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Evaluation of Vector-Enabled Xenosurveillance in Rural Guatemala

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Abstract. Surveillance methods that permit rapid detection of circulating pathogens in low-resource settings are desperately needed. In this study, we evaluated a mosquito bloodmeal-based surveillance method ("xenosurveillance") in rural Guatemala. Twenty households from two villages (Los Encuentros and Chiquirines) in rural southwest Guatemala were enrolled and underwent weekly prospective surveillance from August 2019 to December 2019 (16 weeks). When febrile illness was reported in a household, recently blood-fed mosquitoes were collected from within dwellings and blood samples taken from each member of the household. Mosquitoes were identified to species and blood sources identified by sequencing. Shotgun metagenomic sequencing was used to identify circulating viruses. *Culex pipiens* (60.9%) and *Aedes aegypti* (18.6%) were the most abundant mosquitoes collected. Bloodmeal sources were most commonly human (32.6%) and chicken (31.6%), with various other mammal and avian hosts detected. Several mosquito-specific viruses were detected, including *Culex* orthophasma virus. Human pathogens were not detected. Therefore, xenosurveillance may require more intensive sampling to detect human pathogens in Guatemala and ecologically similar localities in Central America.

INTRODUCTION

The ability to detect circulating pathogens rapidly in remote, resource-limited areas is inhibited by a lack of a surveillance infrastructure. Rural populations with limited access to healthcare and biomedical support are frequently excluded from traditional surveillance methods due to extreme logistical complexities.[1,2] Noninvasive surveillance in these settings could have a significant impact by enabling early detection of viruses. Xenosurveillance is a novel surveillance approach that takes advantage of mosquito feeding behavior to identify bloodborne pathogens that may be circulating in human and animal hosts. This approach circumvents invasive blood sampling of individuals and results in an abundant sample source derived from both humans and animals. We therefore have proposed that xenosurveillance may be a useful method for disease surveillance in rural, resource-limited areas.

The detection of bacteria, viruses, and parasites from mosquito bloodmeals via membrane and animal blood feeding has been previously demonstrated.[3–5] Using field-caught blood-fed *Anopheles* mosquitoes from Liberia and Senegal, we previously detected non–mosquito-borne viruses such as canine distemper virus, Epstein–Barr virus, GB virus C, and hepatitis B virus.[3,6] The utility of this approach in the American tropics, however, where the main human-biting mosquitoes are *Aedes* and *Culex*, not *Anopheles*, has not been addressed.

Accordingly, in the present study, we evaluated the utility of xenosurveillance in rural southwest Guatemala, where *Culex* and *Aedes* mosquitoes are highly abundant. We conducted xenosurveillance and human-directed biosurveillance in parallel to evaluate the logistical and technical feasibility to detect circulating bloodborne pathogens in humans over the course of a 16-week study. We also determined the species composition of mosquitoes collected from within Guatemalan dwellings and their host feeding preferences.

MATERIALS AND METHODS

Study site. This study was performed through the Center for Human Development located in the coastal lowlands of southwestern Guatemala, approximately 20 km from the border with Chiapas, Mexico.[7] The population in the region suffers from high levels of food insecurity, poverty, and poor access to healthcare; diarrheal, respiratory, and other communicable diseases are frequent, especially in children.[7,8] Many households use pit latrines, which can flood during heavy rain and create ideal environments for mosquito breeding. Chickens, ducks, pigs, rats, dogs, and cats are common in households and have close contact with families.

Study design/enrollment. Twenty households from two villages, Los Encuentros and Chiquirines, adjacent to the Center for Human Development, were enrolled in August 2019. Requirements for enrollment included having at least three animals in the household and ability and willingness to consent to participate. Once consent was obtained, capillary blood samples of all enrolled humans in addition to mosquito bloodmeals (described subsequently) were collected at enrollment. We also collected epidemiologic data, including demographics, animals (indoor, outdoor, grazing), and risk factors for infection (water features, septic system, mosquito exposure, contact with animals, etc.) through participant interviews. The study was approved by the Colorado Multiple Institutional Review Board (COMIRB) and the University del Valle de Guatemala (UVG) Ethics Committee. The local Community Advisory Board for Research agreed to the study. The protocols for blood extraction and animal handling were approved by the Colorado State University Institutional Animal

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Care and Use Committee (IACUC; Protocol #1091) and by the UVG IACUC (Protocol #I-2019).

Prospective syndromic surveillance. From August 22, 2019 to December 12, 2019, study nurses contacted (in person or over the phone if not home) enrolled households weekly to survey for the presence of fever, rash, or body aches in household members at any point over the week. If symptoms were present in any household member, human capillary blood and mosquito bloodmeals were collected at time of screening as described subsequently. The study team was prepared to provide supportive medications to symptomatic household members and triage individuals to higher levels of care if they exhibited WHO danger signs.[9] However, no such danger signs were observed over the course of the study.

Sample collection and pre-processing. Human and mosquito samples were collected at enrollment and during symptomatic illness as described earlier.

