A NOVEL PROTEIN-BASED BLOOD MEAL IDENTIFICATION METHOD FOR CHAGAS DISEASE INSECT VECTORS

A proposal presented

By

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Submitted to the dissertation committee members of Judith Ina Keller in partial fulfillment of the requirements for the Ph. D. Proposal Examination.

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Proposal Exam: 30 June 2016
11 am Jeffords 326

Title - A novel protein-based blood meal identification method for Chagas Disease insect vectors

Summary - Chagas disease (CD) is a complex vector borne disease involving blood feeding Triatominae (Hemiptera: Reduviidae) insect vectors, also known as kissing bugs, and insect vector blood meal hosts. This disease has a tremendous impact on millions of people and is a global health problem (1). The etiologial agent of CD, the Trypanosome parasite *Trypanosoma cruzi* (Kinetoplastea: Tripanosomatida), is introduced into the host blood stream from the Triatominae insect's feces during a blood meal. Identifying blood meal sources of Triatominae vectors is critical in understanding CD transmission dynamics, can lead to intermediate host identification, and aids management decisions. Several techniques, mostly DNA-based, are available for blood meal identification. However, identification techniques that can detect blood meal sources of low quality and quantity, and determine multiple blood meals with great confidence, are lacking. The goal of this proposal is to addresses this gap in knowledge with a proteomics-based approach, using liquid chromatography tandem mass spectrometry (LC-MS/MS) host-specific hemoglobin peptides for blood meal identification. Subsequently the following questions are addressed: **Q1) How can protein-based LC-MS/MS blood meal identification techniques aid in the management of CD? Q2) What are the limitations of a protein based method for blood meal identification? For example, how long after a blood meal or insect molt can we detect blood meals? What is our detection ability when it comes to multiple blood meals? What are the optimal conditions for our methods to work? Q3) How are blood meals changing in two endemic regions of Central America, such as Guatemala and El Salvador, and how do they vary in relation to location? How prevalent are blood meals on opossum, a major Triatominae life cycle bridge species in these regions? Q4) Out of various blood proteins, is hemoglobin the most diverse in terms of peptides that can lead to blood meal species identification?** This study is important because it will provide insights into efficient and accurate evaluation of blood meal detection in CD vectors of Latin America where the disease is endemic, and has the potential to be applied to other disease vector systems. Evaluating vector blood meal sources as they relate to disease transmission aids in finding sustainable vector controls (2).

Intellectual Merit - My work will represent the first study using proteomic methods for blood meal identification in CD vectors. The intellectual merit of evaluating these aims is its transformative and innovative approach, comparing protein and DNA techniques, with the goal of enhancing these blood meal detection methods. A second potential far reaching benefit includes the direct application of results to CD prevention, management, and surveillance in endemic areas of Latin America and anywhere CD is present. In a time where environments are drastically changing and climate change influences disease systems, often negatively, identifying vector blood meal hosts prevalence is vital for reducing CD human incidences. This allows for observing changes of hosts in congruence with changes in the environment, and a novel, precise technique for blood meal identification advances our knowledge in this area. This research project excels in its multidisciplinary nature, combining proteomic and genetic approaches to apply directly to disease transmission control in Chagas affected countries throughout Central America. Many areas of expertise are combined such as the ecology and epidemiology of vector-host disease dynamics, as well as the direct application of knowledge to local people living in these conditions. The discoveries from this project will be crucial in furthering the understanding of CD ecology, as well as its methods having the potential for being applied to other vector disease dynamics.

Background

Vector-borne disease systems are some of the most complex disease systems, causing approximately 1.4 million deaths annually worldwide (3). CD, a vector-borne disease, is a neglected tropical disease and is endemic in many parts of Latin America. It mostly occurs in limited resource regions where sub-optimal living conditions are prominent (4-6). CD claims the lives of 23,000 people annually, with 9-10 million infected and over 60 million at risk of infection (4, 7). One-third of those infected with the Chagas parasite develop life-threatening illnesses, and it can take up to 20 years to develop diagnosable symptoms, making treatment difficult (7). CD is transmitted when a blood feeding Triatominae insect vector, also known as a kissing bug, deposits Trypanosome parasite-laden feces on the skin of a mammalian host. The parasite is subsequently introduced into the blood stream from the insect feces when the skin is scratched or through an already existing wound. While congenital transmission occurs in 1-10% of infants born to Chagas parasite positive mothers (7-9), *T. cruzi* transmission to humans occurs primarily during a blood meal from an infected Triatominae vector.

