MartÍnae-Ibarra et al., 2003). In the U.S. the most common vectors are *Triatoma sanguisuga* and *T. gerstaekeri*. *Triatoma sanguisuga* ranges from Texas to the southeast and up to Maryland, and *T. gerstaekeri* is commonly found in Texas and New Mexico (Dorn et al., 2007).

No vaccine exists for *T. cruzi* and there is no consistent policy regarding treatment. However, two drugs, benznidazole and nifurtimox have been shown to have a success rate of approximately 50%. Unfortunately, due to the occurrence of serious side effects, these drugs are not approved for use in all areas. Also, people in many of the endemic areas are not able to afford the medication (WHO, 1997). Therefore, the focus of most control efforts has been vector control (Silviera and Vinhaes, 1999). In 1991 six countries- Argentina, Brazil, Bolivia, Chile, Paraguay and Uruguay began a control program known as the Southern Cone Initiative (SCI) (WHO, 2002). The SCI had three main goals: to eliminate *Triatoma infestans* and other triatomine insects in dwellings and peridomestic ecotopes by use of residual insecticide sprays; to replace adobe walled, palm roofed dwellings with plaster walled, zinc roofed homes in endemic areas; and to

reduce the risk of infection through blood transfusion. A total of $206 million (U.S.) was spent on the SCI, resulting in a $4500 million (U.S.) impact on the economic loss due to Chagas disease. In 2003, the SCI was declared a success (Dias, 2007; WHO, 2002). In 2006 Brazil was declared free from *T. cruzi* transmission by the common vector *Triatoma infestans* (Lima et al., 2008).

Although *T. cruzi* has been described in a wide range of mammalian hosts in the southern United States, it has not yet been reported from the state of Kentucky. In this study we sought to determine if the sylvatic cycle of *T. cruzi* exists in raccoons and opossums in Kentucky, and if so, to determine its prevalence using hemoculture, enzyme-linked immunosorbent assay (ELISA) and an immuno-fluorescence antibody test (IFAT).

MATERIALS AND METHODS

Collection Sites

Animals were trapped at 4 sites in Warren County (see Figure 2). The sites were assigned names according to their geographic locations within the county. The Drakes Creek site (N 36° 57.083 W 086° 22.851') is a 35-40 hectare farm with mostly open, gently rolling farmland planted predominately in native grasses. Wild blackberry bushes are allowed to grow around the pond area and the owner raises fruits and vegetables in a large garden area located near the house. The fields are allowed to grow-up and are surrounded by wooded areas. Drakes Creek runs along the border of this property on two sides and Drakesborough subdivision borders the property on the north side. This property is located less than two miles from Bowling Green and from many well-populated areas. The traps were placed throughout the farm in various locations.

The Barren River site (N 36° 57.989' W 086° 20.230') is located on the banks of the Barren River. While this property may seem secluded, it is located in close proximity to many suburban areas and is only about 2.5 miles from Bowling Green in Warren County. The owners raise cattle, horses, chickens, and fruits and vegetables on the property. Livestock feed is stored in the barn area and there are water sources located throughout the farm. Animals were captured around the barnyard.

The North Campbell site (N 37° 03.780' W 086° 27.940') is located in a rural area about 3.75 miles outside Bowling Green, but only about 0.75 miles from a small populated area and 0.8 miles from the Barren River. The property is mostly open land with clusters of trees located intermittently throughout the farm. This is a cattle farm

with fruits and vegetables raised near the house and barn area on the banks of a small creek that runs through the property. The traps were set in the garden area.

Finally, Anna (N 37° 06.635’ W 086° 24.840’) is located approximately 6.5 miles from the city of Bowling Green. There are many open fields on the property, planted mainly in fescue and surrounded by wooded areas. Traps were set beside a small creek running through the property. Densely populated wooded areas surround the creek.

Four geographically diverse sites were chosen in Barren County. Two of the sites, Bon Ayr (N 37° 00.480’ W 086° 04.756’) and Red Cross (N 37° 00.209’ W 086° 04.731’) represent adjoining farms located approximately 7.5 miles from the city of Glasgow. These are very rural areas and the farms have cattle, grain crops, wild berries, ponds and creeks, fruit trees, gardens, barns and outbuildings. The traps were placed near the garden areas or outbuildings at both sites.

Cedar Grove (N 36° 55.740’ W 085° 55.775’) is located 3.5 miles from Glasgow. This is a 16 hectare farm with four ponds and a small creek running through it. The owner raises cattle, swine, tobacco, and fruit and vegetables, including sweet corn and a large variety of melons. There are also a number of barns and outbuildings on the property. Traps were set in garden area, in the barns or along the creek.

Finally, the Temple Hill site (N 36° 51.712’ W 085° 50.178’) is an 80-90 hectare property located on Skaggs Creek about 9.75 miles southeast of Glasgow, Ky. Formerly a large dairy farm, a number of outbuildings from the dairy operation still remain on the property but it now hosts primarily grain crops and goats. There are large bottoms full of corn bordering Skaggs Creek. There are also ponds and small creeks on the property.

The traps were set near the creeks.

Figure 2. Map showing the counties of Kentucky. Warren and Barren Counties are indicated by arrows.

Trapping of Animals

Raccoons and opossums were trapped between June and December 2007. Animals were collected at 4 sites in Warren County, and 5 sites in Barren County. Havahart® 32”x10”x12” live animal traps were set in the evening and baited primarily with dry cat food, although we occasionally used honey buns, canned sardines, sweet corn or peanut butter. The traps were checked the following morning. Animals were euthanized using an overdose of the inhalant anesthesia, isoflurane. Sterile blood samples were collected into one 10ml serum tube and three 10 ml tubes containing the anticoagulant K_2EDTA using a Vacutainer® system. Samples were taken back to the lab and processed within 24 hours. Serum tubes were centrifuged for five minutes at 1800 xg. Serum was collected and 200 µl aliquots were placed into 1.5ml microfuge tubes and frozen at -80° C for subsequent analysis.