Mosquito bloodmeals. Local technicians were trained on mosquito collection, identification, and sample processing methods before the start of the study. Mosquitoes were collected using an InsectaZooka aspirator (BioQuip Products, Rancho Dominguez, CA) from the indoor areas of enrolled households, specifically targeting resting blood-fed mosquitoes on the walls and surfaces. Attempts to collect mosquitoes were usually made in the morning and placed in a –20°C freezer for at least 2 hours or overnight to ensure death and processed within 24 hours of collection. Blood-fed mosquitoes (female) were identified according to a simplified version of a previously published key for mosquitoes in Guatemala[10] and individual mosquito bloodmeals were expressed onto FTA cards (Whatman, Maidstone, United Kingdom). Forceps were dipped in ethanol and wiped clean between each mosquito. Fifty microliters of RNAlater was added to each mosquito dried blood spot (M-DBS) at the end of each processing session.

Human capillary blood. Blood was collected from all members of the household during enrollment and when a febrile illness occurred in the household. The subject's index finger was disinfected with an alcohol swab before using a sterile lancet to prick the finger. Blood was expressed from the finger and dabbed onto a labeled FTA card, and 100 μL of RNA was added to each human dried blood spot (H-DBS) by the end of the day. FTA cards were stored at –80°C until they were shipped (at room temperature) to Colorado State University, where they were again stored at –80°C until further processing.

RNA/DNA extraction. H-DBS and M-DBS were removed using a Harris 3-mm micro-puncher (GE Healthcare Life Sciences, Chicago, IL) and soaked in 70 μL RNA rapid extract solution for 8 to 18 hours at 4°C. Fifty microliters was subsequently used for nucleic acid extraction using the Mag-Bind Viral DNA/RNA kit (Omega Bio-Tek, Norcross, GA) with the King Fisher Flex Magnetic Particle Processor (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. Nucleic acid was eluted in 50 μL nuclease-free water and stored at –80°C until processing.

Mosquito speciation and bloodmeal identification. Mosquito species was determined for all M-DBS by polymerase chain reaction (PCR) and Sanger sequencing using primers directed against the cytochrome oxidase I (*COI*) gene as described previously.[11] To identify the mosquito bloodmeal host source, *COI* DNA was PCR amplified with M13-tagged primers as previously described.[12] PCR products were separated on an agarose gel, and the corresponding ~800-bp piece was excised. DNA was purified using the NucleoSpin Gel and PCR Clean-Up kit (Machery-Nagel, Allentown, PA) and Sanger sequenced with M13 primers. Obtained sequences were trimmed for quality and blasted against the Barcode of Life *COI* database[13] to identify the bloodmeal source. Samples with no visible band or poor-quality sequencing were rerun before being designated as "No Match."

Shotgun metagenomic sequencing library preparation. A subset of H-DBS and M-DBS from seven symptomatic episodes, comprising 43.6% of all samples, were used to identify circulating viruses using shotgun metagenomic sequencing. Sample sets (all DBS from a household collection event) were chosen based on the week in study, number of symptomatic individuals, number of mosquito samples, and recurrent sampling events. DBS nucleic acid samples were pooled by volume according to time of collection and household. A total of seven human and seven mosquito libraries were generated and sequenced along with water and a blank extracted FTA card as negative controls (Supplemental Table 1). RNA extracted from HeLa cells was used as a positive control for virus detection. Solid phase reversible immobilization (SPRI) bead clean-up was used between each step in the following library preparation protocol. Samples were DNAse-treated and rRNA-depleted using methods described previously.[14] Previously designed mosquito probes[14] were used on M-DBS and human probes (NEB) were used on H-DBS and HeLa cell RNA control. Water and FTA-negative controls received both human and mosquito probes. The sequence-independent, single primer amplification (SISPA) was used to generate and amplify cDNA as described previously.[15] Briefly, cDNA was created via the Superscript IV First-Strand Synthesis System (Invitrogen, Waltham, MA) following the standard protocol, except for the use of tagged random primers: CATAGTCGTACGTATACATC-(Nx12). Second strand synthesis was performed using a Klenow DNA polymerase I fragment (NEB). Primers aligning to the above tag were used to amplify dsDNA fragments and increase sensitivity of detection. Libraries approximately 300 bp in length were prepared using the Nextera XT DNA Sample Preparation kit (Illumina, San Diego, CA). Dual-indexed libraries were pooled together using DNA concentration measured with a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA) and normalized for the number of individual samples included in each library. Size and quality of the pooled libraries were determined using the TapeStation high sensitivity DNA system (Agilent, Santa Clara, CA). A final concentration was determined via library quantification quantitative PCR (qPCR; New England Biolabs, Ipswich, MA) and the pooled library was sent to Genomics and Microarray Core at the University of Colorado–Denver Cancer Center for 2 × 150 paired-end sequencing on an Illumina NovaSeq6000 platform.

Shotgun metagenomic sequencing analysis. Shotgun metagenomic sequencing datasets were processed to identify and tally viral reads. An existing taxonomic identification pipeline was modified for use in this study.[6,16,17] Reads were quality assessed using FastQC.[18] Low-quality, adapter, and SISPA sequences were removed using cutadapt tool version 1.14.[19] The CD-HIT-EST tool version 4.6.8 was used to

