Characterization of Triatomine Bloodmeal Sources Using Direct Sanger Sequencing and Amplicon Deep Sequencing Methods

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Research Article

Keywords: Sanger sequencing, next generation sequencing (NGS), triatomine, bloodmeal analysis, Triatoma, vector

Posted Date: November 29th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1087077/v1

Abstract

Knowledge of host associations of blood-feeding vectors may afford insights into managing disease systems and protecting public health. However, the ability of methods to distinguish bloodmeal sources varies widely. We used two methods—Sanger sequencing and amplicon deep sequencing—to target a 228 bp region of the vertebrate CYTB gene and determine hosts fed upon by triatomines (n = 115) collected primarily in Texas, USA. Direct sanger sequencing of PCR amplicons was successful for 36 samples (31%). Sanger sequencing revealed 15 distinct host species, which included humans, domestic animals (*Canis lupus familiaris*, *Ovis aries*, *Gallus gallus*, *Bos taurus*, *Felis catus*, and *Capra hircus*), wildlife (*Rattus rattus*, *Bufo nebulifer*, *Sciurus carolinensis*, *Sciurus niger*, *Odocoileus virginianus*), and captive animals (*Panthera tigris*, *Colobus* spp., *Chelonoidis carbonarius*). Samples sequenced by the Sanger method were also subjected to Illumina MiSeq amplicon deep sequencing. The amplicon deep sequencing results (average of 302,080 usable reads per sample) replicated the host community revealed using Sanger sequencing, and detected additional hosts in five triatomines (13.9%), including two additional blood sources (*Procyon lotor*, *Bassariscus astutus*). Up to four bloodmeal sources were detected in a single triatomine (*Bufo nebulifer*, *Homo sapiens*, *Canis lupus familiaris*, and *Sciurus carolinensis*). Enhanced understanding of vector-host-parasite networks may allow for integrated vector management programs focusing on highly-utilized and highly-infected host species.

Introduction

Determining bloodmeal sources of arthropod vectors can reveal vector-host interactions critical to understanding networks of pathogen transmission and guiding vector control and disease prevention campaigns. For example, mosquito host feeding patterns vary temporally, which correspond to changes in vertebrate availability and potentially the transmission of mosquito-borne viruses[1,2]. Previous triatomine research has revealed bloodmeal host utilization differences by season and instar stage; for example, in a study in Argentina, *Triatoma infestans* fed on a larger number of different hosts and took mixed meals more often in spring-summer than in winter [3].

Methods of bloodmeal analysis have evolved over time and have included immunological methods (precipitins, ELISA), DNA-based approaches (T-RFLP, PCR and Sanger sequencing, next generation sequencing) and protein characterization (liquid chromatography/mass spectrometry)[4–9]. Over the past two decades, a bloodmeal analysis method used widely across mosquitoes, triatomines, tsetse flies, sandflies, and ticks has been PCR-Sanger sequencing. Briefly, the blooded abdomen is removed, DNA is extracted, PCR amplifies a conserved vertebrate gene, amplicons are directly sequenced by Sanger, and sequences are compared to known sequences using searches such as the Basic Local Alignment Search Tool [BLAST] search with National Center for Biotechnology Information [NCBI] GenBank[10]) to detect residual traces of vertebrate host DNA[5]. While this approach allows for species-specific host identification to the level of resolution afforded by the GenBank sequence database[10], it has posed particular challenges in bloodmeal analysis of vectors with multiple bloodmeals and those that molt through several stages. For example, determination of bloodmeal sources of hard ticks is difficult due to degradation of vertebrate DNA while molting from one life stage to the next [11,12]. Bloodmeal analysis methods which detect only a single host may lead to a biased characterization of the host community. The widely used approach of PCR followed by direct Sanger sequencing does not efficiently detect multiple bloodmeal hosts or any bloodmeal other than the most abundant source at time of sampling. The rapid evolution of next generation sequencing makes it an attractive option for detecting multiple hosts fed upon by an individual vector.

Recently, amplicon deep sequencing has been used as a refinement to PCR-Sanger sequencing methods for identification of bloodmeal hosts[7,13–15]. In this approach, a conserved locus (frequently mitochondrial) that has nucleotide variation among taxa is amplified; amplicons are barcoded and sequenced on next generation sequencing platforms. This approach may yield hundreds of thousands of sequence reads per individual vector, as opposed to the single sequence trace generated using the Sanger approach. The use of amplicon deep sequencing in arthropod vector bloodmeal analysis has afforded the detection of: up to four vertebrate hosts in some *Rhodnius* vectors[14], different individual humans in *Anopheles* mosquitoes[7], non-human feeding in *Aedes aegypti* and *Culex quinquefasciatus*[16], and simultaneous identification of host, ectoparasite, and pathogen DNA in ticks and fleas[17].

Triatomine insects are obligate blood feeding arthropods that feed broadly on mammals (including humans), birds, reptiles, amphibians, and even invertebrates[4,18–22]. Triatomines are distributed throughout the Americas[23], where they are vectors of *Trypanosoma cruzi*, the protozoan parasite responsible for Chagas disease. Over 5.7 million people across the Americas are estimated to suffer from Chagas disease[24]. In the southern United States, where eleven species triatomines are endemic, Chagas disease has been diagnosed in locally-infected humans[25–27] and is recognized as a cause of cardiomyopathy and death in dogs[28–30]. Raccoons (*Procyon lotor* Linnaeus), woodrats (*Neotoma* spp.), and opossums (*Didelphis virginiana* Kerr) have been recognized as reservoir hosts across the southern US[31]. Not only does bloodmeal source affect likelihood of an insect becoming infected with *T. cruzi*, but blood source has also been shown to affect vector life cycle duration and fecundity[32] as well as proclivity to feed and molt[33].