Identifying blood meal sources of Triatominae vectors is critical in understanding CD transmission dynamics as this can lead to vector host prevalence and aid in sound vector management. There is currently no effective vaccine against CD and although two anti-Trypanosomal drugs, Nifurtimox and Benznidazole, are available, these come with considerable side effects and are not always an ideal solution to the overall disease management problem (4, 10). Vector control interventions have previously been effective in decreasing *T. cruzi* occurrence (11, 12). Insect vectors tend to have some flexibility in environmental habitat preference and host species, so changes in climate and environment can increase chances for human contact and CD transmission. *T. cruzi* prevalence in the insect vector is closely tied to the ecology and physiology of the local insect vector species as well as that of the various hosts present (7). Economically, hundreds of millions of dollars are spent annually on vector control (5, 13). Although successful vector control programs throughout Latin America have decreased overall CD occurrence in rural areas, the movement of infected human individuals to cities has led to a movement of parasite and hosts (7).

Triatominae insect vector lifecycles are generally broken down into two types- sylvatic or ‘wild’ bugs and their hosts, and domestic bugs and their hosts (6). There are however, some blood meal host species that can be considered ‘bridge’ species that connect the sylvatic cycles to the domestic cycles. Domestic dogs are considered the most important domestic reservoir for CD, although they may serve as a bridge species as well (14). Opossum and raccoon are recognized important sylvatic blood meal species (2, 7). It has been shown that environmental destruction and habitat changes can affect sylvatic vectors moving into new environments, changing various components of the overall transmission cycle, including the blood meal host species (6, 15-17). For example, deforestation in the Amazon has led to tree-dwelling sylvatic vectors to adapt to a variety of new hosts such as rodents and opossums (17, 18). Therefore, disturbances play a large role in how the vectors move about the landscape, and the artificial ecotope humans have created contributes to these mechanisms (6). Triatominae insect vectors are also frequently attracted to light sources, so sylvatic vectors may move towards domestic habitats at night (6). Information leading to adjusting policies to manage potential animal hosts and insect vectors play increasingly important roles in controlling disease and preventing parasite infection (4, 19).

Critical to aiding in the development of CD management policies is determining the origins of blood meals taken by the Triatominae insect vectors, in order to elucidate intermediate host prevalence. Understanding the blood meal composition of the insect vector populations as it relates

to disease transmission has been challenging due to the complexity of the disease transmission cycle. DNA-based analyses are routinely used to determined blood meal sources from blood-feeding insects (2, 20-33); however, extracting blood meals from insect vectors is challenging and blood meal source identification accuracy is limited (34-38). Although DNA-based techniques are relatively inexpensive and quick, several issues such as contamination arise, particularly when amplification is required. DNA can be easily degraded without proper preservation and a lack of suitable target molecules such as appropriate primers for non-model organisms presents difficulties, possibly leading to misidentification or lack of identification of a blood meal source (34, 35). I propose a more direct identification and quantification of blood meal sources by measuring blood meal proteins. Hemoglobin is the most abundant protein in red blood cells and is important for oxygen transport throughout the body (39). The molecule is made up of two sets of alpha and beta globin chains that combine to form a tetramer. Alpha and beta hemoglobin chain amino acid sequences differ between animal host species and therefore present an excellent target for state of the art proteomic based methods (40, 41). Ultimately, accurately identifying blood meal sources can aid in the tracking of vectors as they move across landscapes and expand their ranges, providing support of the effect of habitat change and destruction as insects feed on novel hosts.