Nomenclature

All animals trapped were assigned a three-part designation for identification purposes. 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 1-29 which represented the order in which the animal was trapped. Opossums were given designations using the same model. For example, OW12 was the 12th opossum caught in Warren County and OB16 was the 16th opossum trapped in Barren County.

Hemoculture

Using a biosafety level II hood, non-coagulated blood samples obtained from each animal were inoculated into two 25cm^2 tissue culture flasks. One ml of whole blood was inoculated into a Liver Infusion Tryptose (LIT) medium containing 10% newborn calf serum (heat inactivated at 56° C for 30 minutes) and 1% penicillin-streptomycin. Flasks were incubated at 27° C for up to three months and were checked weekly under an inverted light microscope at 200X magnification. Positive cultures were expanded into 75cm^2 tissue culture flasks, and all parasite isolates were frozen in cryovials at -80C in a sterile solution of 90% newborn calf serum and 10% dimeththylsulfoxide.

Antigen Preparation

A 50 ml volume of LIT culture medium containing live epimastigote stages of *T. cruzi* in exponential growth phase was subjected to centrifugation at 1100 xg for 10 min. After centrifugation, the supernatant was discarded and the pellet was resuspended in a 30ml volume of DPBS. The sample was placed back into the centrifuge and was spun again at 1100 xg for 10 minutes. Using this same procedure, the pellet was washed a total of 3X in DPBS. After the final wash, the supernatant was discarded and the pellet

was resuspended in 3ml DPBS. An equal volume of 0.5% TritonX lysis buffer was added to the parasite suspension. Parasites in the suspension were disrupted using a Tissue-Tearor (Biospec Products, Inc., Bartlesville, OK) for 60 seconds, followed by a 5 min pause. This homogenization step was repeated 5 times. The sample was then subjected to centrifugation at 1800 x g for 30 min to remove non-solubilized material. The resulting supernatant was collected, and the protein concentration of the sample was determined using the Bio-Rad protein assay, based upon the method of Bradford (1976). Parasite antigen was then stored at –20C until use.

ELISA

A working solution of the parasite antigen extract (10 µl/ml) was prepared using DPBS as the diluent. Fifty µl of the diluted antigen was added to each well of a 96 well flexible microplate (BD Falcon; BD Biosciences, San Jose, CA). The plates were covered and refrigerated overnight. The following day, the plates were washed with three changes of DPBS. A Fisher Scientific Wellwash 4 Mk 2 (Fisher Scientific) automated plate washer was used for all plate washing steps. A blocking solution of nonfat dry milk (NFDM) in DPBS (2.5% w/v) was added to each well (200 µl per well) and the plates were incubated for 30 minutes at 37° C. Following the blocking step, the plates were washed 4 times in DPBS. Opossum serum samples were diluted in NFDM/DPBS (1/20 initial dilution). The samples were then further serially diluted in NFDM/DPBS (2x serial dilution) and assayed in duplicate. Plates were incubated for 1 hour at 37° C. The contents of each well were removed manually using a multichannel pipette and plates were then washed four times in DPBS as described above. A 1/1000 dilution of rabbit anti-opossum Ig in NFDM/DPBS was prepared and was added to all wells of each plate.

The plates were incubated for 1 hour at 37° C. Following this incubation step, plates were washed 4 times as described above. Fifty μl of a 1/1000 dilution of goat anti-rabbit Ig conjugated to horseradish peroxidase (HRPO) in NFMD/DPBS was added to each well and plates were incubated at 37° C. Contents were discarded and plates were washed 4 times in DPBS as described above. Finally, fifty μl of 3,3',5,5'- Tetramethylbenzidine (TMB) liquid substrate was added to each well and plates were kept in the dark for 15 minutes. After the 15 min incubation, the absorbance of each well was determined at a wavelength of 655 nm using a Bio-Rad 550 microplate reader (Bio-Rad Laboratories, Hercules, CA). Negative control wells, exposed to all reagents except opossum sera, were included on each of the assay plates. Samples with titers of 1280 or greater were considered positive.

IFAT

Immuno-fluorescence assay tests (IFAT) were performed on the serum samples obtained from raccoons and opossums by members of Dr. Michael Yabsley's laboratory at the Southeastern Cooperative Wildlife Disease Study located at the University of Georgia in Athens, Georgia. The IFAT procedure (Yabsley et al., 2001) utilized epimastigote stages of *T. cruzi* fixed to double-well glass sides. A commercially available goat anti-raccoon Ig conjugated with fluorescein isothiocyanate (FITC) or a goat-anti-opossum Ig conjugated with FITC (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted in a 1% solution of Evan's blue (Sigma Chemical Co., St. Louis, MO) in phosphate buffered saline were used as secondary antibodies to detect bound raccoon anti-*T. cruzi* antibodies or opossum anti-*T.cruzi* antibodies respectively.

Statistical Analysis

Data were analyzed using the Chi Square Test of Association.