The objective of this study was to explore methods of characterizing the vertebrate host community of triatomines collected across Texas, a state with a high diversity of triatomine species and documented cases of locally-acquired human and canine infection[27,34–36]. We conducted a study of two molecular bloodmeal analysis approaches (direct Sanger sequencing and amplicon deep sequencing) in order to evaluate the application of these two sequencing approaches to the bloodmeal analysis of triatomine vectors of Chagas disease.

Materials And Methods

Specimen collection

From December 2012 to December 2015, we acquired 115 triatomine specimens via two methods: 1) 45 specimens collected by community members across Texas, northern Mexico, and Florida and submitted to our community science program[37], and 2) 70 specimens collected by co-authors and their teams using

standard entomological trapping techniques, including lights, carbon dioxide, and active searching around houses, kennels and wood rat nests [37]. In addition to 60 triatomines that had been previously dissected and tested for *T. cruzi* (see [38–40] for details), we selected 55 additional samples (including 3 from Mexico, 39 from a non-human primate facility, and 13 from a zoological park – intending to test the breadth and resolution of species detection, including distinguishing between *Homo sapiens* and other primate species), for a total of 115 samples. In this study, our main goal was to assess seqeuencing methods; we therefore selected triatomine samples from areas with diverse host communities. This included specimens collected from a zoological park, as well as samples collected from non-human primate facilities. Previous testing of over 1500 triatomines in our collection revealed an overall *T. cruzi* infection prevalence of 54%; *T. cruzi* infection in the specimens in this current study was determined using the same methods[39].

Sample preparation and T. cruzi *molecular typing*

Triatomines were identified [23] and dissected as previously described, including submerging specimens in a 50% bleach solution prior to dissection to mitigate risk of human DNA contamination[41]. Based on visual examination of the dissected gut, evidence of a recent bloodmeal was scored (1 = no blood, desiccated guts; 2 = no blood, guts visible; 3 = traces of blood in gut; 4 = blood present, but either not much or not fresh [dried]; 5 = large amount of fresh blood)[38,41]; for the purposes of this study, these were further classified as 'starved' (scores of 1-3) or fed (scores of 4 or 5). DNA from hindgut tissue was extracted using the Omega E.Z.N.A Tissue DNA kit (Omega Bio-Tek, Norcross, GA). Samples were subjected to multiple PCRs for detection and strain-typing of *T. cruzi* DNA, as previously described[39].

PCR amplification of host CYTB

Extracted hindgut DNA was subjected to PCR amplification of a 228 bp fragment of the vertebrate CYTB gene[42,43]. The primers were originally designed to amplify reptilian hosts but have been reported to amplify a wide range of vertebrate hosts[43,44]. Reactions included 3 µL template DNA, primers at final concentrations of 0.66 µM each, and FailSafe PCR Enzyme Mix with PreMix E (Epicentre, Madison, WI) in a final volume of 50 µL. Primers 'herp1' 5'-GCH GAY ACH WVH HYH GCH TTY TCH TC-3' and 'herp2' 5'-CCC CTC AGA ATG ATA TTT GTC CTC A-3' and previously described cycling conditions for 55 cycles[42]. DNA-negative water controls and positive controls of DNA extracted from cynomolgus macaque (*Macaca fascicularis*) were included in each PCR batch. PCR amplicons were visualized on a 1.5% agarose gel stained with ethidium bromide.

Samples generating a band of ~228 bp, including some that also had an additional band of ~450 bp, were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA). To reduce risk of cross-contamination among samples, triatomine dissection, DNA extraction, PCR, and post-PCR manipulations took place in separate dedicated areas in the laboratory; in addition, three samples (PS334, PS675, PS1306) that revealed a potential human bloodmeal were processed further (see [44] for details of the 'mammal a' primer set and cycling conditions these three samples were subjected to).

Sanger sequencing of CYTB amplicon

An aliquot consisting of 5µl of purified amplicon was sequenced with the 'herp1' primer using Sanger sequencing on a 3730xl DNA Analyzer (ThermoFisher Scientific, Waltham, MA) at Eton Bioscience Inc. (San Diego, CA). The remaining purified amplicon was stored at -20°C until further use. Sequence chromatograms were visually inspected for quality using 4Peaks (version 1.7.1) (Mekentosj, Amsterdam, http://www.nucleobytes.com/4peaks/). Sequences were compared to existing sequences in GenBank (www.ncbi. nlm.nih.gov/genbank/)[10] using BLAST[45] with default parameters to search in the 'nucleotide collection (nr/nt)' database—which includes GenBank, EMBL, DDBJ, PDB, RefSeq and excludes EST, STS, GSS, WGS, TSA—for 'highly similar sequences (megablast)' (https://blast.ncbi.nlm.nih.gov/Blast.cgi). In contrast to a previous study which set a threshold for accepting and reporting a result at ≥95% identities and E-value ~ 0[20], we set a more liberal threshold to include samples with ≥90% identities as a preliminary identification and criterion for moving to amplicon deep sequencing, in order to further evaluate lower identity matches by amplicon deep sequencing. We also considered biological feasibility, defined as the possibility of host presence at the collection site of the triatomine vector, given the current understanding of host species distributions (Texas Parks and Wildlife https://tpwd.texas.gov/ and Austin zoo https://austinzoo.org/).