remove PCR duplicates.[20] FastQC was again used to assess sequence quality post trimming and filtering. Human and mosquito bowtie indices were created to remove host reads using Bowtie version 2.2.9. The human index (GRCh38.p3) was used on H-DBS and HeLa cell libraries. The mosquito index was created using four mosquito genomes (*Ae. aegypti* GCF_002204515.2, *Ae. albopictus* GCA_006496715.1, *Anopheles darlingi* GCA_000211455.3, *C. pipiens* GCF_000209185.1) and was used to filter M-DBS libraries. No host filtering was done on water and FTA negative control libraries. The remaining reads were assembled using SPAdes genome assembler version 3.6.1.[21] Contigs and nonassembled reads were taxonomically assigned using the BLASTn alignment tool version 2.9.0+.[22,23] Taxonomic assignment was based on the highest alignment score and an E value $< 10^{-8}$. If not able to be taxonomically assigned based on nucleotide sequence, DIAMOND version 0.9.30 was used to search reads against the NCBI nr database.[24] Identified viral sequences (excluding phage) were tallied according to NCBI taxonomic ID with a minimum cutoff of 30 hits. Taxonomic identifications were validated by reblasting contigs (NCBI BLAST nucleotide) and mapping reads to indexed reference genomes using Bowtie versus 2.2.9 (Supplemental Table 2).

Sample screening by quantitative PCR. The H-DBS and M-DBS nucleic acid samples were pooled by volume ($n = 8$ per pool) before screening. Quantitative PCR (qPCR) was run using the Express SuperMix Universal One Step system (Invitrogen, Waltham, MA) on a Quant Studio machine (Applied Biosystems, Waltham, MA) according to the manufacturer's instructions. The following primers and probes were used: dengue virus (DENV)-2 primers as previously described,[25] DENV-3 forward TGGCAACAGGTCCCTTTCTG, DENV-3 reverse TGGCGTTGGATGCTAGTCTAAGA, COPV forward TGCAATCAAGAGCCATACAGACT, COPV reverse TCGTCCACACTGGTACCCA. DENV-3 primer sequences were kindly provided by Dr. Laura St. Clair and Dr. Rushika Perrera (Colorado State University, Ft. Collins, CO). DENV-2 and DENV-3 positive controls were provided by Dr. Irma Sanchez (Colorado State University, Fort Collins, CO). Melt curves were used to determine the validity of all amplified products.

RESULTS

Study demographics and household features. Two communities in rural southwest Guatemala were targeted for participation in this study: Los Encuentros and Chiquirines. Of 37 households screened for participation by convenience, 32 (86.5%) were eligible, 20 (62.5%) were enrolled (10 from each village), and 19 (95.0%) completed the study. Households that were not enrolled either did not meet the enrollment requirements (13.5%) or declined to participate (32.4%) largely due to daytime availability. Of enrolled households, nearly all dwellings consisted of cement block structures without screened windows or doors. All households had open water wells or tanks on the property, and many had additional sources of standing water (potted plants, tires, natural water); 50.0% of households did not have a septic system (Supplemental Table 3).

Households had a median of 4.5 people enrolled; 46.7% were under age 18, and 60.2% were female (Table 1). Animals were abundant in households (interquartile range: 16–34 per household) and often found in and around the home. The most abundant animal were chickens (55% of all animals, excluding humans), although pigs, dogs, cats, and horses were also present (Supplemental Table 4).

Symptomatic episodes and samples collected. During 16 weeks of observation, there were 15 symptomatic episodes, all of which included at least one report of fever, rash, or body aches. Seven of 15 episodes reported more than one symptom, and symptoms were reported in multiple household members for four episodes. Two households reported illness in two separate episodes, and a single household reported three instances of febrile illness. Most of the reported illness (15 of 20 reported episodes) occurred in Los Encuentros (Tables 1 and 2). Including enrollment, 1,200 mosquitoes were collected during the study, 507 (42.3%; Figure 1 and Table 2), of which appeared to have taken a bloodmeal in the past 36 hours observed by a dark red/black bloated abdomen.[26,27] Indoor mosquitoes were most abundantly collected at the end of the rainy season (September–October) (Figure 1); 488 of 517 (94.4%) blood-fed mosquito samples were speciated by PCR. *Culex*, *Aedes*, *Mansonia*, *Anopheles*, and *Psorophora* mosquitoes were collected, although *Culex pipiens* was by far the most abundant (60.9%) (Table 3).

Mosquito abundance and bloodmeal identification. Five genera of mosquitoes were identified: *Culex, Aedes, Mansonia, Anopheles,* and *Psorophora. Culex* mosquitoes were most abundantly identified as *Cx. pipiens* though *Cx. nigripalpus* also were collected. *Aedes* mosquitoes included *Ae. aegypti, Ae. taeniorhynchus,* and *Ae. albopictus,* from highest to least abundant (Supplemental Table 5). *Cx. pipiens* and *Ae. aegypti* were the most frequently collected species,

TABLE 1
Demographics and reported symptoms of study participants

Characteristic	Los Encuentros	Chiquirines	Total
Households, *n*	10	10	20
Median participants per household (IQR)	4.5 (3–6)	4.5 (4–6)	4.5 (3–6)
Participants, *n*	49	49	98
Children < 18 years, *n* (%)	25 (51.0)	21 (42.9)	46 (46.9)
Female, *n* (%)	29 (59.2)	30 (61.2)	59 (60.2)
Median age (IQR)	17 (9–32)	19 (7–37)	18.5 (9–36)
Reported symptoms			
Episodes, *n* subjects (*n* households)	15 (10)	5 (5)	20 (15)
Fever, *n* (%)	13 (86.7)	4 (80.0)	17 (85.0)
Rash, *n* (%)	2 (13.3)	1 (20.0)	3 (15.0)
Body aches, *n* (%)	9 (60.0)	1 (20.0)	10 (50.0)

IQR = interquartile range.

