The Use of Xenosurveillance to Detect Human Bacteria, Parasites, and Viruses in Mosquito Bloodmeals

Joseph R. Fauver,¹ Alex Gendernalik,¹ James Weger-Lucarelli,¹ Nathan D. Grubaugh,^{1,2} Doug E. Brackney,³ Brian D. Foy,¹ and Gregory D. Ebel^{1*}

¹Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, Colorado; ²Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, California; ³Center for Vector Biology and Zoonotic Diseases, Connecticut Agricultural Experiment Station, New Haven, Connecticut

Abstract. Infectious disease surveillance is hindered by several factors, including limited infrastructure and geographic isolation of many resource-poor regions. In addition, the complexities of sample acquisition, processing, and analysis, even in developed regions, can be rate limiting. Therefore, new strategies to survey human populations for emerging pathogens are necessary. Xenosurveillance is a method that utilizes mosquitoes as sampling devices to search for genetic signatures of pathogens in vertebrates. Previously we demonstrated that xenosurveillance can detect viral RNA in both laboratory and field settings. However, its ability to detect bacteria and parasites remains to be assessed. Accordingly, we fed *Anopheles gambiae* mosquitoes blood that contained *Trypanosoma brucei gambiense* and *Bacillus anthracis*. In addition, we determined whether two additional emerging viruses, Middle East Respiratory Syndrome Coronavirus and Zika virus could be detected by this method. Pathogen-specific real-time reverse transcription polymerase chain reaction was used to evaluate the sensitivity of xenosurveillance across multiple pathogen taxa and over time. We detected RNA from all pathogens at clinically relevant concentrations from mosquitoes processed up to 1 day postbloodfeeding. These results demonstrate that xenosurveillance may be used as a tool to expand surveillance for viral, parasitic, and bacterial pathogens in resource-limited areas.

INTRODUCTION

More than 400 million acute febrile episodes occur in African children every year, with only a small percentage of them receiving a definitive diagnosis due to proximity of health-care infrastructure, limitations in diagnostic capabilities, and the presumption of a malaria infection.¹ However, a substantial portion of febrile episodes in sub-Saharan Africa are likely caused by pathogens other than *Plasmodium*.² Emerging and reemerging infectious diseases are caused by a variety of pathogenic organisms, are increasing in frequency, and often occur in areas with limited disease surveillance.³ Accordingly, novel surveillance strategies able to detect a wide array of etiological agents could have a considerable public health impact.

The term xenosurveillance refers to a technique that makes use of the hematophagous behavior of some arthropods to survey vertebrates for the presence of infectious disease agents.⁴ Previously, we and others have used xenosurveillance to identify genetic signatures (i.e., genomes) of viruses from *Anopheles gambiae* mosquito bloodmeals in laboratory- and field-based studies.^{4,5} Understanding the natural history of the hematophagous arthropods, including pathogens transmitted, that are used for xenosurveillance is imperative. *Anopheles gambiae* mosquitoes, the main malaria vector in sub-Saharan Africa,^{6,7} are highly anthropophilic,⁸ endophilic,⁹ and endophagic.¹⁰ This behavior makes them important malaria vectors, but also highly efficient, non-invasive samplers of human blood that are relatively simple to collect.

The effectiveness of mosquitoes for sampling vertebrate viruses, including some nonvector-borne agents, is now well

documented. Vertebrate virus nucleic acids from influenza H5N1,¹¹ papillomaviruses,¹² and myxoma virus¹³ have been detected in field-derived mosquito blood meals. Arboviruses also have been detected in the bloodmeal of laboratory reared mosquitoes fed by an artificial feeder⁵ and fed by various viremic animals.¹⁴ Less attention has been paid to the detection of vertebrate-derived bacteria or parasites. Fernandez de Marco and others successfully identified a cow infecting parasite, *Theileria orientalis*, in the bloodmeal of field-caught *Culiseta annulata* mosquitoes.¹⁵ However, the ability of this method to detect human parasites and bacteria remains unknown.