Amplicon deep sequencing and bioinformatics analyses

For all samples yielding ≥90% identity BLAST matches based on direct Sanger sequencing, the remaining volume of purified PCR product (~30 µL) for each sample was subjected to amplicon deep sequencing at Texas A&M AgriLife Research Genomics and Bioinformatics Service (College Station, TX). PCR products from the herp1/herp2 reactions were made into Illumina-compatible sequencing libraries by addition of adapters and indexes in two sequential PCR reactions.

In the first reaction, Illumina-based sequence read primers were added to the initial herp-1 PCR products using a common reverse primer (Herp_2R, 5'-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TCC CCT CAG AAT GAT ATT TGT CCT CA-3') and one of four padded forward primers designed to add diversity for increased data yield and quality. The forward primers used (with bases added for diversity underlined) were Herp_1'A, 5'-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT GCH GAY ACH WVH HYH GCH TTY TCH TC-3'; Herp_1'B, 5'-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT HGC HGA YAC HWV HHY HGC HTT YTC HTC-3'; Herp_1'C, 5'-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT HWG CHG AYA CHW VHH YHG CHT TYT CHT C-3'; and Herp_1'D, 5'-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT HWW GCH GAY ACH WVH HYH GCH TTY TCH TC-3'. Each 25 µl reaction contained 1 ng herp1 PCR product, 0.5 µM each forward and reverse primer, 200 µM dNTPS, and 0.02 U/µl Phusion Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA) in 1X Phusion reaction buffer. Samples were initially denatured at 98°C for 30 sec, then cycled eight times at 98°C for 10 sec, 58°C for 20 sec, and 72°C for 30 sec. Samples were held at 10°C following a final elongation for 5 min at 72°C, bead purified with 1X AMPure XP beads (Beckman Coulter Indianapolis, IN, USA), quantified with picogreen reagent (ThermoFisher Scientific Waltham, MA, USA), and checked for size on a fragment analyzer (Agilent, Santa Clara, CA, USA).

The second PCR added combinatorial dual indexes with the following sequences (where X represents barcode bases): P5_Index_Primer, 5'-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACX XXX XXX XAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T-3' and P7_Index_Primer, 5'-CAA GCA GAA GAC GGC ATA CGA

GAT XXX XXX GTC TCG TGG GCT CGG-3'. PCR reaction components and thermocycling conditions were similar to the first round, but unique combinations of a P5 and P7 index primer were used instead of Illumina-herp1 primers. PCR products were cleaned, quantified, and visualized as described above, and equimolar amounts of barcoded final PCR products were pooled and quantified using the Kappa library quantification qPCR kit. Pooled libraries were sequenced on a MiSeq (Illumina, San Diego, CA, USA).

Demultiplexed data were filtered and clustered to remove errors using SeekDeep bioinformatics pipeline (version 2.5.0)[46]. After merging forward and reverse reads, sequences for each sample were filtered for minimal length (228 bp), 97% identity and quality (Phred score of above 25 across 75% of the read length). Clusters with reads less than 0.5% relative abundance were rejected. The rest of the output consensus sequences were subjected to taxonomy search using nucleotide BLAST[45] using default parameters to search in the 'nucleotide collection (nr/nt)' database for 'highly similar sequences (megablast)'; we considered accepted matches (here referred to as 'usable clusters') generated by amplicon deep sequencing to be those with >99% identity and 100% query cover. As with the Sanger sequencing analyses, we considered the biological feasibility of the results.

Results

Triatomine collection and T. cruzi results

The 115 triatomines in this study were collected between June 2013 and October 2015 and included seven species of *Triatoma*: *T. gerstaeckeri* (Stål) (84; 73.0%), *T. sanguisuga* (Leconte) (16; 13.9%), *T. lecticularia* (Stål) (6; 5.2%), *T. indictiva* Neiva (4; 3.5%), *T. mexicana* (Herrich-Schaeffer)(1; 0.9%), *T. neotomae* Neiva (1; 0.9%), 1 unidentified *Triatoma* adult (0.9%), and 2 *Triatoma* nymphs (unknown species; 1.7%). These samples were collected primarily across 22 counties in Texas, with the exception of one sample from Florida (Lake County) and three samples from Mexico (states of Hidalgo and Tamaulipas; USDA Import Permit 123470). Habitats from which insects were collected included the grounds of a zoo, a nonhuman primate research facility, in and around private residences, dog kennels, chicken coops, farms, and tents. Samples represented different bloodmeal conditions identified at the time of dissection: 44 samples (38.3%) had a bloodmeal score of 4 or 5 and were considered 'fed'. Of the 115 triatomines, 64 (55.7%) were infected with *T. cruzi*. *T. cruzi* strains in infected triatomines were: 35 TcI (54.7%), 23 TcIV (35.9%), and 6 (9.4%) TcI/TcIV mixed infections.