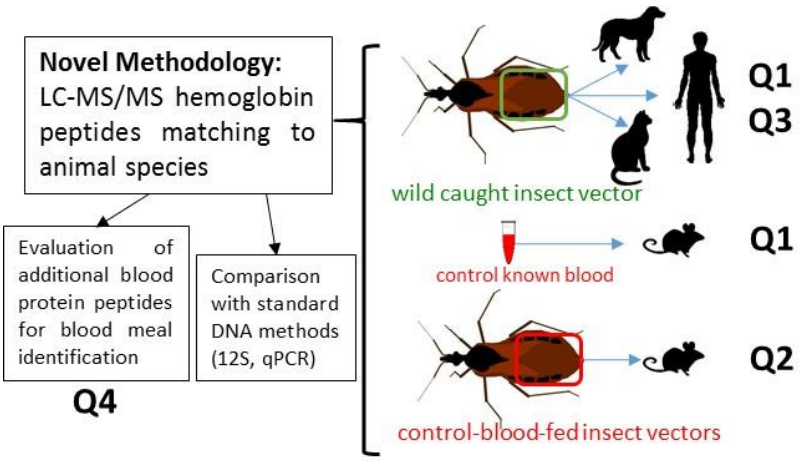

Figure 1. General schematic outlining aims with their experimental counterparts.

Research Approach

My general research approach is to evaluate blood meal composition of wild caught insect vectors and control-blood-fed insect vectors with LC-MS/MS, using hemoglobin peptides for blood meal source species identification (**Figure 1**). I will use CD as a study system for these novel techniques (**Q1**). I will determine how robust using LC-MS/MS to identify animal specific hemoglobin protein peptides is in comparison to routinely-used DNA techniques (**Q2**). I will quantify blood meals using absolute abundances obtained through the aid of synthetic AQUA peptides. This will aid in the determination of multiple blood meals by quantifying blood meal source components and comparing their abundance to the whole blood meal. I will also investigate how long and successfully we can determine blood meals in Triatominae insect vectors with the help of control experiments and known blood meals, in order to ultimately apply this methodology to the CD system (**Q3**). Furthermore, I will evaluate the application of using additional blood proteins for blood meal identification (**Q4**). All proteomic analyses will take place at the University of Vermont with the help of the Vermont Genetics Network Proteomics Core. All blood meal control experiments will take place at the Southwestern Biological Institute in Arizona. Triatominae insect vectors will be collected in Latin America in collaboration with the Chagas EcoHealth Initiative.

Q1) How can protein-based LC-MS/MS blood meal identification techniques aid in the management of CD?

AIM 1: Using a protein mass spectrometry approach, I aim to identify Chagas vector blood meal sources, and validate the technique with direct blood sample analysis. Comparisons to blood meal identification by 12S mitochondrial sequencing and probe-based quantitative polymerase chain reaction (qPCR) will be made for validation.

Understanding blood meal composition can lead to the development of holistic control strategies for CD transmission, aiding in understanding and managing disease dynamics. A host of molecular and serological techniques are available for blood meal detection to identify preferred host species without collecting the vector directly from the host. Previously employed antibody-based techniques such as the precipitin and antisera tests are labor intensive and require specific antibodies of possible host species in an area (18, 42-47). Extensive molecular techniques focusing on mitochondrial DNA (ie. cytochrome b or cytochrome c oxidase) have been employed with varying degrees of success (48, 49). Large databases, previously published universal primer sequences, and the large copy number of mitochondria per cell are advantageous (29-32, 48, 49); however, species-specific classification, ambiguity in blood meal identification, problems identifying blood meals in older bugs, and the need for high quality and quantity DNA can be challenging (23, 24, 28, 34). In many cases, such as with 12S sequencing, specific primers require development, are tailored to possible host species in an area and can often only detect higher taxonomic groupings. Others have employed mass spectrometry-based techniques in other vector systems, however these often rely on known species spectra for comparison (34-36, 40, 41, 50).

