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DETERMINING THE PREVALENCE OF *TRYPANOSOMA CRUZI* IN ROAD-KILLED OPOSSUMS (*DIDELPHIS VIRGINIANA*) FROM BALDWIN COUNTY, GEORGIA, USING POLYMERASE CHAIN REACTION

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ABSTRACT

Twenty-nine road-killed opossums (*Didelphis virginiana*) collected from Baldwin County, central Georgia, were tested for the presence of *Trypanosoma cruzi* with polymerase chain reaction (PCR) analysis. Utilizing a cell culture containing *T. cruzi* epimastigotes, a positive control was established by injecting parasites into opossum heart tissue. DNA was extracted from both the control and sample heart tissues using a Proteinase K protocol. PCR analysis of the isolated DNA with *T. cruzi*-specific primers indicated the presence of parasite DNA in at least 10.3% of the opossums in the Baldwin County sample. This study demonstrates that PCR of extracted DNA from road-killed specimens is an effective method for detecting the presence of *T. cruzi* in opossums.

Key words: *Trypanosoma cruzi*, American trypanosomiasis, PCR, *Didelphis virginiana*

INTRODUCTION

American trypanosomiasis, caused by the parasite *Trypanosoma cruzi* and commonly known as Chagas' disease, has plagued South American cultures since at least 7050 B.C (1). The World Health Organization considers American trypanosomiasis to be the most serious parasitic disease in Latin America (2). Globally, it may be the third largest parasitic disease burden, after malaria and schistosomiasis (3). Despite major advances against the vectors of *T. cruzi*, the impacts of the protozoan are staggering: 16–20 million people infected and 40 million at risk in Central and South America, with an estimated 200,000 new cases and 21,000 deaths associated with this disease annually (4, 5). While autochthonous vector-borne cases of infection are rare in the United States (6), recent estimates indicate that over 300,000 individuals within the Latin American immigrant populations are infected with *T. cruzi* (7).

Trypanosoma cruzi is a heteroxenous flagellate exhibiting a sylvatic transmission cycle vectored to mammalian reservoir species by triatomine insects, blood-feeding reduviids which defecate infective trypomastigotes onto the skin, coat, or nests of host species. The trypanosome may enter the mammalian host through mucous membranes or an open wound; once circulating in the animal's bloodstream, it is capable of invading any tissue (8). Humans are rarely aware that they have had contact with the vector, and if the initial parasitemia is symptomatic, the conditions are similar enough to common viral infections to be dismissed (9). *Trypanosoma cruzi* is cardiomyotropic, and infected individuals may be unaware of the parasite's presence for thirty years before symptoms of heart damage appear. Once heart disease is evident, treatment only alleviates the symptoms; the disease is ultimately fatal (4, 5).

Because autochthonous vector-borne cases of American trypanosomiasis are relatively rare in the United States, only limited population studies on the presence of *T. cruzi* in host species have been conducted. The parasite has been reported in both triatomine insects and mammalian hosts across the southern half of the United States below latitude 42N – from northern California to Maryland (10). The parasite was not accidentally imported to the United States, but has moved concurrently with native marsupial reservoirs and insect vectors (11) from South to North America. Reports of *T. cruzi* infections of wild and domestic mammals of the United States were reviewed by John and Hoppe (12). In the Southeast, opossums (*Didelphis virginiana*) and raccoons (*Procyon lotor*) appear to be the most commonly infected mammalian hosts. McKeever et al. (13) reported *T. cruzi* infections in 88 of 552 (15.9%) opossums examined from southwestern Georgia and northwestern Florida. Olsen *et al.* (14) found *T. cruzi* in 17 of 126 (13.5%) opossums and 5 of 35 (14.3%) raccoons from east-central Alabama. Barr *et al.* (15) identified *T. cruzi* in 18 of 48 (37.5%) opossums from southern Louisiana. Pung *et al.* (16) observed *T. cruzi* in 6 of 39 (15.4%) opossums and 12 of 54 (22.2%) raccoons from southeastern Georgia. Pietrzak and Pung (17) found *T. cruzi* in 13 of 30 (43.3%) raccoons examined from St. Catherines Island, Georgia. Yabsley and Noblet (18) reported *T. cruzi* infections in 104 of 221 (47.0%) raccoons from South Carolina and the Piedmont of Georgia.

In the previous research on mammalian reservoir species, the presence of the parasite has been detected either by microscopic examination of blood or blood culture, or by serologic testing for antibodies to the parasite (13,16,18,19). Although commonly used, these methods are not entirely reliable. Trypanosomes are difficult to find in the bloodstream after the initial stages of infection. A small number of the parasites may complete the entire life cycle instead of encysting in tissue, but the probability that these trypanosomes will be taken in a blood sample is low. Testing for antibodies is sensitive and reliable, as long as no other pathogens, such as *Leishmania*, are present to cause a false-positive result (20, 21). Polymerase chain reaction (PCR) is more sensitive than the previously mentioned methods, as it enables

the amplification of DNA sequences that are unique to the parasite itself. Also, PCR does not involve the difficulties of obtaining fresh blood samples required for use in other methods. Kirchhoff *et al.* (22) demonstrated the greater sensitivity of PCR compared to microscopic methods of detecting *T. cruzi*. PCR has been used to confirm the presence of *T. cruzi* in an 18-month-old boy in Tennssee (23) and in dead triatomines collected from the residence of an infected individual in Louisiana (6). The caveat to PCR analysis is that it alerts only to the presence of *T. cruzi* DNA. It is not indicative of a live infection, though one supposes that a successful immune reaction against *T. cruzi* would destroy all aspects of the foreign material (20, 21, 22).