TABLE 2
Reported symptoms and obtained samples

Week	Village	Household ID	Subjects with fever	Subjects with rash	Subjects with body aches	H-DBS obtained	M-DBS obtained
Enroll	Los Encuentros	All	NS	NS	NS	49	105
	Chiquirines	All	NS	NS	NS	49	84
1	Los Encuentros	1007	1	0	0	6	13
	Chiquirines	2013	1	0	1	5	13
2	Los Encuentros	1001	1	0	0	4	9
	Chiquirines	2002	1	0	0	7	3
3	Los Encuentros	1016	1	0	1	6	9
4	Los Encuentros	1019	0	0	1	6	51
	Chiquirines	2012	0	1	0	4	32
	Chiquirines	2014	1	0	0	2	14
5	Los Encuentros	1003	1	0	1	3	21
6	Los Encuentros	1002	2	1	1	7	31
7	NR	NR	NR	NR	NR	0	0
8	Los Encuentros	1001	2	0	2	4	30
9	NR	NR	NR	NR	NR	0	0
10	NR	NR	NR	NR	NR	0	0
11	Los Encuentros	1019	0	1	0	6	35
12	Chiquirines	2002	1	0	0	4	28
13	NR	NR	NR	NR	NR	0	0
14	NR	NR	NR	NR	NR	0	0
15	Los Encuentros	1020	2	0	1	4	12
16	Los Encuentros	1019	3	0	2	5	17
Total			17	3	10	171	507

H-DBS = human dried blood spots; M-DBS = mosquito dried blood spots; NR = none reported; NS = not surveyed.

accounting for 60.9% and 18.6% of identified mosquitoes respectively (Table 3). To identify mosquito bloodmeal sources, we used a previously described protocol to amplify and sequence vertebrate *cytochrome oxidase 1* (CO1) sequences. We successfully amplified 464 of 488 (95.1%) total bloodmeal samples, indicated by the presence of a band corresponding to ~800 base pairs on an agarose gel. In total, 343 of 488 (70.3%) amplified samples were able to be host-identified. Unidentified samples were a result of poor sequencing results, indicating potential mixed hosts (i.e., mosquito feeding on multiple species). Of the 343 identified mosquito bloodmeal samples, our results demonstrate that humans and chickens were the most common sources of blood, accounting for 32.6% and 31.8% of identified bloodmeals respectively. All mosquito genera (excluding *Psorophora*, which had no identified bloodmeals) fed on humans. *Cx. pipiens* fed most abundantly on chickens and other avian hosts (63.6%), whereas *Ae. aegypti* fed mainly upon humans (91.7%) (Figure 2 and Supplemental Table 5).

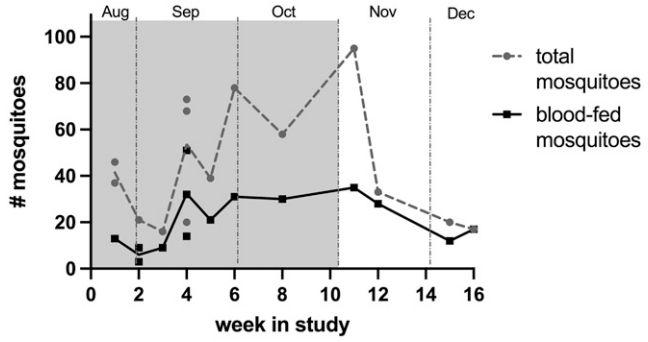

FIGURE 1. Total and blood-fed mosquitoes collected increases over rainy season. Total mosquitoes collected per household during symptomatic episodes over the 16-week study (gray dashed line). Blood-fed mosquitoes collected per household (black solid line). Rainy season indicated by shaded background.

Virus identification in human and mosquito dried blood spots. Human and mosquito dried blood spots (H-DBS, M-DBS) collected during seven symptomatic episodes were used to identify viral agents using a shotgun metagenomic sequencing approach (Supplemental Table 1). Retrospective bloodmeal analysis of the mosquitoes used for sequencing revealed 33.0% M-DBS derived from human hosts. To increase our sensitivity of detection using the SISPA method, all libraries underwent a high degree of amplification, thus even FTA and water control libraries produced reads from spurious sequence contaminants (Figure 3A). No human or animal viruses were detected in any library except in HeLa cells, which are stably infected with human papilloma virus and act as a control for virus detection. Insect-specific viruses were detected in H-DBS, water, and FTA control libraries though did not exceed 27 identified reads. Thus, a threshold of 30 reads was used to identify viruses that were present above background. Many insect-specific viruses were detected at high levels in M-DBS (Figure 3B and C). The most abundantly identified insect-specific viruses (ISVs) were *Culex* orthophasma virus (COPV;

TABLE 3
Mosquito species identified during course of study. 488 of 507 (96.3%) were able to be species-identified by cytochrome oxidase I sequencing

Species	Los Encuentros	Chiquirines	Sum (% of identified)
Aedes aegypti	61	30	91 (18.6)
Aedes albopictus	2	1	3 (0.6)
Aedes taeniorhynchus	0	3	3 (0.6)
Anopheles sp.	0	2	2 (0.4)
Anopheles albimanus	3	5	8 (1.6)
Culex sp.	36	14	50 (10.2)
Culex nigripalpus	9	1	10 (2.0)
Culex pipiens	194	103	297 (60.9)
Mansonia dyari	11	9	20 (4.1)
Mansonia titilans	1	1	2 (0.4)
Psorophora ferox	2	0	2 (0.4)
Total identified	319	169	488

