Identifying Chagas' Disease Reservoirs with PCR and Next-Generation DNA Sequencing

Nicolas J. Means[1], Darlisha Owens[2], Troy Kieran[3], Travis C. Glenn[3], Nicole Gottdenker[3]

Oklahoma State University, Stillwater, OK[1] Grambling State University, Grambling, LA[2] University of Georgia, Athens, GA[3]

Abstract

American trypanosomiasis (Chagas' Disease) is a zoonotic vector-borne disease caused by the protozoan parasite *Trypanosoma cruzi*, and is an important cause of morbidity and mortality in Latin America. *T. cruzi* circulates between reservoir hosts (wild and domestic mammals) and hematophagous triatomine insect vectors. Humans are susceptible to the disease once infected with the parasite by contact with the infected insect vector, ingestion of food or drink contaminated with the pathogen, transplacental transmission, or by transfusion with infected blood or tissue transplants. Research has shown that blood meal analysis, via standard PCR and sequencing, are capable of identifying host reservoirs down to the species level, but these techniques are limited because: 1) they often cannot identify multiple blood meals within a vector, 2) they cannot be used to simultaneously detect vector infection with trypanosomes or coinfection with other pathogens, and 3) they may require a relatively large amount of vertebrate reservoir DNA, which may be degraded in the insect vector. The objective of this study is to standardize next generation sequence methodologies for simultaneous blood meal species identification and trypanosome infection within kissing bugs, *Rhodnius pallescens,* a triatomine vector of Chagas' disease.

To prepare samples for next generation sequencing, we had to quantify the amount of DNA extracted from *R. pallescens*, normalize the DNA concentrations, and optimize the PCR conditions for each portion of the next generation 'Taggimatrix' technique. The samples were put in three different groups consisting of high, medium, and low concentration DNA. For optimization of the PCR, there were a series of tests with a known insect and mammal DNA. Conditions such as number of cycles, temperature and time changed throughout each experiment. We then used the optimized PCR conditions on the DNA from the three groups (high, medium and low) to obtain amplification of mammal and trypanosome DNA. From the tests, we found that 30% of the new samples that were collected had the trypanosome parasite within the DNA of the insect while 68% showed mammalian DNA within the blood meal.

Introduction

- Chagas' Disease- a tropical disease caused by *Trypanosoma cruzi*.
- This parasite's life cycle includes reduviid bug vectors, (the kissing bugs). This insect transmits the parasite infection between many of the mammal species from which it feeds. Although vectors can feed from non-competent species (avian, reptile, and amphibian), only mammals are can transmit *T. cruzi* to vectors.
- Research has shown that blood meal analysis, via standard PCR and sequencing techniques, are able to identify host reservoirs down to the species level, but only if the amount of host DNA is in a high enough concentration in relation to the insect DNA. Furthermore, these techniques generally only work when a vector only feeds from a single species, but one insect vector may potentially feed from more than one vertebrate reservoir.
- **Next generation sequencing techniques** have the potential to revolutionize blood meal analysis and pathogen detection in triatomine vectors, allowing for better understanding of relationships between these mammalian reservoirs and their hosts, and to simultaneously detect a wide variety of pathogens within the insects, and to evaluate potential interactions between these pathogens and *T. cruzi* transmission.
- **Taggimatrix** is a series of PCR reactions that allow the use of multiple tags, which are used for identification purposes. With these tags and sequencing methods, we can pool many samples and **identify blood meal species**, despite a small amount of DNA in relation to the kissing bug, as well as identify trypanosome and other infections.