In the present study of road-killed wild opossums (*Didelphis virginiana*), we tested tissue from the heart muscles (where the amastigote form of the parasite prefers to encyst) for the presence of *T. cruzi* DNA utilizing PCR. It was expected that approximately 15% of these specimens would prove positive for *T. cruzi*, assuming that the Baldwin County opossum population exhibits the average rate of infection as determined by previous studies conducted in Georgia (13, 16).

MATERIALS AND METHODS

Collection of Specimens: During the winter months of 2004, twenty-nine road-killed (7 females, 20 males, 2 sex-undetermined) opossums were collected from Baldwin County, Georgia, as part of a morphological study of the species (24, 25, 26). None of the carcasses showed evidence of significant decomposition. The opossums were fresh frozen as found at -20°C and collectively skinned and eviscerated in early April. At that time, the heart, liver, and spleen, as available, were collected from each specimen and fresh frozen at -20°C until removed for DNA extraction in March 2005.

Establishment of Controls: A sample of live *T. cruzi* Brazilian epimastigotes in liver-infusion tryptose was obtained from the Department of Emerging and Tropical Diseases at the University of Georgia and used to make a positive control. One milliliter of culture was aseptically transferred to a microcentrifuge tube. The epimastigotes were spun out of the culture at 3000 rpm for five minutes and resuspended in phosphate buffered saline (0.2g KH_2PO4, 1.41g Na_2HPO_4, 0.2g KCl, 8g NaCl, pH 7.4, sterile). A visual count under the microscope determined an estimate of the number of epimastigotes per microliter of solution. A serial dilution was performed to reduce the concentration of epimastigotes. One opossum heart was randomly chosen to provide fifty 4 mm^3 control samples. Each sample was injected with approximately 500 live epimastigotes suspended in 5µL of phosphate buffered saline. A pork heart was obtained from a local slaughterhouse to act as a negative control. An additional sample with no living tissue or DNA was also run to check for contamination and also serve as an absolute negative control.

DNA Extraction: The A DNA extraction method was adapted from a protocol in the Molecular Cloning manual (27). Two pieces of tissue

approximately 4mm^3 were cut from the left ventricle apex wall of each heart, cut into fine pieces with a disposable razor blade, and deposited into a 1.5 mL microfuge tube with 600 µL of mammalian cell lysis buffer (10 mM Tris HCl, pH 8.0; 1 mM EDTA, pH 8.0; 0.1% SDS). To aid in breakdown of proteins and enzymes, 20 µL of Proteinase K were added and the contents were mixed by vortexing. The tubes were placed in a 55°C incubator for a minimum of five hours. The tubes were vortexed occasionally during the incubation period. The tubes were allowed to cool to room temperature for twenty minutes after removal from the incubator. To precipitate the proteins and cell lysate, 200 µL of potassium actetate (60 mL 5M potassium acetate; 11.5 mL glacial acetic acid; 28.5 mL H_2O) were added to each tube, and vortexed for twenty seconds. The tubes were then spun in a Sorvall RMC14 4°C centrifuge at ~12.5 x 1000 rpm for six minutes. The supernatant was poured into a fresh tube with 600 µL of 100% isopropanol. Each tube was vortexed briefly and centrifuged in a Labnet International Spectrafuge 24D at room temperature at 13.3 x 1000 rpm for two minutes. The supernatant was discarded and the remaining DNA pellet was washed with 600 µL of 95% ethanol. The tubes were inverted five times and the DNA pellet was spun down at room temperature at full speed for two minutes. The ethanol wash was repeated, the supernatant discarded, and the tubes set to air dry on their sides for fifteen minutes. The DNA was resuspended in 50 µL H_2O, allowed to sit at room temperature for five minutes, then placed in the freezer at -20°C.

Polymerase Chain Reaction: Each tube contained 25 µL of PCR reaction mix based on Promega *Taq* DNA polymerase in storage buffer B (20 mM Tris HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween 20, and 0.5% Nonidet-P40). The reactions contained 1X Magnesium-free Promega buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100), 0.2 mM dNTP, 2.2 mM of each primer, and 2.5 units/100 µL total volume *Taq* DNA polymerase. Template DNA (2.5 µL) and $MgCl_2$ (3.2 mM) were added to each tube. The primers employed were S-35 and S-36, *T. cruzi* specific primers flanking a 330 bp length of kinetoplast minicircle DNA (22, 23, 28). Amplification was carried out in a Techne TC-312 thermocycler. Initial denaturation was set at 94°C for 10 minutes. The reaction lasted 40 cycles, each of which included one minute of denaturation at 94°C, 2.5 minutes of annealing at 65°C, and 3 minutes of elongation at 72°C. Final elongation lasted 10 minutes at 72°C (29). Upon completion, the reaction tubes were held in the thermocycler at 4°C until removed for electrophoresis. Two tubes, one containing naked *T. cruzi* DNA as template and the second substituting H_20 were added to the reaction to act as absolute references when judging bands in the gel. As previously mentioned, positive and negative controls derived from heart tissue were also included to add the element of mammalian DNA to the reaction.