RESULTS

Raccoon Trapping Results

A total of 44 raccoons were captured from nine sites in Warren and Barren Counties of Kentucky. There were 42 adult animals and two juveniles. Warren County produced 25 (57%) of the raccoons (from four sites), while Barren County yielded 19 (43%) of the raccoons in this study (from five sites). Sixteen of the 25 raccoons (64%) were taken from the Drakes Creek site in Warren County. Seven of the animals from that site were male (43.7%), nine were female (56.3%), 19 were adults (95%) and one was a juvenile (5%). Five of the Warren County animals (20%) came from Anna. Two of the five (40%) were male, three were female (60%), and there were four adults (80%) and one juvenile (20%). The Barren River site contributed three raccoons (12%). All of these were adult males. One of the 25 (4%) was taken from the North Campbell site and it was an adult female. Table 1 shows detailed descriptions of each raccoon trapped in Warren.

Table 2 shows detailed descriptions of each of the 19 raccoons taken from Barren County including sex, life stage (adult/juvenile) and trapping site. Ten raccoons were taken from the Cedar Grove site. Eight of the 10 (80%) were males and 2/10 (20%) were females. One female raccoon was taken from the Red Cross site. Five male raccoons (83%) and one female (17%) were caught at Bon Ayr. Two females came from the Temple Hill site. All raccoons taken from Barren County were adults.

Opossum Trapping Results

Forty-eight opossums, including two juveniles and seven nursing opossums were captured for this study. Of the 48 opossums captured, five (10.5%) were captured from

five sites in Barren County and 43 from the four sites in Warren County. A total of 31 opossums were captured at the Drakes Creek site. Twelve (38.7%) were male, 12 (38.7%) were female, and seven (22.6%) were kits taken from the marsupium, thus the sex was indeterminable. One of the 31 (3%) was a juvenile, 23 (74%) were adults, and seven (22.5%) were kits. Six opossums were taken from the Barren River site. Five (83%) were males and one (17%) was a female. All of the animals from this site were adults.

The North Campbell site yielded two opossums, one was a male and one female, and both were adults. Four Virginia opossums were captured at the Anna site. Three of the four were male and one was female. All four were adult animals. Table three shows detailed descriptions of each opossum trapped in Warren, including sex, life stage (adult/juvenile) and collection site. Table four shows a detailed description of the opossums caught in Barren County. Both of the opossums collected at the Cedar Grove site were males. One was a juvenile and one was an adult. Two adult opossums came from the Bon Ayr site. One was male and one female. A single adult female opossum was taken from Red Cross.

Animal	Sex	Life Stage	Site	Date Trapped	Latitude	Longitude
RW1	M	A	Barren River	7-13-07	N-36° 57.989'	W-086° 20.230'
RW2	F	A	Drakes Creek	7-17-07	N-36° 57.083'	W-086° 22.851'
RW3	M	A	Drakes Creek	7-19-07	N-36° 57.962'	W-086° 22.674'
RW4	M	A	Barren River	7-22-07	N-36° 57.989'	W-086° 20.230'
RW5	M	A	Barren River	7-25-07	N-36° 57.989'	W-086° 20.230'
RW6	M	A	Drakes Creek	7-25-07	N-36° 57.962'	W-086° 22.674'
RW7	F	A	Drakes Creek	7-27-07	N-36° 57.083'	W-086° 22.851'
RW8	F	A	North Campbell	8-01-07	N-37° 03.780'	W-086° 27.940'
RW9	F	A	Drakes Creek	9-8-07	N-36° 57.047'	W-086° 22.730'
RW10	F	J	Drakes Creek	9-8-07	N-36° 57.962'	W-086° 22.674'
RW11	M	A	Drakes Creek	9-14-07	N-36° 57.083'	W-086° 22.851'
RW12	F	A	Drakes Creek	9-18-07	N-36° 57.962'	W-086° 22.674'
RW13	M	A	Drakes Creek	9-18-07	N-36° 57.083'	W-086° 22.851'
RW14	M	A	Drakes Creek	9-18-07	N-36° 57.047'	W-086° 22.730'
RW15	F	A	Drakes Creek	9-19-07	N-36° 57.962'	W-086° 22.674'
RW16	M	A	Drakes Creek	9-20-07	N-36° 57.083'	W-086° 22.851'
RW17	F	A	Drakes Creek	9-25-07	N-36° 57.962'	W-086° 22.674'
RW18	F	A	Anna	9-26-07	N-37° 06.635'	W-086° 24.840'
RW19	M	A	Anna	9-27-07	N-37° 06.635'	W-086° 24.840'
RW20	F	A	Anna	10-1-07	N-37° 06.635'	W-086° 24.840'
RW21	F	A	Drakes Creek	10-1-07	N-36° 57.083'	W-086° 22.851'
RW22	M	J	Anna	10-1-07	N-37° 06.635'	W-086° 24.840'
RW23	F	A	Anna	10-2-07	N-37° 06.635'	W-086° 24.840'
RW24	F	A	Drakes Creek	10-2-07	N-36° 57.083'	W-086° 22.851'
RW25	M	A	Drakes Creek	10-1-07	N-37° 06.635'	W-086° 24.840'

Table 1. Raccoons trapped from Warren County, Kentucky between June 2007-December 2007. M=Male, F=Female, A=Adult, J=Juvenile.