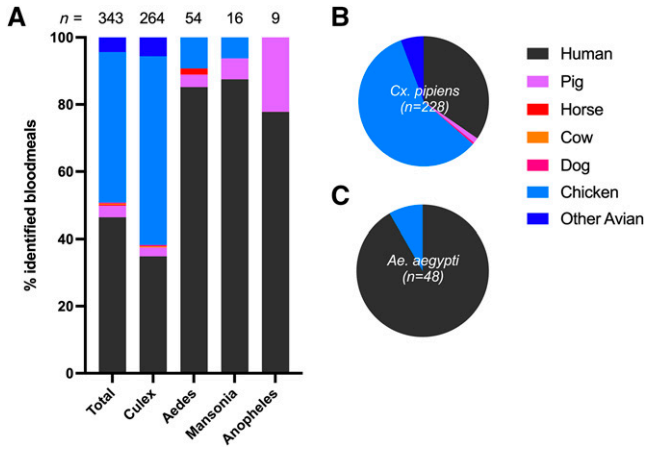

FIGURE 2. Many bloodmeals obtained from avian species due to large numbers of *Culex pipiens*. The *cytochrome oxidase 1* gene was amplified and sequenced to perform a BLAST search using the Barcode of Life database. A total of 343 of 488 (70.3%) species-identified mosquito samples were able to be traced to a single host source. (**A**) Percent of mosquito bloodmeals identified as mammal or avian host sources for each mosquito species. (**B**) Percent of all identified host sources *Cx. pipiens* mosquito bloodmeals. (**C**) Percent of all identified host sources of *Ae. aegypti* mosquito bloodmeals.

Phasmaviridae, previously known as *Culex* phasma-like virus[28]) and Hubei reo-like virus 7 (HRLV7; Reoviridae-like). Terena virus (TV; unclassified Bunyavirales-like) was also abundantly detected. However, upon validation of identified ISVs, we found 100% of reads identified as TV mapped to the COPV reference genome with 97.7% identity. We then discovered the S and M genes of TV are 99.1% and 99.3% identical to COPV, respectively. Reads identified as TV were therefore reattributed to COPV. Pooled reads from M-DBS resulted in 98.6% identity (98.9% coverage) to the COPV reference genome (Supplemental Table 2). qPCR for COPV was used to validate these results and screen the remaining M-DBS samples. Estimated prevalence for COPV was 24.6% (95% CI: 18.0–32.5) based on 60 or 67 positive pools (507 total samples).[29] H-DBS were negative for COPV by qPCR. During the time of our study, DENV 2/3 were circulating in southwest Guatemala. However, DENV 2/3 was not detected in either H-DBS or M-DBS via shotgun metagenomic sequencing or qPCR.

DISCUSSION

The rise of global pandemics, coupled with the complexity and uncertainty associated with predicting pathogen emergence, emphasizes the need for minimally invasive, accessible, low-cost surveillance methods that are applicable under field conditions. We therefore evaluated xenosurveillance in rural Guatemala, a region that is of interest for three principal reasons. First, it is a region that has a high burden of infectious diseases with significant needs for and barriers to effective health surveillance.[7] Second, there is a high rate of human migration within and through the region, providing an important sentinel population for emerging infectious disease surveillance.[7] Finally, and perhaps most important, the region is quite different from those where we have previously conducted pilot studies of xenosurveillance, in that *Culex* and *Aedes* mosquitoes, which tend to be less anthropophilic compared with *Anopheles gambiae*, are the main mosquitoes that fed on humans indoors. Therefore, we sought to assess the extent to which xenosurveillance may be effective in this type of environment.

We collected and identified several mosquito genera, including *Culex*, *Aedes*, *Mansonia*, *Anopeheles*, and *Psorophora*. *Culex pipiens* (60.9%) were most abundant and fed mainly on birds, whereas *Ae. aegypti* (18.6%) fed mostly on humans. Thus, the feeding patterns of *Cx. pipiens* and *Ae. aegypti* at our site are typical for these species.[30,31] The disproportionate number of *Culex* samples skewed our blood samples toward those derived from birds, in contrast to our prior study in which *An. gambiae*[6] accounted for 80% of the collection and strongly prefer to feed on humans. These observations present challenges and opportunities for implementation of xenosurveillance in Latin America; more intensive sampling may be required to capture human infection adequately, but surveillance of domestic and peridomestic animals may be enhanced.

Analysis of M-DBS and H-DBS revealed no human viruses present, in contrast to prior detection of human viruses (GB virus C, Epstein–Barr virus, and hepatitis B virus) from *Anopheles* bloodmeals in Liberia.[3,6] Many factors likely contributed to the lack of human virus detection in the present study. First, the number of blood-fed mosquitoes was not sufficient for virus detection in humans. As previously described, the abundance of *Cx. pipiens* in this study limited the retrieval of human-sourced mosquito bloodmeals because the majority were derived from chickens and other birds. In addition, multiple samples are likely needed for adequate detection of bloodborne viruses from any one individual,[32] although the absence of viruses detected in H-DBS suggests circulating bloodborne viruses were not present at the time of collection. Second, symptom surveillance was done once weekly, resulting in samples that were not collected at the time of illness. We may therefore have missed critical windows where mosquitoes may have sampled circulating viremia.[33] Finally, virus discovery was performed on a chosen subset of samples and thus could result in missed human viruses. Collectively, these results suggest that efficient detection of human pathogens in this setting may require more frequent and abundant sampling, or that human pathogens were not circulating at detectable levels.