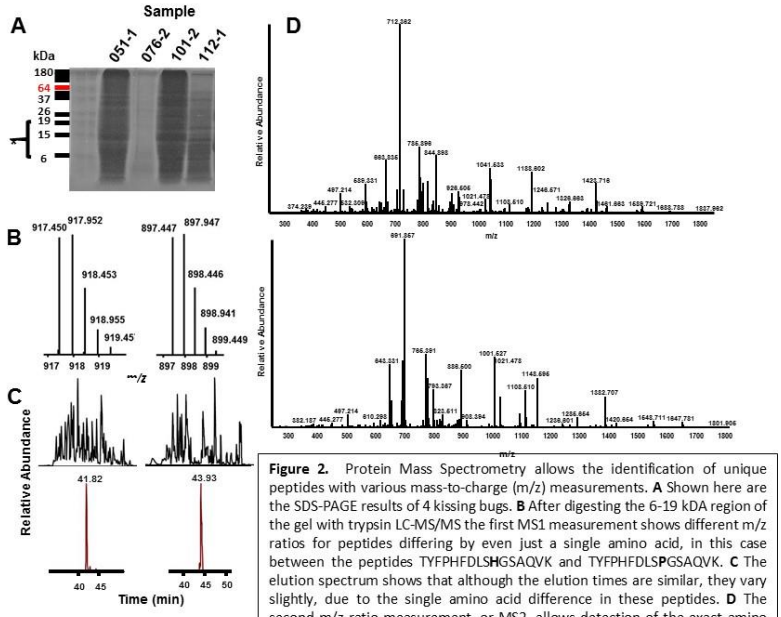

Figure 2. Protein Mass Spectrometry allows the identification of unique peptides with various mass-to-charge (m/z) measurements. **A** Shown here are the SDS-PAGE results of 4 kissing bugs. **B** After digesting the 6-19 kDA region of the gel with trypsin LC-MS/MS the first MS1 measurement shows different m/z ratios for peptides differing by even just a single amino acid, in this case between the peptides TYFPHFDLS**H**GSAQVK and TYFPHFDLS**P**GSAQVK. **C** The elution spectrum shows that although the elution times are similar, they vary slightly, due to the single amino acid difference in these peptides. **D** The second m/z ratio measurement, or MS2, allows detection of the exact amino acid sequence of the peptide by fragmenting the peptide.

I propose a hemoglobin protein based approach, using animal species-specific peptides to identify blood meals in the insect gut. This approach allows me to identify blood meal proteins directly using proteomic techniques, such as LC-MS/MS. As a control, I will identify peptides directly from known blood sources. While DNA techniques can theoretically measure a single molecule for blood meal source identification, the challenge lies in the fact that this single molecule needs to have all required blood meal information and needs to amplify correctly. My new approach measures billions of protein molecules smartly, potentially leading to a more comprehensive blood meal source analysis, complementing already existing DNA techniques.

Different studies have shown various insect host blood meal preferences, ranging from animals such as raccoons, rats, pigs, dogs, opossum, birds such as chickens, and even amphibians such as frogs (2, 6, 21, 25, 27, 43, 51, 52). Mass spectrometry allows us to identify peptides unambiguously (**Figure 2**), and therefore amino acid sequences can be matched to hemoglobin

sequences currently in GenBank (41). Similar approaches using mass spectrometry have been used successfully in other disease systems, such as those of ticks and mosquitos (34-36, 40, 41, 50, 53).

Although experiments using various DNA techniques have shown a range of sensitivities, there is additional ambiguity of what an insect vector fed on, especially when it comes to multiple blood meals (20). Preliminary results show that when dividing kissing bug abdomens into 2 halves, using one for DNA and one for protein blood meal identification, we are able to identify 100% of samples (n=4) at least to blood meal family level using hemoglobin peptides, while only one sample was identified to the correct species with 12S sequencing. Few studies have evaluated the use of probe-based qPCR for blood meal identification (20, 54). Species specific primers and probes are needed, which are a limiting factor for this technique. In addition, it has been previously shown that human DNA contamination on samples can interfere with accurate blood meal identification (20). Our preliminary results show a similar trend where qPCR amplification with human specific probes and primers amplifies human DNA in negative control samples even with great efforts to minimize contamination.

My goal is to successfully use hemoglobin peptide-based mass spectrometry to identify blood meal sources. Due to the stable nature of hemoglobin, I expect the versatility of this methodology to allow us to identify insect samples of low quality, which has previously been an issue. Ultimately, this novel methodology will be a valuable complementary tool for already existing DNA techniques, having the potential to be directly applied to CD management areas.