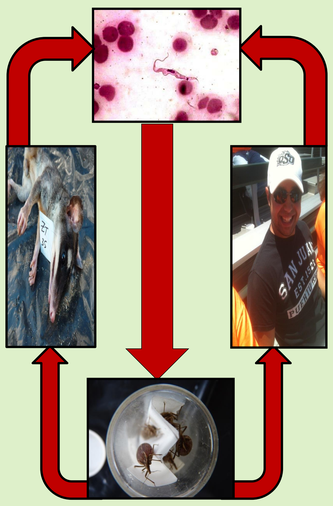
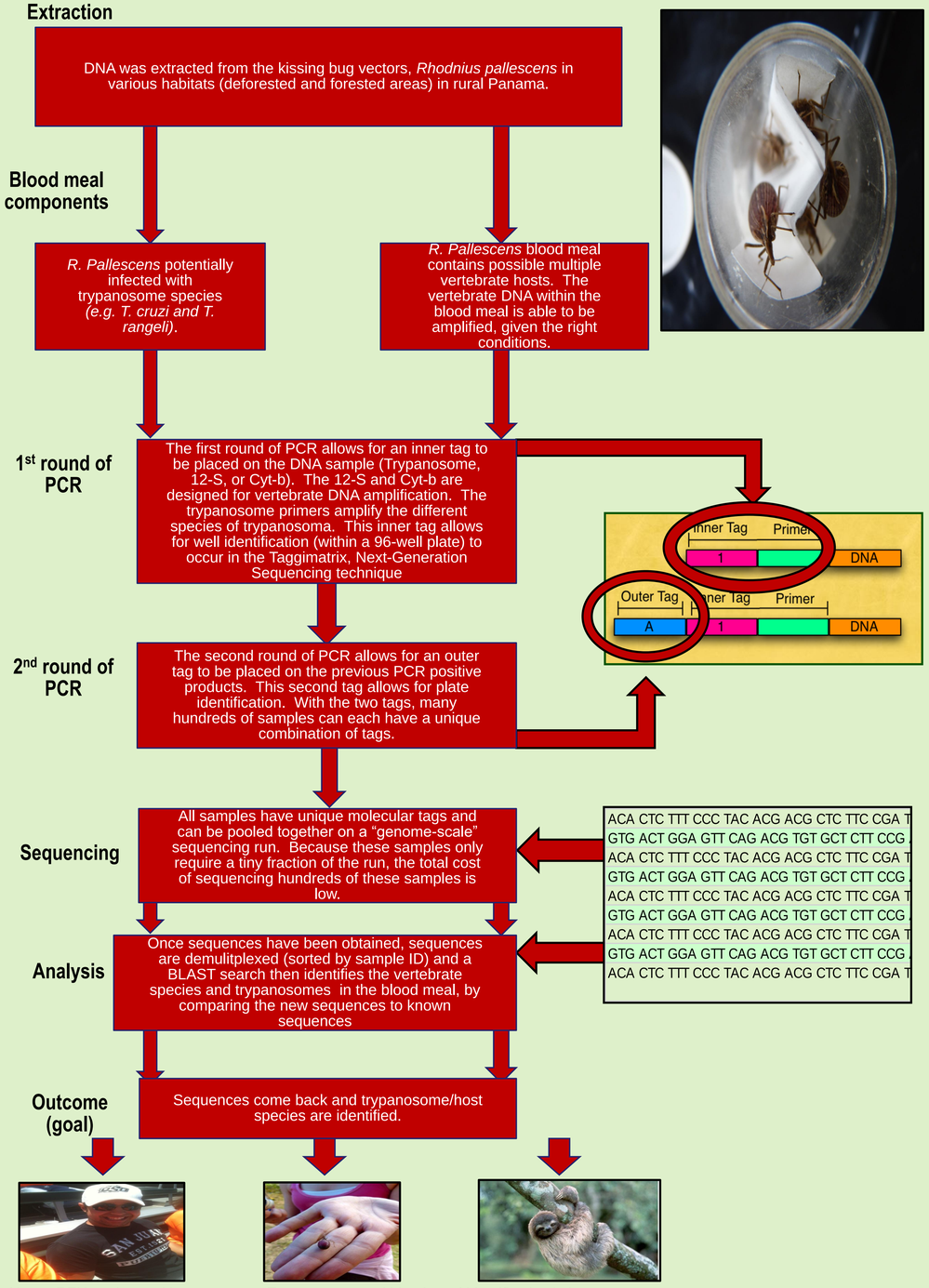


Figure 1: A general schematic of linkages of the trypanosome lifecycle with vectors, potential mammal reservoirs, and humans

Objectives

- Optimize PCR procedures for vertebrate blood meal amplification in insect vectors while excluding insect DNA
- Run PCR for identification of *Trypanosoma spp.* Presence
- Run PCR for the 12-S and Cyt-b amplification of mammalian DNA in sampled blood meals
- Perform Next-Generation Sequencing for Cytochrome-b and 12-S to identify blood meal contents for mammalian species
- Analyze sequences of mammalian hosts and perform a BLAST search for species identification
- Correlate results with the environmental habitat of the insect sample