Animal	Sex	Life Stage	Site	Date Trapped	Latitude	Longitude
RB1	F	A	Bon Ayr	6/9/2007	N-37° 00.480'	W-086° 04.756'
RB2	M	A	Red Cross	6/9/2007	N-37° 00.209'	W-086° 04.731'
RB3	M	A	Bon Ayr	6/12/2007	N-37° 00.480'	W-086° 04.756'
RB4	M	A	Bon Ayr	6/16/2007	N-37° 00.480'	W-086° 04.756'
RB5	M	A	Cedar Grove	6/22/2007	N-36° 55.740'	W-085° 55.775'
RB6	M	A	Cedar Grove	6/26/2007	N-36° 55.740'	W-085° 55.775'
RB7	M	A	Bon Ayr	6/30/2007	N-37° 00.480'	W-086° 04.756'
RB8	M	A	Bon Ayr	7/6/2007	N-37° 00.480'	W-086° 04.756'
RB9	M	A	Cedar Grove	7/9/2007	N-36° 55.740'	W-085° 55.775'
RB10	M	A	Cedar Grove	7/19/2007	N-36° 55.740'	W-085° 55.775'
RB11	M	A	Cedar Grove	7/23/2007	N-36° 55.740'	W-085° 55.775'
RB12	F	A	Cedar Grove	7/23/2007	N-36° 55.740'	W-085° 55.775'
RB13	F	A	Cedar Grove	7/26/2007	N-36° 55.740'	W-085° 55.775'
RB14	M	A	Cedar Grove	7/30/2007	N-36° 55.740'	W-085° 55.775'
RB15	M	A	Cedar Grove	8/7/2007	N-36° 55.740'	W-085° 55.775'
RB16	M	A	Cedar Grove	8/7/2007	N-36° 51.712'	W-085° 50.178'
RB17	F	A	Temple Hill	10/4/2007	N-36° 51.712'	W-085° 50.178'
RB18	M	A	Bon Ayr	10/4/2007	N-37° 00.480'	W-086° 04.756'
RB19	F	A	Temple Hill	10/22/2007	N-36° 55.740'	W-085° 55.775'

Table 2. Raccoons trapped from Barren County, Kentucky, between June 2007 – December 2007. M=Male, F=Female, A=Adult.

Animal	Sex	Life Stage	Site	Date Trapped	Latitude	Longitude
OW1	M	A	Drakes Creek	7/13/2007	N-36° 57.962'	W-086° 22.674'
OW2	F	A	Drakes Creek	7/13/2007	N-36° 57.047'	W-086° 22.730'
OW3	F	A	Drakes Creek	7/13/2007	N-36° 57.962'	W-086° 22.674'
OW4	F	A	Drakes Creek	7/13/2007	N-36° 57.083'	W-086° 22.851'
OW5	F	A	Drakes Creek	7/14/2007	N-36° 57.047'	W-086° 22.730'
OW6	F	A	Drakes Creek	7/16/2007	N-36° 57.083'	W-086° 22.851'
OW6-1	--	K	Drakes Creek	7/16/2007	N-36° 57.083'	W-086° 22.851'
OW6-2	--	K	Drakes Creek	7/16/2007	N-36° 57.083'	W-086° 22.851'
OW6-3	--	K	Drakes Creek	7/16/2007	N-36° 57.083'	W-086° 22.851'
OW6-4	--	K	Drakes Creek	7/16/2007	N-36° 57.083'	W-086° 22.851'
OW6-5	--	K	Drakes Creek	7/16/2007	N-36° 57.083'	W-086° 22.851'
OW6-6	--	K	Drakes Creek	7/16/2007	N-36° 57.083'	W-086° 22.851'
OW6-7	--	K	Drakes Creek	7/16/2007	N-36° 57.083'	W-086° 22.851'
OW7	M	A	Drakes Creek	7/20/2007	N-36° 57.962'	W-086° 22.674'
OW8	M	A	Drakes Creek	7/22/2007	N-36° 57.083'	W-086° 22.851'
OW9	M	A	Barren River	7/24/2007	N-36° 57.989'	W-086° 20.230'
OW10	M	A	Barren River	7/26/2007	N-36° 57.962'	W-086° 22.674'
OW11	F	A	Drakes Creek	7/28/2007	N-36° 57.083'	W-086° 22.851'
OW12	M	A	North Campbell	7/30/2007	N-37° 03.780'	W-086° 27.940'
OW13	F	A	North Campbell	7/30/2007	N-37° 03.780'	W-086° 27.940'
OW14	F	A	Drakes Creek	7/31/2007	N-36° 57.083'	W-086° 22.851'
OW15	M	A	Drakes Creek	7/31/2007	N-36° 57.047'	W-086° 22.730'
OW16	M	A	Drakes Creek	9/6/2007	N-36° 57.962'	W-086° 22.674'
OW17	M	A	Drakes Creek	9/6/2007	N-36° 57.047'	W-086° 22.730'
OW18	F	A	Drakes Creek	9/6/2007	N-36° 57.083'	W-086° 22.851'
OW19	M	A	Drakes Creek	9/6/2007	N-36° 57.962'	W-086° 22.674'
OW20	F	A	Drakes Creek	9/6/2007	N-36° 57.083'	W-086° 22.851'
OW21	F	A	Drakes Creek	9/18/2007	N-36° 57.047'	W-086° 22.730'
OW22	F	J	Drakes Creek	9/18/2007	N-36° 57.962'	W-086° 22.674'
OW23	M	A	Drakes Creek	9/18/2007	N-36° 57.083'	W-086° 22.851'
OW24	F	A	Drakes Creek	9/20/2007	N-36° 57.047'	W-086° 22.730'
OW25	M	A	Drakes Creek	9/25/2007	N-36° 57.962'	W-086° 22.674'
OW26	M	A	Drakes Creek	9/26/2007	N-36° 57.083'	W-086° 22.851'
OW27	M	A	Anna	9/26/2007	N-37° 06.635'	W-086° 24.840'
OW28	M	A	Drakes Creek	9/27/2007	N-36° 57.047'	W-086° 22.730'
OW29	M	A	Anna	10/1/2007	N-37° 06.635'	W-086° 24.840'
OW30	M	A	Barren River	10/2/2007	N-36° 57.989'	W-086° 20.230'
OW31	F	A	Anna	10/2/2007	N-37° 06.635'	W-086° 24.840'
OW32	F	A	Barren River	10/2/2007	N-36° 57.989'	W-086° 20.230'
OW33	M	A	Drakes Creek	10/2/2007	N-36° 57.083'	W-086° 22.851'
OW34	M	A	Barren River	10/9/2007	N-36° 57.989'	W-086° 20.230'
OW35	M	A	Anna	10/11/2007	N-37° 06.635'	W-086° 24.840'
OW36	M	A	Barren River	10/16/2007	N-36° 57.989'	W-086° 20.230'

Table 3. Opossums trapped from Warren County, Kentucky between June 2007-December 2007. M=Male, F=Female, A=Adult, J=Juvenile, K=kit.