Gel Electrophoresis: Electrophoresis was carried out on a Promega agarose 2% gel at 50 volts, and viewed at 20, 30 and 40 minutes. Ethidium

bromide was the intercalating agent that illuminated bands of amplified DNA. Each well carried 10 µL of PCR product, with the exception of the amplified naked *T. cruzi* DNA, of which only 5 µL were used to reduce the intensity of that band. Three lanes spaced across the gel were designated to carry naked *T. cruzi* DNA to serve as an absolute reference for the 330 bp kDNA band and also to correct slant, if necessary. The gels also included an absolute negative control with water substituted for the DNA template, a negative control with pork heart DNA, and a positive control in which opossum heart samples were injected with *T. cruzi*. The results were viewed with a BioChemi UVP BioImaging System. For samples that produced inconclusive results, further testing with careful increase of *Taq* to 5 units/ 100 µL total volume and associated increase of $MgCl_2$ to 3.6 mM yielded more conclusive data.

RESULTS

Three of twenty-nine hearts, one female and two males, were positive for the 330 bp kDNA minicircle sequence, indicating a 10.3% infection rate in the Baldwin County opossum population. The three positive specimens were collected from widely separated localities that when plotted on a map are arranged in a triangle around Milledgeville. The distance from locality A to B is approximately 2 miles, from A to C is approximately 4 miles, and from B to C is approximately 5 miles. This pattern suggests three independent infection events; it is unlikely that these individuals were litter mates exposed to an infected triatomine in the same nest. A litter-mate scenario also would have required dispersal across a major waterway (Oconee River).

In the gels, the lanes that served as an absolute reference for the 330 bp kDNA band also showed characteristic 660 bp and 990 bp bands in which the amplified DNA was linked (21). The positive control carried a 330 bp band as prominent as the naked *T. cruzi* band, and also showed a 660 bp band. The pork heart negative control was dubious as to its usefulness, as it carried a band slightly smaller than the 330bp parasite DNA. Upon intensification of the light in the BioImiaging unit, this smaller band would be transformed into a smear that encompassed the area where the 330 bp band would have been, had it been there. It was also entirely possible that the pig whose heart was used could have come in contact with the triatomine insects that carry *T. cruzi*, as it was a locally grown pig living in elements similar to those of the opossum. Fortunately, the absolute negative control could be relied upon with confidence in this situation. All of the lanes carried a low molecular weight primer-dimer band, and many carried a second slightly larger low-weight band, including the absolute negative control. This second band was probably another manifestation of primer-dimer in which the two short sequences bind at their 3' ends. Subsequent viewings of the gel after additional electrophoresis showed no further separation of these two bands, indicating that they were very close in size.

DISCUSSION

Urbanization of Georgia's rural areas has placed residents into closer contact with known reservoirs of the protozoal parasite *Trypanosoma cruzi* and the triatomine insects that serve as vectors. Previous studies of reservoir hosts relied on microscopic examination of blood or serologic tests for antibodies. These methods are not entirely reliable, as trypanosomes invade tissues and are rarely found in the bloodstream after the initial stages of infection. Serologic tests are more reliable, but cross-reactive antigens may result in false-positives. With both of these methods, fresh blood is required. This study tested heart muscle tissue, where the parasite prefers to encyst, and does not require a sample of fresh blood. As previously stated, this procedure eliminates the difficulty involved in trapping live animals and taking blood samples.

Results of the present study demonstrate that collection of road-killed mammals and PCR of extracted DNA is an appropriate and effective method for detecting the presence of *T. cruzi* in opossums. The 10.3% infection rate of Baldwin County opossums is similar to the infection rates of opossums obtained in studies conducted on the Coastal Plain of Georgia and Piedmont of Alabama using other diagnostic techniques (13, 14, 16). The PCR obtained results appear to be as reliable as immunohistochemical indicators of *T. cruzi* infection. PCR analysis has the benefit of being both quicker and simpler. Other methods take longer, require more reagents, and/or are more difficult to carry out.

This method of collection and testing may be useful in conducting comprehensive surveys of *T. cruzi* prevalence throughout large areas and in multiple mammalian hosts. Collection of road-killed mammals is less difficult and requires less training than trapping live animals and collecting blood samples. Although badly decomposed specimens may not be suitable for testing, in most cases encysted *T. cruzi* DNA should be detectable in the heart muscle. This is certainly the case if the road-killed mammals were collected during periods of freezing temperatures.

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