PCR amplification of CYTB genetic region

We attempted to amplify the CYTB genetic region from hindgut DNA extracts of 115 triatomines. Overall, 56 generated bands—38 samples (11 'starved' triatomines and 27 'fed' triatomines) generated single bands of the target amplicon size (228 bp); an additional 18 samples (16 'starved' triatomines and 2 'fed' triatomines) generated a band of approximately 450 bp along with the expected band of the 228 bp target amplicon size.

Sanger Sequencing

All 38 samples generating a single band were submitted for Sanger sequencing, 36 of the samples generated a sequence that generated a preliminary identification (³ 90% identities and expect (E) values of ~ 0) in NCBI BLAST (Table 1). We attempted Sanger sequencing of 15 of the 18 samples that had generated two bands; only 2 samples of these samples were successfully sequenced via Sanger. Attempts to cut only the ~450 bp products from the gels with two bands and generate sequences were unsuccessful.

Of the 44 samples from triatomines that appeared 'fed' at time of dissection, 26 (59.1%; 95% CI 44.4-72.3%) yielded identifiable host results via PCR and Sanger sequencing. In contrast, only 10 of the 71 triatomines that appeared 'starved' yielded identifiable host results (14.1%; 95% CI 7.8-24.0%) (df = 1, Pearson $\chi^2 = 25.59$, $p < 0.001$).

In the 36 samples that generated a preliminary identification by Sanger sequencing, fifteen vertebrate species were identified, including 12 mammalian species, one bird, one reptile, and one amphibian (Table 2). In order of frequency detected: *Canis lupus familiaris* (domestic dog - 11), *Homo sapiens* (human - 5), *Gallus gallus* (chicken - 3), *Ovis aries* (sheep - 3), *Bufo nebulifer* (Gulf Coast toad - 2), *Panthera tigris* (tiger - 2), *Rattus rattus* (black/roof rat - 2), *Bos taurus* (cattle - 1), *Capra hircus* (goat - 1), *Chelonoidis carbonarius* (red-footed tortoise - 1), *Colobus* spp (colobus monkey - 1), *Felis catus* (cat - 1), *Odocoileus virginianus* (white-tailed deer - 1), *Sciurus carolinensis* (eastern gray squirrel - 1), *Sciurus niger* (fox squirrel - 1).

The three samples (PS334, PS675, PS1306) that revealed a potential human bloodmeal using the 'herp' primers and subjected also to the 'mammal a' primer PCR revealed human DNA results as well (E-values of ~0 and 99% identities in NCBI BLAST matches for all samples).

Amplicon deep sequencing

Amplicon deep sequencing analysis was performed on 36 samples for which Sanger sequences were obtained (32 samples with ³ 95% identities discovered by BLAST on Sanger sequencing products, and an additional 4 samples with < 95% identities—ranging from 92% to 94%—in order to evaluate lower identity matches using amplicon deep sequencing). We obtained 22,155,506 reads (Supplemental Table 1) from the MiSeq run, of which 18,263,359 reads matched Illumina adapters and were used in further filtering. When filtered by size and quality scores, 11,023,747 sequences were available for clustering; of these, 10,874,884 reads matched a vertebrate host species in GenBank. In total, 49.8% of the raw reads and 60.4% of the adapter matched reads were usable, with an average of 598,764 raw and 307,215 usable reads per sample.

Seventeen vertebrate host species were identified by amplicon deep sequencing (Table 2). In order of frequency detected: *Canis lupus familiaris* (dog - 12), *Homo sapiens* (human - 8), *Gallus gallus* (chicken - 3), *Ovis aries* (sheep - 3), *Bufo nebulifer* (Gulf Coast toad - 2), *Panthera tigris* (tiger - 2), *Rattus rattus* (black/roof rat - 2), *Bassariscus astutus* (ringtail - 1), *Bos taurus* (cow - 1), *Capra hircus* (goat - 1), *Chelonoidis carbonarius* (red-footed tortoise - 1), *Colobus guereza* (guereza - 1), *Felis catus* (cat - 1), *Odocoileus virginianus* (white-tailed deer - 1), *Procyon lotor* (raccoon - 1), *Sciurus carolinensis* (eastern gray squirrel - 1), *Sciurus niger* (fox squirrel - 1). Of the 12 samples that indicated dog as a blood source, 11 were infected with *T. cruzi* – 5 with TcI, 4 with TcIV, and 2 with TcI/TcIV mixed infections. Of the 8 samples that indicated human as a blood source, 3 (33%) were infected with *T. cruzi* – 1 with TcI and 2 with TcIV (Table 2).

In the BLAST matches, the output consensus sequences matched perfectly (100% identity) across the entire target in all but three instances (99% for *Sciurus carolinensis* [Eastern gray squirrel; AZ-085 and PS706] and *Odocoileus virginianus* [white-tailed deer; SNBL005]; Table 2). Samples rejected on the basis of a poor match showed 53% or lower coverage (data not shown). A finding from one sample was rejected on the basis of a lack of biological feasibility (PS502) in which 998 (1.38%) of 72,493 reads matched with 100% identity to *Colobus guereza* (eastern black and white colobus); the other two hosts identified for this sample were *Ovis aries* (domestic sheep; 93.09% of reads and 100% identity match) and *Bassaricus astutus* (ringtail; 5.54% of reads and 100% identity match). This triatomine was found on a rural ranch in Gillespie County, Texas, where the likelihood of finding the *Colobus* host was remote. The reads matching the *Colobus*, were, therefore curated out of further analysis on the basis of biological implausibility.