Animal	Sex	Life Stage	Site	Date Trapped	Latitude	Longitude
OB1	M	A	Bon Ayr	6/7/2007	N-37° 00.480'	W-086° 04.756'
OB2	F	A	Red Cross	6/8/2007	N-37° 00.209'	W-086° 04.731'
OB3	M	A	Cedar Grove	6/22/2007	N-36° 55.740'	W-085° 55.775'
OB4	F	A	Bon Ayr	7/7/2007	N-37° 00.480'	W-086° 04.756'
OB5	M	A	Cedar Grove	7/9/2007	N-36° 55.740'	W-085° 55.775'

Table 4. Opossums trapped from Barren County, Kentucky, between June 2007 and December 2007. M=Male, F=Female, A=Adult, J=Juvenile.

Hemoculture and Serology

A total of 17 isolates of *T. cruzi* were successfully established by hemoculture of whole blood from raccoons in complete LIT medium. The length of incubation time between the initiation of the hemocultures and the first appearance of epimastigote stages of the parasite ranged from two to six weeks. Figure 3 shows epimastigote stages present in one of the positive hemocultures (RW10).

Figure 3. *T. cruzi* cultured from a whole blood sample taken from raccoon, RW10.

A total of 19/44 (43%) raccoon sera were found to be positive for anti-*T. cruzi* antibodies by IFAT. Table 5 lists the raccoons from Warren County, along with their hemoculture and IFAT results. Serological and hemoculture results for Barren County raccoons are summarized in Table 6. Fourteen of 25 raccoons (56%) from Warren County showed positive IFAT results and 14 (56%) were hemoculture positive for *T. cruzi*. Four of 19 raccoons (21%) from Barren County showed positive IFAT results, while only 3/19 (15.7%) showed positive hemoculture results.

Serological and hemoculture results from Warren County opossums are summarized in Table 7. Thirteen of the 43 opossums (30%) tested from Warren County were IFAT positive and 9/43 (21%) showed positive ELISA results; however, no *T. cruzi* isolates were successfully cultured from opossum blood samples. The serological results of opossums taken from Barren County are shown in Table 8. Figure 4 is a representative graph showing the ELISA data obtained for opossums OW2, OW4, OW11, and OB3. None of the opossum blood samples obtained from Barren County produced positive hemoculture results, however, 2/5 (40%) were IFAT positive and 2/5 (40%) were positive by ELISA.

Statistical Analysis

The Chi Square Test of Association showed no significant difference in *T. cruzi* infection rates between male and female raccoons. There were also no significant differences between infection rates in raccoons indicated by IFAT in this study and infection rates reported in previous studies from South Carolina and Georgia using IFAT (Yabsley et al., 2001; Yabsley and Noblet, 2002.)

Animal	Hemoculture	IFAT
RW1	-	-
RW2	+	+
RW3	-	-
RW4	-	-
RW5	+	-
RW6	-	-
RW7	+	+
RW8	-	+
RW9	+	+
RW10	+	+
RW11	+	+
RW12	-	-
RW13	+	+
RW14	+	+
RW15	+	+
RW16	-	+
RW17	-	-
RW18	+	+
RW19	-	-
RW20	+	-
RW21	+	+
RW22	+	-
RW23	-	+
RW24	+	-
RW25	-	+

Table 5. Hemoculture and IFAT results for raccoons trapped from Warren County, Kentucky.

Animal	Hemoculture	IFAT
RB1	--	+
RB2	--	+
RB3	--	-
RB4	--	+
RB5	--	-
RB6	--	-
RB7	--	-
RB8	--	+
RB9	--	-
RB10	--	-
RB11	+	-
RB12	+	-
RB13	-	-
RB14	+	-
RB15	--	-
RB16	--	-
RB17	--	-
RB18	--	-
RB19	--	-

Table 6. Hemoculture and IFAT results for raccoons trapped from Barren County, Kentucky.

Animal	Hemoculture	IFAT	ELISA	Titer	Maximum Abs
OW1	--	-	-	640	0.278
OW2	--	-	-	640	0.159
OW3	--	+	+	1280	0.446
OW4	--	+	-	640	0.55
OW5	--	+	+	2560	1.123
OW6	--	+	+	2560	1.404
OW6-1	N/A	-	N/A	N/A	N/A
OW6-2	N/A	-	N/A	N/A	N/A
OW6-3	N/A	-	N/A	N/A	N/A
OW6-4	N/A	-	N/A	N/A	N/A
OW6-5	N/A	-	N/A	N/A	N/A
OW6-6	N/A	-	N/A	N/A	N/A
OW6-7	N/A	-	N/A	N/A	N/A
OW7	--	+	+	2560	0.966
OW8	--	+	+	2560	0.4
OW9	--	-	+	2560	1.067
OW10	--	-	-	1280	0.245
OW11	--	-	-	640	0.637
OW12	--	+	-	10,240	0.477
OW13	--	-	-	640	0.302
OW14	--	-	+	10,240	1.256
OW15	--	-	+	5120	0.834
OW16	--	-	-	2560	0.698
OW17	--	-	-	640	0.650
OW18	--	-	-	10, 240	0.683
OW19	--	+	-	640	0.826
OW20	--	-	-	5120	0.826
OW21	--	+	-	5120	0.793
OW22	--	+	-	1280	0.539
OW23	--	-	-	2560	0.654
OW24	--	-	-	640	0.298
OW25	--	-	-	2560	0.462
OW26	--	-	-	10,240	0.498
OW27	--	-	-	1280	0.341
OW28	--	-	-	640	0.186
OW29	--	+	-	640	0.316
OW30	--	+	-	160	0.560
OW31	--	-	-	2560	0.337
OW32	--	-	-	320	0.438
OW33	--	-	+	320	0.9
OW34	--	-	-	81,920	0.256
OW35	--	+	-	640	0.494
OW36	--	-	-	640	0.356