Although no human viruses were detected, analysis of M-DBS revealed the presence of ISVs. The detection of ISVs was expected because excretion of the mosquito bloodmeal onto FTA cards is accompanied by mosquito tissues (e.g., the midgut) that may be infected by ISVs. ISVs have been described in xenosurveillance-based studies published previously[17] and provide an additional internal control for our ability to detect viral RNA by our sample processing and analysis procedures. We found several ISVs that were highly abundant in M-DBS, including COPV and HRLV7. TV was also identified, although on further analysis, we found reads mapped solely to regions that were highly identical to COPV; thus, we reattributed TV-identified reads as COPV. COPV has been identified in a number of *Culex* mosquito collections in Australia,[34] Brazil,[35,36] and Grenada.[34] Similarly, HRLV7 has been described in both *Aedes* and *Culex* mosquitoes on three continents, as well as in this study.[37,38] There have been a few recent studies showing decreased vector competence and replication of pathogenic flaviviruses

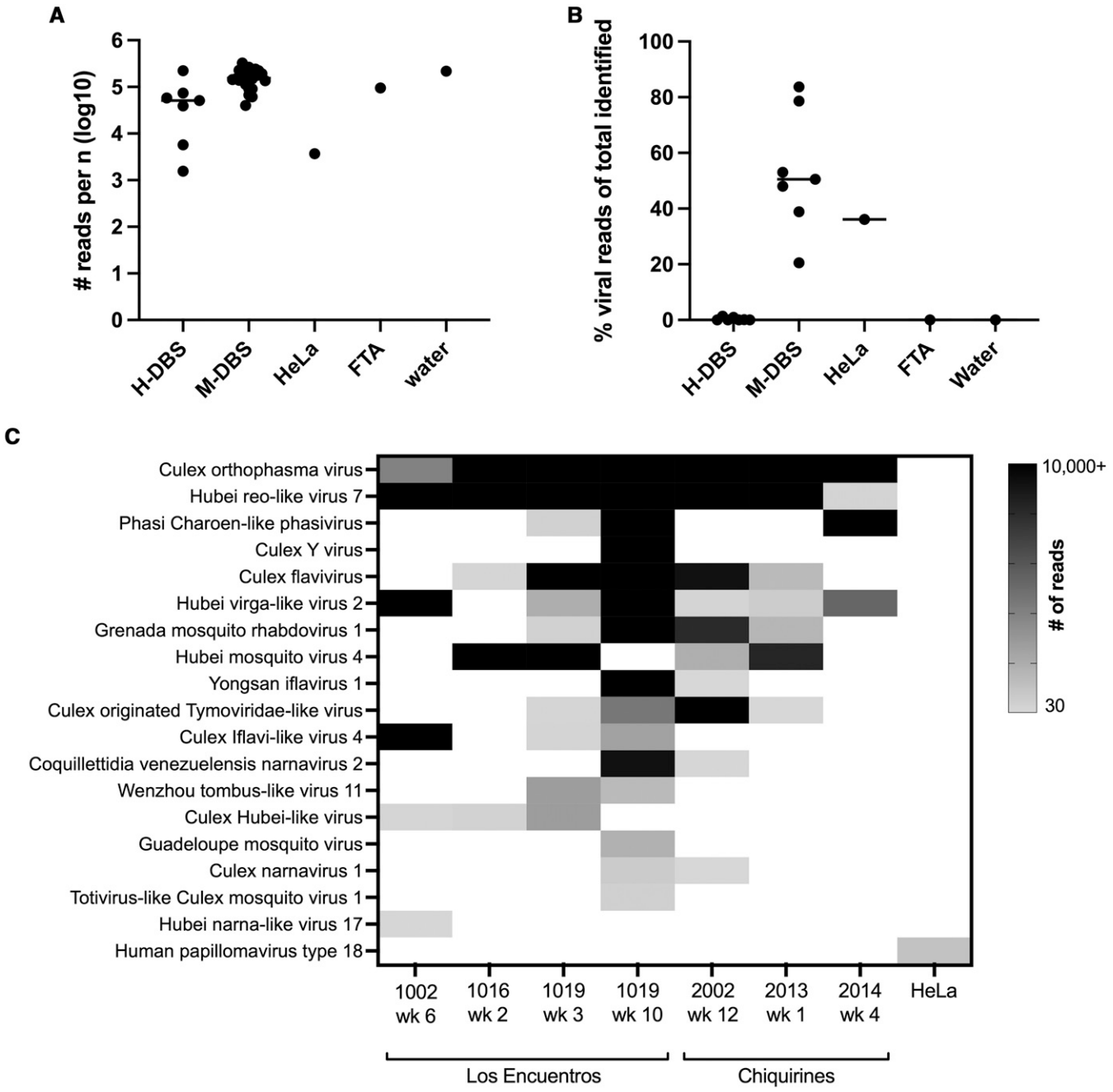

FIGURE 3. Shotgun metagenomic sequencing reveals many insect-specific viruses but no human viruses. Extracted nucleic acid from DBS were pooled by volume according to household, collection time, and type (human or mosquito) in addition to water, FTA card, and HeLa cell controls. Shotgun metagenomic sequencing libraries were created and obtained sequencing reads were run through a taxonomic assessment pipeline as described. Viral reads were tallied by unique taxonomic ID. (**A**) Total number of reads obtained per n in sample for each library type. (**B**) Proportion of identified reads aligning at the super-kingdom level to viruses. (**C**) Virus read tallies (excluding phage) in mosquito libraries with a cutoff value of 30 hits.

after cells or mosquitoes undergo primary infection with ISVs.[39,40] Despite the clear abundance of these ISVs around the world, there is currently comparatively little information on how they affect mosquito biology, arthropod-borne virus evolution, and pathogen transmission.