Q2) What are the limitations of a protein based method for blood meal identification? For example, how long after a blood meal or insect molt can we detect blood meals? What is our detection ability when it comes to multiple blood meals? What are the optimal conditions for our methods to work?

AIM 2: I aim to use protein-based LC-MS/MS methods and DNA methods to detect blood meals in known experimentally fed Triatominae insect vectors, with the aid of a controlled feeding study.

Triatominae insect vectors and the *T. cruzi* parasite, the etiological agent of CD, form an intriguing relationship. Although it is generally accepted that the *T. cruzi* parasite and Triatominae insect relationship is commensal, some studies suggest that insects infected with the parasite need to take in larger blood meals, or that feeding behavior is modified in other ways such as feeding more aggressively (6, 42). Information about blood meal perseverance in the insect gut and therefore longevity of detection ability in Chagas vectors is lacking, even though this is critical information to aid in the knowledge of the parasite transmission and overall feeding habits of the insect.

To explore the robustness of the aforementioned novel protein-based blood meal identification technique, I will use an experimental feeding study. Triatominae vectors are thought to digest their blood meals slowly (up to 10 weeks) (21, 23, 33, 42). In ticks, hemoglobin was detected 11 months post blood meal using mass spectrometry based methods (50). Evaluating the blood persistence in the insect gut is an important factor that can aid in determining the efficacy of protein-based versus DNA-based techniques by comparing the results of both methodologies. This can shed light on some of the feeding habits of these insect vectors. In order to investigate blood meal persistence, I will use adult *Triatoma protracta* laboratory colonies fed exclusively on mice. Insects will be preserved in ethanol 1, 2, 4, and 8 weeks post mouse blood meal. This will allow me to determine the ability to detect hemoglobin protein peptides using mass spectrometry at various time points after a blood meal. I will also evaluate the blood meals using standard 12S mitochondrial sequencing frequently used for blood meal identification (25-28) as well as probe-

based qPCR (20) to compare results to my novel detection methods. An additional component of interest is the location of the blood meal post ingestion. Absorption of blood meals in Hemiptera kissing bugs takes place in the midgut via intestinal epithelial cells (55). Different researchers have used different techniques and different parts of the insect to detect blood meals (ie. 31). I will have an undergraduate honors thesis student work on identifying blood meals in different anatomical structures of control mouse-fed bugs, and evaluating the aforementioned protein and DNA-based techniques.

Molting behavior of insects can also affect blood meal detection. Kissing bugs undergo hemimetaboly, meaning that they emerge wingless from an egg, successively molt through 2, 3, 4, and 5th instars, until they molt into winged adults (56). It has been debated how much of the intestinal system of the insect changes as these bugs undergo their molts, therefore evaluating blood meal detection post molt is an important factor. In this case mass spectrometry may be more comprehensive as preliminary experiments suggest we can detect smaller amounts of blood meal. If we can identify blood meals with mass spectrometry post-molt, this can offer information on insect host blood prevalence where DNA methods may not be as effectual. Therefore, I will collect insects 1, 2, 4, and 8 weeks post-molt from the aforementioned experimental colonies and identify blood meals using protein-based mass spectrometry and standard DNA methods. The insects will be fed exclusively on mice pre-molt. By using both, DNA and LC-MS/MS protein-based techniques for blood meal identification, the measurement systems can be compared on accuracy and detection ability of the known blood meals.