Table 7. Hemoculture, ELISA and IFAT results from opossums trapped in Warren County.

Animal	Hemoculture	IFAT	ELISA	Titer	Maximum abs
OB1	--	-	-	320	0.251
OB2	--	-	-	5120	0.508
OB3	--	+	-	20480	0.517
OB4	--	-	+	5120	1.24
OB5	--	+	+	5120	0.864

Table 8. Hemoculture, ELISA and IFAT results from opossums trapped in Barren County.

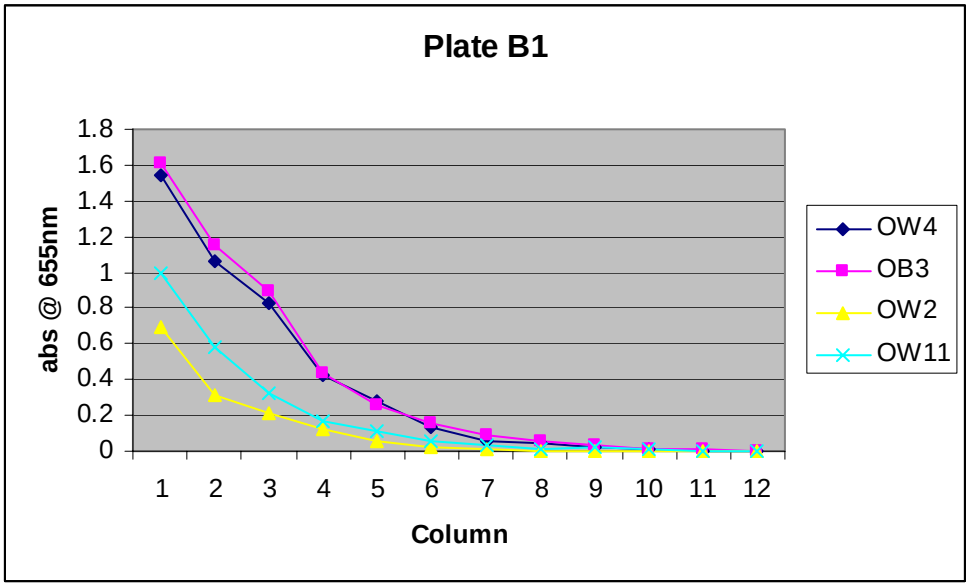

Figure 4. A representative graph showing antibody detection by ELISA for four opossum sera. A two-fold serial dilution of each serum sample was performed, starting with a 1/20 dilution of serum in column 1. Sera were assayed in duplicate, points indicate average absorbance at each dilution.

DISCUSSION

Despite the potential for autochthonous transmission to humans, *Trypanosoma cruzi* infection in wild mammals and triatomine bugs in the United States has not been well studied. However, the presence of the sylvatic cycle of *T. cruzi* has been documented in the following states: Alabama, California, Florida, Georgia, Louisiana, Maryland, Oklahoma, North Carolina, South Carolina, Tennessee, Texas and Virginia (Olsen et al., 1964; John and Hoppe, et al., 1986; Barr et al., 1991; Karsten et al., 1992; Pung et al., 1995; Pung et al., 1998; Pietrzak et al., 1998; Herwaldt et al., 2000; Yabsley et al., 2001; Yabsley and Noblet, 2002; James et al., 2002; Yabsley and Noblet, 2002; Hancock et al., 2005; Dorn et al., 2007; Hall et al., 2007). Although *T. cruzi* has been isolated from a variety of wild mammals, particularly in the southeastern United States, the present study represents the first report of the sylvatic cycle of *T. cruzi* infection in the state of Kentucky.

Although a wide variety of mammals are known to serve as hosts including, gray foxes (*Urocyon cineroargenteus*), striped skunks (*Mephitis mephitis*), macaques (*Macaca silenus*), lemurs (*Lemur catta*), Allegheny woodrats (*Neatoma magister*), nine-banded armadillos (*Dasypus novemcincthus*), big brown bats (*Eptiscus fuscus*), moles (*Neurotrichus gibbsii*), and dogs (*Canis familiaris*); *T. cruzi* infections in the northern raccoon *(Procyon* lotor) and the Virginia opossum (*Didelphis virginiana*) have been studied far more extensively than in any other mammal in the United States (Yabsley et al, 2001; James et al, 2002; Hall et al, 2007). This is likely due to a number of factors, such as the ubiquitous distribution of these animals, their abundance, their nuisance status in most areas, and the relative ease with which they can be trapped and handled.

Whereas raccoons are representative of the placental mammals, the opossum is the only marsupial that exists in the United States (Lotze and Anderson, 1979; McManus, 1974).