Therefore, we sought to determine whether xenosurveillance may be used to detect genetic signatures of bacteria and parasites, in addition to medically relevant viruses. Specifically, *An. gambiae* mosquitoes were fed bloodmeals containing *Trypanosoma brucei gambiense*, *Bacillus anthracis*, Middle East Respiratory Syndrome Coronavirus (MERS-CoV), or Zika virus (ZIKAV). To assess the sensitivity of xenosurveillance, bloodmeals containing serial 10-fold dilutions of each pathogen were fed to mosquitoes. Mosquitoes were also fed and held for up to 24 hours to determine the sensitivity of xenosurveillance over time. Specific real-time reverse transcription polymerase chain reaction (qRT-PCR) assays were used to detect RNA from each pathogen. Using this technique, we could detect RNA at or below typical clinical concentrations for up to 24 hours. These results indicate that xenosurveillance is a sensitive and effective means of detecting pathogens in blood samples collected by mosquitoes at clinically and operationally relevant concentrations and timescales.

MATERIALS AND METHODS

Mosquitoes and microbes. *Anopheles gambiae* sensu stricto mosquitoes were used in all experiments. Mosquitoes were derived from the laboratory G3 strain (origin The Gambia)

*Address correspondence to Gregory D. Ebel, Department of Microbiology, Immunology, and Pathology, Colorado State University, 1692 Campus Delivery, Fort Collins, CO 80523. E-mail: gregory.ebel@colostate.edu

or from a recently colonized field strain from Burkina Faso.¹⁶ Larvae were reared at 28–31°C and fed fish food daily. Adults were held in 80% relative humidity on a 14:10 light:dark photoperiod and were provided with water and a 10% sucrose solution ad libitum. Adult mosquitoes used for experiments were 3–7 days' postemergence. *Bacillus anthracis* Sterne 34F2 strain bacteria and *T. b. gambiense* STIB 386 strain was obtained through the National Institute of Health (NIH) Bio-defense and Emerging Infections Research Resources Repository, National Institute of Allergy and Infectious Disease, NIH. Bacteria were propagated a day prior to feeding in tryptic soy broth at 37°C. Parasites were maintained in a T-75 tissue culture flask with HMI-9 culture media.¹⁷ Trypanosomes were passaged weekly by removing media, centrifuging at 350 × *g* for 5 minutes, and resuspending parasites in fresh culture media in a new flask. Stocks of MERS-CoV (obtained from Tony Schountz) and ZIKAV (strain PRVABC59) were grown on Vero cells as previously described.¹⁸

Serial dilution bloodfeed. To determine limits of detection for each pathogen, various dilutions of a blood/pathogen mixture were used; 500 µL of live cultures (*B. anthracis*/*T. b. gambiense*) or stocks of virus (MERS-CoV/ZIKAV) were diluted with 500 µL of defibrinated sheep blood and successive serial dilutions were made to a final concentration of one volume pathogen to 10,000 volumes blood. Cartons of mosquitoes were exposed to the blood/pathogen mixture using a water-jacketed membrane feeding apparatus and held for 12 hours postbloodfeed (Figure 1). Mosquito bloodmeals were stored on CloneSaver Flinders Technology Associates cards (GE Healthcare, Pittsburgh, PA) and processed as previously described with slight modification.⁴ Briefly, bloodfed mosquitoes were anesthetized with triethylamine and bloodmeals were removed from the mosquito abdomen with forceps, placing the anterior end of the abdomen to the FTA card, and squeezing out the blood bolus. Forceps were used to push the blood bolus onto the FTA card and up to 20 µL of RNA Later (ThermoFisher, Waltham, MA) was added to the mosquito dried blood spots (M-DBS) to enhance diffusion into the card and stabilize nucleic acid. Forceps were cleaned with 70% ethanol between processing of each sample. Each FTA card was left to dry overnight at room temperature and moved to storage in a –80°C freezer for up to 2 weeks.

The starting concentration of each pathogen was determined by qRT-PCR analysis of RNA extracted from 50 µL of undiluted stock agent. In addition, RNA from 2 µL of each blood/pathogen mixture was pipetted directly onto FTA cards (referred to as DBS) and tested by qRT-PCR as earlier. The volume of 2 µL was chosen because it is the approximate volume of a mosquito bloodmeal. DBS samples served as a positive control, as well as a measure for how much pathogen RNA was lost during the process of bloodfeeding.