During this study, we identified several benefits and challenges in conducting xenosurveillance in Guatemala. We were successful in enrolling and maintaining a household cohort for this 16-week study. This was facilitated by The University of Colorado Center for Human Development, which has developed a good relationship with the surrounding communities. Notably, all household enrollments, interviews, sampling of blood and mosquitoes, and mosquito identification was performed by locally trained Guatemalan study nurses, highlighting the importance of developing local connections and expertise to conduct surveillance efficiently. We collected numerous blood-fed mosquitoes for this study using the Insectazooka, although mosquitoes were somewhat damaged, which makes

species identification difficult and error-prone without extensive training and processing time. We were successful in detecting viral RNA from M-DBS; however, more frequent sampling or targeted detection of human pathogens, is needed.

In summary, xenosurveillance has some potential as a useful surveillance strategy in low- to middle-income countries; however, mitigating the described challenges and maximizing the unique opportunities presented by the lack of host-specificity of most human-biting mosquitoes in Guatemala requires further work and development. In particular, the frequency of mosquito collection and feeding behavior (including the peak feeding times and host preferences) should be carefully considered. Building local capacity for beginning to end sample processing and analysis would ultimately benefit the application of this surveillance method. Technologies such as nanopore sequencing (Oxford Nanopore Technologies, Oxford, United Kingdom) and BioFire (BioFire Diagnostics, Salt Lake City, UT) could be useful tools for on-site virus identification. Our evidence suggests that abundant and frequent sample sources are essential for early detection of circulating pathogens. We therefore foresee xenosurveillance being useful as a targeted approach in regions where zoonotic outbreaks are inevitable.

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REFERENCES

1. Halliday JEB, Hampson K, Hanley N, Lembo T, Sharp JP, Haydon DT, Cleaveland S, 2017. Driving improvements in emerging disease surveillance through locally relevant capacity strengthening. *Science 357:* 146–148.
2. Jayatilleke K, 2020. Challenges in implementing surveillance tools of high-income countries (HICs) in low middle income countries (LMICs). *Curr Treat Options Infect Dis* 123*:* 191–201.
3. Grubaugh ND et al., 2015. Xenosurveillance: a novel mosquito-based approach for examining the human-pathogen landscape. *PLoS Negl Trop Dis* 9: e0003628.
4. Fauver JR, Gendernalik A, Weger-Lucarelli J, Grubaugh ND, Brackney DE, Foy BD, Ebel GD, 2017. The use of xenosurveillance to detect human bacteria, parasites, and viruses in mosquito bloodmeals. *Am J Trop Med Hyg 97:* 324–329.
5. Yang Y, Garver LS, Bingham KM, Hang J, Jochim RC, Davidson SA, Richardson JH, Jarman RG, 2015. Feasibility of using the mosquito blood meal for rapid and efficient human and animal virus surveillance and discovery. *Am J Trop Med Hyg 93:* 1377–1382.
6. Fauver JR, Weger-Lucarelli J, Fakoli III LS, Bolay K, Bolay FK, Diclaro JW, Brackney DE, Foy BD, Stenglein MD, Ebel GD, 2018. Xenosurveillance reflects traditional sampling techniques for the identification of human pathogens: a comparative study in West Africa. *PLoS Negl Trop Dis 12:* 0006348.
7. Asturias EJ et al., 2016. The Center for Human Development in Guatemala: an innovative model for global population health. *Adv Pediatr 63:* 357–387.
8. Olson D et al., 2017. Performance of a mobile phone app-based participatory syndromic surveillance system for acute febrile illness and acute gastroenteritis in rural Guatemala. *J Med Internet Res 19*. e368.
9. World Health Organization, 2005. *Handbook: IMCI Integrated Management of Childhood Illness.* Geneva, Switzerland: WHO. Available at: https://apps.who.int/iris/handle/10665/42939. Accessed July 28, 2021.
10. Cark-Gil S, Darsie F, 1983. The mosquitoes of Guatemala. Their identification, distribution and bionomics, with keys to adult females and larvae in English and Spanish. *Mosq Syst 15:* 151–284.
11. Hemmerter S, Šlapeta J, Van Den Hurk AF, Cooper RD, Whelan PI, Russell RC, Johansen CA, Beebe NW, 2007. A curious coincidence: mosquito biodiversity and the limits of the Japanese encephalitis virus in Australasia. *BMC Evol Biol 7*, doi: 10.1186/1471-2148-7-100.
12. Ivanova NV, Zemlak TS, Hanner RH, Hebert PDN, 2007. Universal primer cocktails for fish DNA barcoding. *Mol Ecol Notes 7:* 544–548.
13. Ratnasingham S, Hebert PDN, 2016. BOLD: the barcode of life data system (www.barcodinglife.org). *Mol Ecol Notes 2007:* 355–364.
14. Fauver JR, Akter S, Morales AIO, Black WC, Rodriguez AD, Stenglein MD, Ebel GD, Weger-Lucarelli J, 2019. A reverse-transcription/RNase H based protocol for depletion of mosquito ribosomal RNA facilitates viral intrahost evolution analysis, transcriptomics and pathogen discovery. *Virology 528:* 181–197.
15. Chrzastek K, Lee DH, Smith D, Sharma P, Suarez DL, Pantin-Jackwood M, Kapczynski DR, 2017. Use of sequence-independent, single-primer-amplification (SISPA) for rapid detection, identification, and characterization of avian RNA viruses. *Virology 509:* 159–166.
16. Cross S, Kapuscinski ML, Perino J, Maertens BL, Weger-Lucarelli J, Ebel GD, Stenglein MD, 2018. Co-infection patterns in individual *Ixodes scapularis* ticks reveal associations between viral, eukaryotic and bacterial microorganisms. *Viruses 10:* 388.
17. Fauver JR et al., 2016. West African *Anopheles gambiae* mosquitoes harbor a taxonomically diverse virome including new insect-specific flaviviruses, mononegaviruses, and totiviruses. *Virology 498:* 288–299.
18. Babraham Bioinformatics. *FastQC. A Quality Control Tool for High Throughput Sequence Data*. Available at: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/. Accessed June 16, 2021.
19. Martin M, 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J 17:* 10.