The location of the trapping sites and availability of resources and mates likely played a role in shaping the population densities of raccoons and opossums in this study. The entrance to the Drakes Creek site in Warren County is located in the Drakesborough subdivision, a residential area with over 100 homes and a commons area with a playground and tennis court. This site borders Drakes Creek and is between 1.5 and two miles from the city of Bowling Green, KY. Two of the traps at this site were placed approximately 100 meters from homes within the subdivision. The availability of water, anthropogenic food sources and shelter led to a high population of raccoons. There were 16 raccoons captured at this site, seven males (44%) and nine females (56%). The Anna site has an abundance of shelter and water but is in a more rural setting. It is located 6.5 miles from the city of Bowling Green, KY. There were five raccoons captured from this site, three females and two males. The Cedar Grove site has abundant shelter, water and anthropogenic food sources in terms of fruits, vegetables and livestock feed. There were eight male and two female raccoons captured at this site. These results suggest that where resources are abundant, males are much less territorial and competition among males is limited. Some of these males may have been litter-mates; however, considering that all but one of the males was an adult this is an unlikely explanation for the high concentration of males. The results for the opossums were very similar, Drakes Creek produced 24 opossums, 12 males (50%) and 12 females (50%) and Anna yielded four opossums, three males (75%) and one female (25%).

Trypanosoma cruzi is most commonly transmitted via interaction with the

invertebrate host, either during blood meals by the insect or from animals feeding on an infected bug. However, *T. cruzi* is also reported to be transmitted transplacentally (in eutherians) Gürtler et al. (2003) estimated that almost 850 congenital human cases of *T. cruzi* infection occurred in Argentina in 1993. Moreno et al. (2003) reported vertical transmission of *T. cruzi* in Wistar rats. Transmission has also been reported through the genitalia of mice (Herrera and Urdaneta-Morales, 2001). The role (if any) of vertical transmission in the sylvatic cycle of *T. cruzi* infection in the United States has not been determined. However, the results of genotypic analysis of a variety of raccoon isolates of *T. cruzi* have provided evidence that vertical transmission may be common in raccoon populations in the southeast.

In the present study, a total of 25/44 (57%) raccoons were positive for *T. cruzi*. Infection rates were highest in Warren County, where 18 of 25 raccoons (72%) were shown to be positive for *T. cruzi* by hemoculture or serological analysis. Fourteen of the animals were hemoculture positive and 14 were positive by IFAT. However, only 10/18 (56%) of the raccoons were positive by both hemoculture and IFAT. Four of the 18 raccoons (22%) were hemoculture positive only and four (22%) were IFAT positive only. In Barren County 7/19 raccoons (37%) were found to be positive. Four (21%) were positive by IFAT and three (16%) were positive by hemoculture.

The overall prevalence rate of 72% observed for Warren county is higher than has been reported in similar studies performed in other areas of the United States; whereas, results from Barren County (37%) are more consistent with previously reported infection rates (John and Hoppe 1986; Karsten et al., 1992; Pung et al., 1995; Yabsley et al 2001; Hancock et al., 2005). John and Hoppe (1986), who trapped raccoons in Oklahoma,

found 5/8 animals (63%) were infected when blood was cultured in diphasic blood agar medium (NNN medium). Karsten et al. (1992) reported a prevalence of 3/20 (15%) in raccoons from North Carolina, when blood was cultured in LIT medium. In 1995, Pietrzak and Pung (1998) found *T. cruzi* infections in 13/30 raccoons (43%) trapped from St Catherine's Island; a barrier island in Liberty County, Georgia. Eleven of the 30 (37%) were found to be positive by hemoculture in LIT medium. Twelve of 54 raccoons (22.2%) trapped in southeastern Georgia were positive by hemoculture in LIT medium (Pung et al., 1995). Therefore, the positive hemoculture results obtained for raccoons trapped from Warren County (56%) and Barren County (16%) fall within the range of hemoculture results reported from similar studies conducted in the southern United States.

Analysis of the sera collected from raccoons in this study showed a *T. cruzi* seroprevalence of 41% (18/44). This is consistent with the seroprevalence found in other parts of the United States. Yabsley et al. (2001) reported a seroprevalence in raccoons from southeast Georgia of 47/83 (57%) by IFAT and 45/83 (54%) by ELISA. Hancock et al. (2005) found 154/464 (33%) of serum samples from raccoons in Northern Virginia contained anti-*T. cruzi* antibodies by IFAT. Similarly, Yabsley and Noblet (2002b) reported that 104/221 (47%) of raccoons in South Carolina and Georgia were seropositive based on IFAT results.

There were no obvious trends observed in the infection rates of males vs. females or adults vs. juveniles. A Chi Square Test of Association confirmed there was no significant difference in *T. cruzi* infection rates between male and female raccoons. When the same analysis was performed on the data provided in Pung et al. (1995),

Yabsley and Noblet (2002a), there were also no significant differences observed between male and female infection rates. Comparisons were not made on the opossum samples because available results from other studies do not provide data from serological analyses to be compared with our results.

The high degree of sensitivity and specificity of the ELISA and the IFAT in humans is well known, with sensitivity rates for the IFAT reported to be around 95%-96% and 98%-100% for the ELISA (Partel and Rossi, 1998). When Yabsley et al. (2001) compared the use of ELISA and IFAT techniques to hemoculture for detecting *T. cruzi* infections in 83 raccoon samples, they found the IFAT and ELISA to have a sensitivity rate of 96%, while hemoculture was only 30% sensitive. Jansen et al. (1985) found the IFAT to be 97% sensitive when testing for *T. cruzi* in experimentally infected opossums (*Didelphis marsupialis*). In the present study, the highest overall percentage of IFAT positive raccoons was in Warren County, with a prevalence of 56%, compared to 21% in Barren County. Chi Square analysis of the data showed no significant differences between infection rates indicated by IFAT in this study and infection rates reported in raccoons from South Carolina and Georgia using IFAT (Yabsley et al., 2001; Yabsley and Noblet, 2002b).