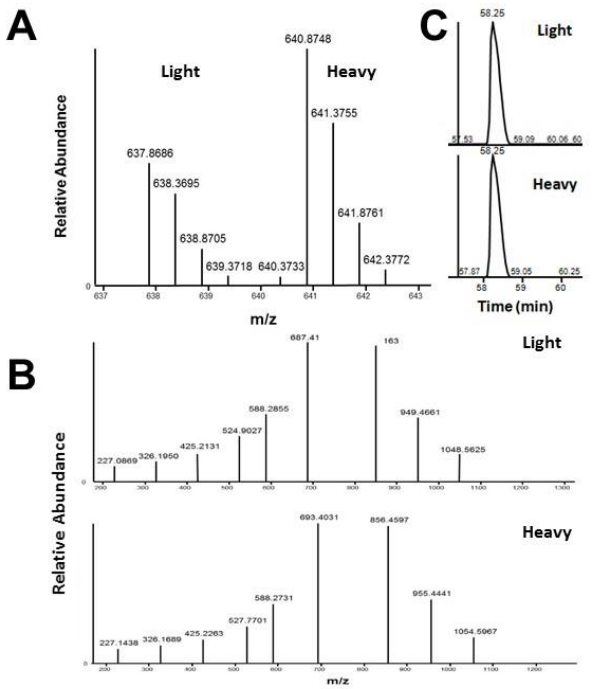

Figure 3. A Peptides can be quantified with the help of synthetic, or 'AQUA' peptides. These peptides have an identical amino acid sequence as our native or light peptide, however, they are heavily labeled on one amino acid. The MS1 measurement between light and heavy peptide is slightly shifted due to the difference in mass of the heavily labeled amino acid. **B** This can be further seen in the MS2 fragmentation spectrum where peaks that include the heavy-labeled amino acid are shifted by the m/z difference as compared to the native peptide. **C** The elution spectra, the time point at which the peptides elute off the column occur at the same time point, further supporting that the sequence of the native light peptide and the synthetic heavy peptide are the same.

With 12S mitochondrial sequencing, multiple blood meals are often detected in Triatominae vectors (27). Detecting multiple blood meals with 12S sequencing however proves challenging and often an additional cloning step is required (20, 27). I will use the experimental colonies of kissing bugs to determine our ability to detect multiple blood meals with LC-MS/MS. By experimentally offering laboratory colonies of *T. protracta* multiple hosts sequentially (a different host every 2 weeks), I will be able to elucidate if we can detect multiple blood meals. In addition, this blood protein peptide approach will allow me to use absolute abundances of blood proteins in order to quantify blood meals using synthetic AQUA peptides (**Figure 3**) (57-59). Furthermore, the spectra and particularly the b and y ions used to establish the amino acid sequence of the synthetic versus the native peptide will allow me to investigate if there are blood meals not identified through the National Center for Biotechnology Information (NCBI) protein Blast database.

Q3) How are blood meals changing in two endemic regions of Central America, such as Guatemala and El Salvador, and how do they vary in relation to location? How prevalent are blood meals on opossum, a major Triatominae life cycle bridge species in these regions?

AIM 3: I aim to identify sylvatic and domestic Chagas vector blood meal sources in endemic regions of Central America by evaluating samples previously identified with DNA-based detection methods, offering a complementary picture of the blood meal source composition.

Based on what is known in the field, blood meal sources are not detected in upwards of 50% of Chagas insect vectors, even in larger scale studies (29-31, 42-44, 46, 47) (Lima et al. 2016 *unpublished data*). The need for accurate blood meal detection is great in terms of vector and host management. Although incidence of CD is decreasing with intervention methods, (2, 4, 11, 13) knowing potential hosts in an area is still of great significance. Moreover, knowing potential 'bridge' species- species that can connect sylvatic to domestic disease systems- is critical. An important species connecting sylvatic and domestic environments appears to be the opossum, which is also a natural host for the *T. cruzi* parasite (60). Previous studies have shown high opossum blood meal prevalence (43) in locations such as Guatemala (48% detection, n=45), El Salvador (28% detection, n=39), and Honduras (35% detection, n= 46) (Lima et al. 2016 *unpublished data*). However, in samples currently analyzed with mass spectrometry from El Salvador (n=6), opossum was not detected. Evaluating the accuracy of previously established blood meals in these samples with mass spectrometry analysis is of value, aiding in establishing a comprehensive and accurate representation of blood meal sources in the region.