Comparison of Sanger sequencing and amplicon deep sequencing results

While some differences were observed in the frequency of occurrence of hosts between Sanger sequencing and amplicon deep sequencing, the most abundant host identified by both methods was domestic dog (Table 2). The next most frequent host was human. Amplicon deep sequencing not only replicated the host dataset as determined by Sanger sequencing, but also afforded the detection of additional hosts within a subset of triatomines (Table 2). Five triatomines (13.9% of the 36 vectors for which host was determined) had evidence of multiple hosts; four showed evidence of blood from two species and one showed evidence of blood from four species (Figure 1). In all samples with multiple hosts detected, a dominant amplicon represented > 90% of reads (Table 2), which was also the taxa identified by Sanger sequencing. Species represented in triatomines with evidence of multiple bloodmeals included domestic (human, domestic dog, sheep) and wildlife species (Gulf coast toad, tiger[from a zoo], Eastern gray squirrel, fox squirrel, ringtail, raccoon).

Discussion

We used two techniques - direct Sanger sequencing and amplicon deep sequencing - to identify hosts fed upon by triatomines collected primarily from Texas. Our findings of diverse wildlife species, domestic dog, and human bloodmeal sources corroborate previous findings of triatomines as opportunistic feeders. Amplicon deep sequencing was able to identify up to 4 different blood sources within one triatomine, compared to Sanger sequencing alone only identifying a single likely most recent source within each triatomine.

Triatomines are long-lived insects that feed multiple times in each of five nymphal instars and throughout the adult life stage[23,47]. For triatomine bloodmeal analysis, direct Sanger sequencing of a single PCR product—a method which typically reveals only the most abundant host taxa—may fail to provide the information necessary to learn about transmission networks. PCR followed by cloning and sequencing has offered more success detecting multiple bloodmeals[18,48,49]. Amplicon deep sequencing fills the need for a powerful method that detects not only the most recent/abundant host DNA, but also older and partially degraded bloodmeals as well. This method is becoming increasingly used in the detection of bloodmeals from triatomines yielding multiple host information from individual bugs allowing comparison of triatomines from different habitats, understanding their behaviors and controlling the spread of Chagas disease[13–15, 50].

Despite many advantages, the limits of amplification-based methods include that a single primer pair may not capture and amplify from all existing taxa with equal efficiency. For this reason, the ratios of the reads may not represent the ratio of the host abundance within the bloodmeal and conclusions to this effect are to be drawn with caution. The primers used in this study amplify a small region (228 bp) which helps increase likelihood of detecting older, degraded bloodmeals. However, even with this small target, only 56 of 115 (48.7%) of samples produced an amplicon of the expected 228 bp size, and only 36 of those (31.3% overall) resulted in a sequenced host identification. As noted previously[20], samples with noticeable blood were more likely to result in host identification. Although we regularly use primer sets for other vertebrate genes in our vector bloodmeal analysis work, our prior (unpublished) data have shown greatest success with the herp primer set which was used in the current study. Future studies might explore success using other primer sets.

An additional consideration when choosing a bloodmeal analysis method is cost per sample. In general, next generation sequencing methods require more expensive consumables, more intricate instruments, and bioinformatics training to analyze the resulting data, making them more expensive than many other methods. As technology becomes more accessible and affordable, amplicon deep sequencing may be useful for addressing research questions focusing on host-vector interactions in systems where vectors feed on multiple hosts over time.

Triatomines require multiple bloodmeals to molt and reach adulthood. Evidence of multiple host taxa within individual triatomines—including up to four different bloodmeal sources in one triatomine—have been detected using antisera[3,6], ELISAs[51,52], species-specific primer sets[53,54], and PCR and cloning[18,48]. Detection of multiple bloodmeal sources using PCR and direct sequencing is rare, although has occurred in at least one triatomine collected in Texas[20]. Next generation sequencing has recently been shown to be a sensitive method for detecting an average of 4.9 bloodmeal sources per triatomine [15]. Much remains to be explored regarding how long evidence of a bloodmeal source can be found in a triatomine, and whether detection of multiple bloodmeal sources is indicative of meals over several stages or partial/incomplete feeding attempts during one life stage of a specimen. The five individuals generating multiple host identifications (4.3%) in this study had a variety of fed and starved statuses. Continued development of more sensitive and high-throughput methods is needed, as well as controlled studies investigating detection rates after known/controlled feeding of triatomines.