Five (12%) of the opossum samples from Warren County showed positive results for *T. cruzi* antibodies using the ELISA method but were not judged to be positive by IFAT. Six opossums (14%) from Warren County were positive by IFAT but did not show positive results using the ELISA. There was a negative control included on each plate for the opossum ELISA, the values for the negative controls from each plate were averaged together and that value was used as the baseline in determining the titers.

However, no positive control was used because we did not have an opossum sample that was known to contain high levels of anti- *T. cruzi* antibodies. This made it difficult to interpret the ELISA data because there was some variation observed among plates. It would be useful to repeat the ELISA for each of the opossum samples and include a positive control to verify the results obtained so far, particularly since the IFAT results were not completely consistent with the ELISA results.

There were 7/25 (28%) raccoon samples that produced positive hemocultures but tested negative by IFAT. This could be due to the raccoon being captured in the early stages of infection before measurable levels of anti-*T. cruzi* antibodies appear in the serum. Barr et al. (1991) determined that the first detectable antibody response was 27 days postinfection when dogs were experimentally infected with *T. cruzi*. Jansen et al. (1991) determined that antibody levels were not detectable until 4-5 weeks postinfection, while parasitemia levels were found to peak 2-3 weeks after experimental infection of opossums with *T. cruzi*. Another possible explanation for the discordant results may be errors in the interpretation of IFAT results. It will be necessary to perform the ELISA on the raccoon samples to confirm the IFAT tests.

There were no positive hemoculture results from any opossums in this study. This is difficult to explain given that the protocol for culturing the opossum samples was the same as that for culturing the raccoon samples. The cultures were maintained in the same incubator as the raccoon samples at 27°C. On one occasion the temperature of the incubator rose unexpectedly to 35°C for up to 48 hours. This fluctuation may have caused problems for the cultures; however, there were raccoon samples in the incubator at the same time that subsequently showed positive results. Also, it had been five months

since the first samples had been placed in the incubator and there had been no positive results, so it is unlikely that the temperature fluctuation was responsible for the lack of positive cultures. In addition, there was a greater tendency for the culture flasks inoculated with opossum blood to show bacterial or fungal contamination. Even when following the same protocol for removing blood from the raccoons and opossums, it was more difficult to obtain a sterile blood sample from opossums. Another possible explanation for the lack of positive hemoculture results from opossums is that the immune system of the opossum is highly efficient (Belov et al., 2007). The normal values which have been reported for numbers of circulating leucocytes in the circulatory system of the opossum is much higher than that of the raccoon (Strolle et al., 1978). This may support the possibility that the opossums are able to clear the parasites from the bloodstream more efficiently than raccoons.

More samples from rural sites to increase the sample size would be useful in further analysis of rural vs. peridomestic prevalence rates. In addition, studies involving the capture of triatomine vectors and the capture of juvenile animals would be useful in explaining the apparent high rate of transmission of the parasite in the southeastern United States.

The increased population density of raccoons and opossums in peri-domestic areas would be expected to increase the possibility of zoonotic transmission of *T. cruzi* to humans. The common vector for *T. cruzi* in the southeastern U.S., *Triatoma sanguisuga*, is most often found in animal nests and burrows (Pung et al., 1995). However, the bug is known to occasionally inhabit human dwellings (Zeledon, 1974; Beard et al., 1988; Pung et al., 1995; Herwaldt et al., 2000; Dorn et al., 2007). The likelihood of transmission of

T. cruzi from triatomine bugs to humans in the United States is thought to be very low There are only seven documented indigenous cases of Chagas disease in the country, and vector transmission to humans may be rare due to the sylvatic habits of *T. sanguisuga* and their delayed defecation behavior (Zeledon, 1974, Pung et al., 1995, Dorn et al., 2007). In contrast, there have been numerous reports of *T. cruzi* infection in domestic dogs in the United States (Barr et al., 1991; Meurs et al., 1998; Herwaldt et al. 2000; Dorn et al., 2007). With the increased migration of humans into the habitats of animals known to harbor *T. cruzi,* there are increased opportunities for humans and their pets that may feed on infected animals to come into contact with the parasite. The findings of this study extend the range of *T. cruzi* into the southcentral region of Kentucky, and, to my knowledge, this is the first report of the parasite in the state. This finding is important because domestic dogs in rural areas commonly feed upon raccoons and opossums, and raccoon hunting is a common sport in the region. There are ample opportunities for people to come in contact with infected animals and potentially infected triatomine bugs.

The virulence of parasite strains found in mammals in the United States reportedly varies from the sylvatic strains known to exist in Central and South America (Lisboa et al., 2007; Hall et al., 2007; Roellig et al., 2008). Although beyond the scope of the present study, additional experiments are needed to investigate the selective infectivity and virulence of type I and II strains (and substrains) of *T. cruzi* in laboratory bred eutherians and metatherians. In addition, specimens in this study were only collected in two counties in south central Kentucky. Future studies will be required to determine the prevalence of *T. cruzi* infection in other parts of the state, and to determine the prevalence of the parasite in other mammals and triatomine insects. The public health

significance of *T. cruzi* in the United States has been ignored for too long. Further studies are urgently needed to fully assess the presence and prevalence of *T. cruzi* in other areas of the United States where surveys have not yet been performed.

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