Since blood meal sources such as opossum were detected with various DNA techniques in samples from our collaborators, I will use the remaining half of the abdomen of a subset of these samples to identify blood meals with mass spectrometry, and subsequently compare them to vector blood meal preference previously identified with DNA techniques. As I am able to identify peptides unambiguously and am able to detect blood sources of hemoglobin sequences not in NCBI with the use of AQUA peptides, I expect to shed light on the efficacy of currently used DNA methods (12S sequencing, taxa specific primers). Some of my preliminary experiments have shown the ability to detect blood meals even in low quality samples. I was able to identify blood meals to at least family level of insects that had been collected dead, and having been stored in ethanol/glycerol. Both of these factors- state of the bug (collected alive or dead), and storage conditions such as using ethanol can interfere with DNA-based blood meal identification methods. Consequently, applying protein based methods has potential for a more complete insect host preference profile of a region. These methods could subsequently be applied to other insect vector systems, especially ones where blood meal detection has been challenging and sensitivity to small amounts of blood is important. My work will represent the first study of CD vector blood meals using LC-MS/MS and protein peptide-based methods.

Q4) Out of various blood proteins, is hemoglobin the most diverse in terms of peptides that can lead to blood meal species identification?

AIM 4: I aim to evaluate the peptide diversity of lower abundance blood proteins, such as serum albumin.

Blood accounts for approximately 7-8% of human body volume and is made up platelets, and red (erythrocytes) and white blood cells suspended in blood plasma (61). Erythrocytes made up in part of hemoglobin molecules are highly abundant molecules responsible for oxygen transport in the body. Preliminary data shows that hemoglobin is a great target for protein mass

spectrometry based blood meal identification. However, in some cases resolution cannot be made to the species level. For example, one blood meal was only identified to the family level. In this case, evaluation of additional blood proteins may give resolution to a more taxonomically specific grouping, ideally a specific species, or lend support to existing hemoglobin-based identification.

Serum albumin is the most abundant blood protein in blood plasma. It is widely occurring in mammals and this presents an additional target for protein-based proteomic techniques in order to identify blood meals. A major benefit of hemoglobin is its large record within GenBank of sequences of various species (17,000+ records containing the word 'hemoglobin' in any search field), presenting a thorough underlying database. Although serum albumin has significantly fewer entries (2,640 records containing the word 'albumin' in any search field, 1,017 records containing the word 'serum albumin' in any search field), the molecule's amino acid (AA) sequence is longer (~608 AA). Hemoglobin is made up of alpha (142 AA) and beta chains (147 AA). When comparing sequences of cat, dog, and human, alpha and beta chains are approximately 20% variable, suggesting the conservability of the molecule, but also variability between species. Albumin is comparable in variability (~18%) between these three species. Other proteins have been identified in tick blood meals by LC-MS/MS (50) such as immunoglobulins and histones matching to specific species. Preliminary work with Serum Albumin proteins shows support for blood meals identified in CD vectors (n=4). Therefore, using additional blood proteins has the potential to benefit overall blood meal identification techniques using state of the art mass spectrometry based proteomic techniques.

Broader Impacts - We need to understand the results of this study because my work has the potential to be directly applied to areas where CD is prevalent. The broader impacts of this research project are immediate benefits to society, having the potential to save lives through vector control measures. Through dissemination of research findings through publication in journals and direct contact with various villages throughout regions such as Guatemala and El Salvador, outreach efforts will be made to directly apply the findings. We will inform locals of the preferred blood meal sources in their regions. Although Chagas prevalence decreases with sound prevention methods, as environments change, insects have the potential to move around the landscape from sylvatic to domestic ecotopes. Identifying blood vector source 'bridge' species between these habitats with the aid of state of the art proteomic techniques can hone in on suggesting further management implications.

The direct application of results to CD prevention, management, and surveillance has the power to dramatically change lives of people living in endemic areas. In addition to this project being directly involved with people living in these Chagas endemic areas, I have also mentored students at the University of Vermont. Innovative research is critical to disease management and mentoring students paves the way for the researchers of tomorrow. I have trained an undergraduate student for over a year in the proposed techniques and this has led her to develop her own honors thesis project involving blood meal detection in various anatomical parts of the bug. I will continue to mentor this student as well as take on further undergraduate students. My student and I have already started to disseminated our work through various and on-going conference presentations. Identifying blood meal sources is a critical step in an already complex disease cycle. These activities are potentially transformative, as the CD burden in Latin America is a major one. In the future, studies such as this one will continue to be increasingly more important, aiding in our understanding of vector-borne diseases and how to control them (3).

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