Our findings add to the evidence of triatomines as opportunistic and indiscriminate feeders. Triatomines used in this study were mainly collected by community scientists in/near homes, and those samples predictably revealed bloodmeals including human and animals associated with domestic and peridomestic environments (dog, cow, cat, chicken, sheep, and goat). The most frequently identified bloodmeal source was dog (33.3% of triatomines with any host detected contained dog blood); 11 of the 12 triatomines with dog blood revealed had only dog blood. The second most frequently identified bloodmeal source was human; this included 5 triatomines with only human blood and 3 triatomines with human blood and another species. The 3 triatomines with a small percentage of reads (3.4 - 9.4%) indicating human results, as well as the biologically implausible finding of *Colobus* DNA in a sample found in rural Texas (PS 502), raise concern for potential contamination of samples during processing. The possibility of contamination was much reduced by strict procedures to minimize and monitor for contamination, including separate pre- and post-PCR processing areas, bleaching of triatomines prior to dissection to remove exogenous DNA, and inclusion of negative controls in PCRs. These findings of small amounts of human DNA in samples may reflect a less abundant or less recent feeding on humans. Using highly-sensitive techniques, such as the methods used here, increases the likelihood of detecting contamination in samples; all results should be considered with respect to the actual probability that the bloodmeal hosts revealed by the methods are biologically feasible.

The most frequently identified bloodmeal source was dogs, which are recognized reservoirs of *T. cruzi* in Texas[29,36]. Of the 12 samples that indicated dog as a blood source, 11 were infected with *T. cruzi* – 5 with TcI, 4 with TcIV, and 2 with TcI/TcIV mixed infections. Of the 8 samples that indicated human as a blood source, 3 (33%) were infected with *T. cruzi* – 1 with TcI and 2 with TcIV – representing potential infection risk if the person had been exposed to the triatomine feces (alternately, it could indicate the triatomine had fed on an infected human, but this likelihood is low in the US). Known wildlife reservoirs of *T. cruzi* – squirrels, ringtails, raccoons (Hodo & Hamer, 2017) – were determined to be bloodmeal sources in this sample set. Several triatomines from a zoo harbored a variety of mammalian hosts – tiger, colobus monkey, roof rat, humans and dogs – capable of being infected by *T. cruzi*, as well as non-mammalian hosts – Gulf coast toad, red-footed tortoise, chicken – refractory to *T. cruzi* infection. Two triatomines that had fed on red-footed tortoise and Gulf coast toad were infected with *T. cruzi*, indicating these triatomines had meals from reservoirs prior to feeding on these refractory species. Feeding studies of laboratory-reared bugs with known and changing bloodmeals over the triatomines lifecycle will be key to interpretation of future findings [20]. Advances in bloodmeal determination are needed to further explore the wild sources of *T. cruzi* infection in triatomines prior to their dispersal to human houses and their surrounding where they pose public and veterinary health risk.

Amplicon deep sequencing is a powerful technique for elucidating the feeding patterns of triatomines than direct Sanger sequencing methods alone. As additional knowledge of blood feeding patterns is generated, a more intricate understanding of vector ecology and bloodmeal sources can be coupled with *T. cruzi* reservoir infection data, which may be useful for designing disease risk reduction interventions.

Declarations

Author Contributions

S.B.: Designed research, performed research, analyzed data, wrote manuscript. R.C-R.: Designed research, performed research, sample and data collection, analyzed data, wrote manuscript. B.C. and A.M.: Performed research, analyzed data. L.D.A.: Performed research, data management, analyzed data. V.B-G., P.C., W.C., M.C., and G.F.: Sample and data collection. C.D.J., R.P.M, and S.W.: Performed research. N.J.H.: wrote/updates/maintains SeekDeep. J.A.B.: Contributed reagents and/or analytical tools. G.L.H.: Designed research, contributed reagents and/or analytical tools. S.A.H.: Designed research, contributed reagents and/or analytical tools, wrote paper. All authors reviewed the manuscript.

Acknowledgements

We are grateful for triatomines collected by community science program participants and by staff at the Michale E. Keeling Center for Comparative Medicine and Research of MD Anderson – Stanton Gray, Carolyn Hodo, Monica Mitchell, and Greg Wilkerson. We are appreciative of the field and laboratory contributions assistance of Taylor Hollmann Britten, Adam Curtis, Raven Forrest Fruscalzo, Connor Grantham, Gaston I. Jofre, Hee Kim, Bryant McDowell, Sarah Noe McCullough, Jéssica Caroline Gomes Noll, Ryan Pugh, Michael Sanders, and Faith Weeks, as well as the drawing of a ringtail by Tara Roth. Funding was provided by Texas A&M AgriLife Research, the Texas A&M University and Consejo Nacional de Ciencia y Tecnología (CONACYT) Collaborative Research Grant Progam, Texas EcoLab Program, the Wild Animal Health Fund of the American Association of Zoo Veterinarians, and the Harry Willet Foundation.

Data Availability Statement

Data from next generation sequencing will be deposited into the SRA database (NCBI).

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Tables

Table 1. Triatomine bloodmeal scores, number of samples with hosts identified, and detection of multiple bloodmeal hosts via next generation sequencing. During dissection, triatomines were assigned bloodmeal scores of 1 (no blood, desiccated guts), 2 (no blood, guts visible), 3 (traces of blood in gut), 4 (blood present, but either not much or not fresh [dried]), or 5 (large amount of fresh blood); these were then classified as 'starved' (scores of 1-3) or fed (scores of 4 or 5). The herp1/herp1 primer set was used to amplify a 228bp fragment of DNA (some samples also produced a second band of ~450bp). Sanger sequencing and BLAST matching were used to determine bloodmeal hosts. Triatomines of 'fed' statuses more frequently had a bloodmeal host characterized via Sanger sequencing that triatomines of 'starved' statuses. In addition to Sanger sequencing, amplicons were subjected to next generation sequencing methods. Five triatomines had multiple hosts detected via next generation sequencing methods.