20. Li W, Godzik A, 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics 22:* 1658–1659.
21. Bankevich A et al., 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol 19:* 455–477.
22. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL, 2009. BLAST+: architecture and applications. *BMC Bioinformatics 10:* 1–9.
23. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ, 1990. Basic local alignment search tool. *J Mol Biol 215:* 403–410.
24. Buchfink B, Xie C, Huson DH, 2014. Fast and sensitive protein alignment using DIAMOND. *Nat Methods 12:* 59–60.
25. Laue T, Emmerich P, Schmitz H, 1999. Detection of dengue virus RNA in patients after primary or secondary dengue infection by using the TaqMan automated amplification system. *J Clin Microbiol 37:* 2543–2547.
26. Gyawali N, Murphy AK, Hugo LE, Devine GJ, 2020. A micro-PRNT for the detection of Ross River virus antibodies in mosquito blood meals: a useful tool for inferring transmission pathways. *PLoS One 15:* e0229314.
27. Reeves LE, Gillett-Kaufman JL, Kawahara AY, Kaufman PE, 2018. Barcoding blood meals: New vertebrate-specific primer sets for assigning taxonomic identities to host DNA from mosquito blood meals. *PLoS Negl Trop Dis 12:* e0006767.
28. Abudurexiti A et al., 2019. Taxonomy of the order Bunyavirales: update 2019. *Arch Virol 164:* 1949–1965.
29. Cowling DW, Gardner IA, Johnson WO, 1999. Comparison of methods for estimation of individual-level prevalence based on pooled samples. *Prev Vet Med 39:* 211–225.
30. Barr AR et al., 1975. Review article: host-feeding patterns of mosquitoes, with a review of advances in analysis of blood meals by serology. *J Med Entomol 11:* 635–653.
31. Takken W, Verhulst NO, 2013. Host preferences of blood-feeding mosquitoes. *Annu Rev Entomol 58:* 433–453.
32. Kading RC, Biggerstaff BJ, Young G, Komar N, 2014. Mosquitoes used to draw blood for arbovirus viremia determinations in small vertebrates. *PLoS One 9:* e99342.
33. Guzman MG et al., 2010. Dengue: a continuing global threat. *Nat Rev Microbiol 8:* S7–S16.
34. Ramos-Nino ME, Fitzpatrick DM, Eckstrom KM, Tighe S, Hattaway LM, Hsueh AN, Stone DM, Dragon JA, Cheetham S, 2020. Metagenomic analysis of *Aedes aegypti* and *Culex* quinquefasciatus mosquitoes from Grenada, West Indies. *PLoS One 15:* e0231047.
35. da Silva Ferreira R et al., 2020. Insect-specific viruses and arboviruses in adult male culicids from midwestern Brazil. *Infect Genet Evol 85:* 104561.
36. Pauvolid-Corrêa C, Solberg A, Couto-Lima O, Nogueira DM, Langevin RM, Komar S, 2016. Novel viruses isolated from mosquitoes in Pantanal, Brazil. *Genome Announc 4:* 1195–1211.
37. Ribeiro GdO et al., 2019. Detection of RNA-dependent RNA polymerase of hubei reo-like virus 7 by next-generation sequencing in *Aedes aegypti* and *Culex* quinquefasciatus Mosquitoes from Brazil. *Viruses 11:* 147.
38. Vibin J, Chamings A, Collier F, Klaassen M, Nelson TM, Alexandersen S, 2018. Metagenomics detection and characterisation of viruses in faecal samples from Australian wild birds. *Sci Rep 8:* 8686
39. Vasilakis N, Tesh RB, 2015. Insect-specific viruses and their potential impact on arbovirus transmission. *Curr Opin Virol 15:* 69–74.
40. Öhlund P, Lundén H, Blomström AL, 2019. Insect-specific virus evolution and potential effects on vector competence. *Virus Genes 55:* 127.