Methods



Happy REU students with expertise in NGS techniques

Trypanosome and blood meal identification in insect vectors

Results

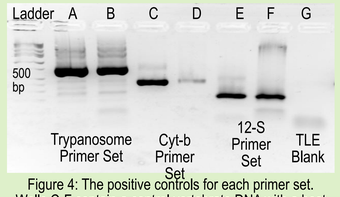


Figure 4: The positive controls for each primer set. Wells C-F contain a control vertebrate DNA with a host reservoir DNA sample. Wells A-B contain two control positive Trypanosome insects. Well G is the standard blank negative

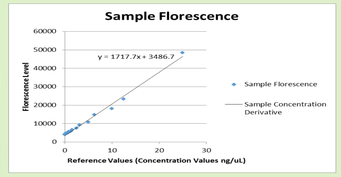


Figure 5: A concentration calculation from the plate reader data of fluorescence. Fluorescence values over the reference concentrations gave the linear regression formula used to calculate sample concentration

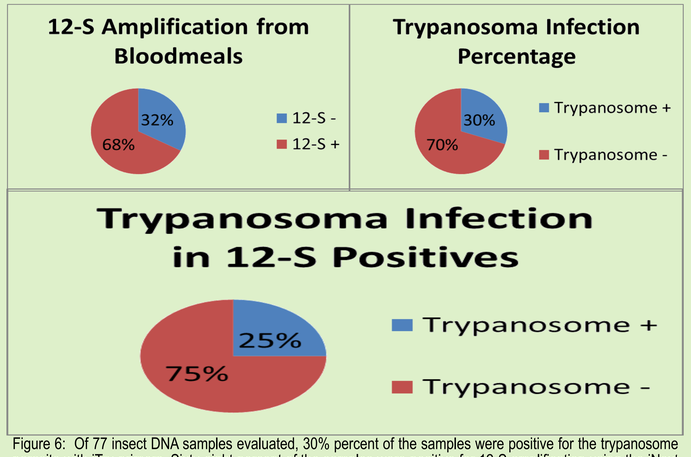


Figure 6: Of 77 insect DNA samples evaluated, 30% percent of the samples were positive for the trypanosome parasite with iTru primers. Sixty-eight percent of the samples were positive for 12-S amplification using the iNext primers. From the positive 12-S samples, 25 percent of the samples were also infected with the trypanosome parasite.

Discussion

- Most of the DNA that was worked with had very low concentrations
- With test samples of pure mammal DNA, amplification was easily achieved for both cytochome b and 12S, without amplifing contaminating insect DNA. However, the amount of mammalian DNA in the insect blood meals required modified procedures (increased cycle numbers). Cytochrome b was not successfully amplified from insects with blood meals and 12-S amplification was possible mainly in fresh insect samples.
- iNext primers were used to amplify the 12-S samples, a note-worthy observation. We were using iTru primers to test the original samples, which did not amplify these same samples. Primer design is extremely important.
- The percentage of trypanosome infection was much lower than previous studies have shown, the trypanosome primers may not be the exact primers that would work best on all the samples. This is also a significant reduction in sample size from the previous studies.

Future Directions

- Continue to perfect the PCR settings for the iTru primers and start to experiment with the different iNext primers. The iNext primers seem to work better with the 12-S amplification of the insects.
- Since all results are based on the new gourp of samples, tests with the older group can begin. PCR conditions and primer design can be optimized to deal with these old DNA samples.
- Preliminary tests can be done with the trypanosome primers with the old group of samples. Several control positives for these samples did produce PCR products when the amplifications were repeated.
- Bioinformatic analysis of the sequences can occur once the samples are amplified. The sequences can be analyzed and compared to known sequences through a BLAST search.

Acknowledgements

We would like to give special thanks to the University of Georgia, The College of Veterinary Medicine, The College of Public Health for letting us use their facilities and resources. Also, a special thanks to the National Science Foundation for their funding of the REU program and the project for the summer of 2014 at the University of Georgia. Funding for laboratory work was provided by an OVPR Faculty Seed Grant from the University of Georgia.