Bloodmeal status	n	~228bp band on PCR	Sanger sequencing produced appropriately sized fragment that resulted in a BLAST match	Single host detected by NGS	Multiple hosts detected by NGS*	Identifiable host results	95% confidence interval of identifiable host results
Starved	71	27	10	8	3	14.1%	7.8-24.0%
Fed	44	29	26	24	2	59.1%	44.4-72.3%
Total	115	56	36	31	5	31.3%	23.6-40.3%

Table 2. Triatomine sample information for specimens with bloodmeal host result determined by direct Sanger and NGS methods. Ecological data and vertebrate bloodmeal host identifications based on direct Sanger sequencing versus amplicon deep sequencing for 36 triatomines collected across Texas and Florida.

	Sample ID	*Triatoma* species	Collection Area	*T. cruzi* status	*T. cruzi* DTU	Blood meal score	Sanger Sequencing			Next Generation Sequ	
							Sanger Sequencing Result	E-value	% Identity	Next Generation Sequencing Result	Taxon assig reads
Triatomines with only one bloodmeal host revealed	AZ-011	*T. gerstaeckeri*	Zoo - redfooted tortoise area	Positive	TcI/TcIV	4	*Chelonoidis carbonarius*	3.0E-93	98	*Chelonoidis carbonarius*	164,3
	AZ-036	*T. gerstaeckeri*	Zoo - leopard area	Positive	TcIV	2	*Rattus rattus*	2.0E-94	99	*Rattus rattus*	309,8
	AZ-043	*T. sanguisuga*	Zoo - wolf hybrid area	Negative	-	1	*Gallus gallus*	2.0E-94	98	*Gallus gallus*	226,0
	AZ-082	*T. sanguisuga*	Zoo - lion area	Negative	-	4	*Rattus rattus*	5.0E-96	99	*Rattus rattus*	470,3
	AZ-084	*T. gerstaeckeri*	Zoo - unknown exact area	Negative	-	5	*Colobus spp*	7.0E-95	98	*Colobus guereza*	453,7
	AZ-118	*T. indictiva*	Zoo - tiger area	Negative	-	3	*Panthera tigris*	2.0E-79	96	*Panthera tigris*	91,50
	EL016	*T. gerstaeckeri*	Near house with dog	Positive	TcI	3	*Canis lupus familiaris*	8.0E-69	93	*Canis lupus familiaris*	57,65
	PS195	*T. gerstaeckeri*	Dog kennel	Positive	TcI	4	*Canis lupus familiaris*	9.0E-94	98	*Canis lupus familiaris*	511,5
	PS253	*T. gerstaeckeri*	Dog kennel	Positive	TcI	5	*Canis lupus familiaris*	2.0E-96	97	*Canis lupus familiaris*	453,8
	PS334	*T. sanguisuga*	On person outdoors	Positive	TcIV	5	*Homo sapiens*	1.0E-97	99	*Homo sapiens*	511,7
	PS403	*T. sanguisuga*	In house with dog	Positive	TcIV	4	*Canis lupus familiaris*	6.0E-95	100	*Canis lupus familiaris*	471,4
	PS449	*T. gerstaeckeri*	In house - in bed	Positive	TcI	5	*Homo sapiens*	3.0E-88	98	*Homo sapiens*	269,2
	PS498	*T. gerstaeckeri*	Farm	Positive	TcI	4	*Bufo nebulifer*	3.0E-98	99	*Bufo nebulifer*	525,1
	PS501	*T. gerstaeckeri*	On house on farm with sheep	Positive	TcI	2	*Ovis aries*	5.0E-86	97	*Ovis aries*	260,9
	PS503	*T. gerstaeckeri*	Farm with sheep	Positive	TcI	4	*Ovis aries*	2.0E-85	97	*Ovis aries*	406,2
	PS507	*T. gerstaeckeri*	Farm with goats	Positive	TcIV	4	*Capra hircus*	4.0E-87	98	*Capra hircus*	124,9
	PS675	Unk (nymph)	House	Negative	-	5	*Homo sapiens*	3.0E-93	99	*Homo sapiens*	475,7
	PS888	*T. gerstaeckeri*	Farm with chickens	Negative	-	4	*Gallus gallus*	8.0E-89	98	*Gallus gallus*	536,5
	PS889	*T. gerstaeckeri*	Farm with cats	Negative	-	3	*Felis catus*	1.0E-66	99	*Felis catus*	59,19
	PS1035	*T. gerstaeckeri*	Unknown	Positive	TcIV	5	*Canis lupus familiaris*	1.0E-87	99	*Canis lupus familiaris*	357,2
	PS1102	*T. gerstaeckeri*	Outside of house with dog	Positive	TcIV	5	*Canis lupus familiaris*	3.0E-93	98	*Canis lupus familiaris*	357,5
	PS1119	*T. lecticularia*	Chicken coop	Negative	-	4	*Gallus gallus*	1.0E-87	98	*Gallus gallus*	272,7

	PS1122	*T. gerstaeckeri*	House with dog	Positive	TclV	4	*Canis lupus familiaris*	4.0E-97	99	*Canis lupus familiaris*	357,7
	PS1156	*T. gerstaeckeri*	Dog bed	Positive	Tcl/TclV	4	*Canis lupus familiaris*	2.0E-96	100	*Canis lupus familiaris*	257,6
	PS1164	*T. gerstaeckeri*	Farm with cattle	Positive	Tcl	5	*Bos taurus*	5.0E-86	98	*Bos taurus*	326,4
	PS1256	*T. indictiva*	House with dog	Positive	Tcl	5	*Canis lupus familiaris*	2.0E-95	99	*Canis lupus familiaris*	481,2
	PS1257	*T. gerstaeckeri*	Near dog	Positive	Tcl/TclV	4	*Canis lupus familiaris*	5.0E-91	98	*Canis lupus familiaris*	443,3
	PS1281	*T. gerstaeckeri*	In house with dog	Positive	Tcl	5	*Canis lupus familiaris*	3.0E-93	98	*Canis lupus familiaris*	500,9
	PS1301	*T. lecticularia*	House	Negative	-	5	*Homo sapiens*	1.0E-91	99	*Homo sapiens*	340,1
	PS1306	*T. sanguisuga*	House	Negative	-	5	*Homo sapiens*	1.0E-96	98	*Homo sapiens*	402,4
	SNBL005	*T. gerstaeckeri*	Non-human primate research center	Negative	-	2	*Odocoileus virginianus*	7.0E-55	96	*Odocoileus virginianus*	8,453
Triatomines with more than one bloodmeal host revealed	AZ-040	*T. indictiva*	Zoo - tiger area	Negative	-	1	*Panthera tigris*	2.0E-69	94	*Panthera tigris*	47708
										Homo sapiens	1818
	AZ-085	*T. sanguisuga*	Zoo - fox area	Negative	-	3	*Bufo nebulifer*	2.0E-48	92	*Bufo nebulifer*	1021
										Homo sapiens	36
										Canis lupus familiaris	24
										Sciurus carolinensis	22
	PS393	*T. sanguisuga*	On outside of house	Positive	TclV	4	*Sciurus niger*	1.0E-76	97	*Sciurus niger*	1562
										Homo sapiens	1625
	PS502*	*T. gerstaeckeri*	Farm with sheep	Positive	Tcl	2	*Ovis aries*	2.0E-75	94	*Ovis aries*	6748
										Bassariscus astutus	4014
	PS706	*T. sanguisuga*	On outside of house	Positive	TclV	4	*Sciurus carolinensis*	8.0E-89	98	*Sciurus carolinensis*	2474
										Procyon lotor	1762

* 998 reads in this sample identified *Colobus guereza*. The plausibility of this host in this sample is not verifiable and therefore, these are set aside from consideration

Figures

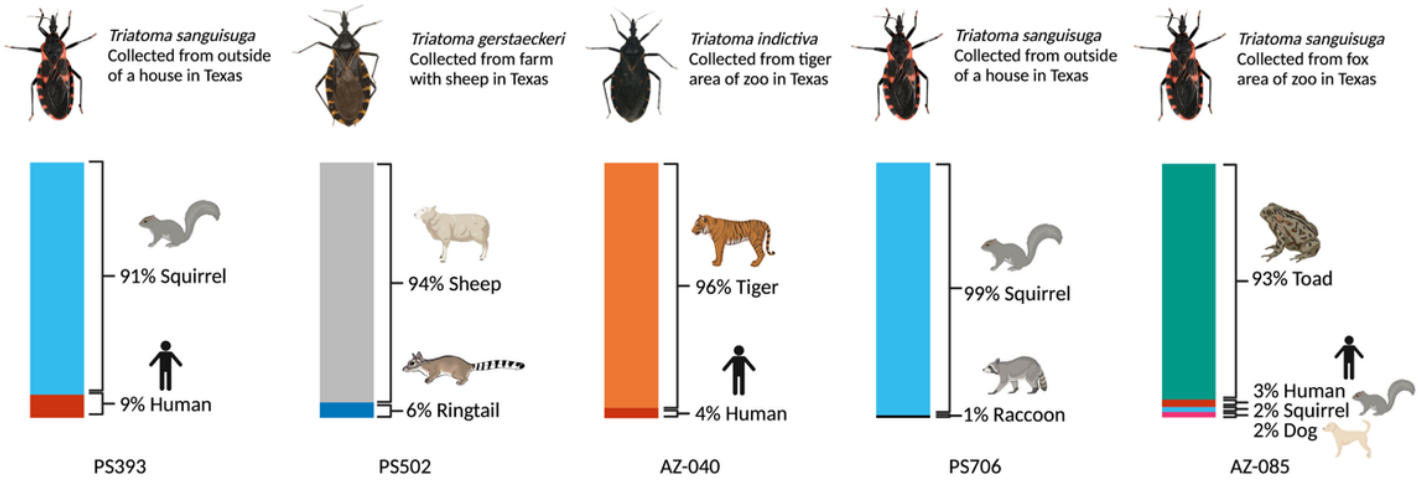

Figure 1

Amplicon deep sequencing revealed multiple bloodmeal sources in several triatomines. DNA extracted from triatomine hind guts was subjected to amplicon deep sequencing. Of the 36 samples generating amplicon deep sequencing results, 5 samples generated results revealing more than one bloodmeal source. One of the five samples had four bloodmeal sources identified. Figure created with BioRender.com.

Supplementary Files

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