Time series bloodfeed. After pathogen exposure, mosquitoes were sampled at 6-hour time points up to 24 hours to determine how long pathogen RNA could be detected. Cartons of mosquitoes were exposed to 500 µL of culture/stock mixed with 500 µL of defibrinated sheep blood and held until sampling (Figure 1).

Sample processing. A single punch was removed from each DBS and M-DBS card using a Harris 3 mm micro-puncher (GE Healthcare) and placed into an 8-strip PCR tube containing 70 µL of RNA Rapid Extraction Solution (ThermoFisher) supplemented with 1% 0.5-mm ethylenediaminetetraacetic acid. A total of 5 M-DBS per pathogen

were used for each dilution experiment and time point. PCR tubes were placed in a Talboys Standard Microplate Vortex Mixer (Southern Labware, Cumming, GA) at 800 rpm for up to 16 hours at 4°C to elute nucleic acids; 50 µL of elution was used for DNA/RNA extraction with the Mag-Bind Viral DNA/RNA kit (Omega Bio-Tek, Norcross, GA) according to manufacturer protocol.

qRT-PCR analysis. Because of specific knowledge about pathogens spiked into mosquito bloodmeals, we opted to develop pathogen-specific qRT-PCR approaches opposed to a more unbiased approach (e.g., Next-Generation Sequencing). As well, qRT-PCR was chosen over quantitative PCR to detect all pathogens assessed using the same methodology. To prepare PCR standards, extracted RNA from each pathogen culture/stock was reverse transcribed with a forward primer containing a T7 transcription site on the 5' end. Subsequent DNA was transcribed using the MEGAscript T7 Transcription kit (ThermoFisher) (Supplemental Figure 1). Transcripts were diluted to 1×10^8 transcripts per reaction, and further log-serially diluted to 1×10^2 transcripts per reaction. Specific primer and FAM probe sets were used for each individual pathogen (Table 1). Primers and probes were designed with the Primer3 software using Geneious, version 9.0.3 (Auckland, New Zealand). For *T. b. gambiense* and *B. anthracis*, primers were designed to highly and constitutively expressed genes, Alpha tubulin (TriTrypDB no. Tb927.1.2340) and the RNA polymerase beta subunit (GenBank no. AF205325.1)

FIGURE 1. Methodology of xenosurveillance in a laboratory setting. Mosquitoes were allowed to feed on pathogenemic bloodmeals. Engorged mosquitoes were held for a specified period of time and bloodmeals were expelled onto FTA cards (mosquito dried blood spots [M-DBS]). DBS consisted of 2 µL of the pathogenemic bloodmeals. FTA cards were allowed to dry at room temperature overnight, and were then transferred to a –80°C freezer for storage. M-DBS and DBS were eluted off of FTA cards for RNA extraction. Extracted RNA was used for pathogen specific real-time reverse transcription polymerase chain reaction (Figure adapted from Grubaugh and others⁴).

TABLE 1
Primer and probe sequences for qRT-PCR analysis

Primer	5'-3' sequence	Product size
T.b.g. Alpha tubulin F	AAGTCCAAGCTCGGCTACAC	182
T.b.g. Alpha tubulin R	TACGTGGGCGCTCAATATC	
T.b.g. Alpha tubulin P	ACCGCAGGTGTCGACGGCTGTCGTGG	
<i>Bacillus anthracis</i> RNA Pol Beta Subunit F	CCACCAACAGTAGAAAAATGCC	175
<i>B. anthracis</i> RNA Pol Beta Subunit R	AAATTTACCAGTTTCTGGATCT	
<i>B. anthracis</i> RNA Pol Beta Subunit P	ACTTGTGTCTCGTTCTTCGATCCAAAGCG	
MERS-CoV Nucleocapsid F	GGCACTGAGGACCCACGTT	75 ²¹
MERS-CoV Nucleocapsid R	TTGCGACATACCCATAAAAGCA	
MERS-CoV Nucleocapsid P	CCCAAATTGCTGAGCTTGCTCCTACA	
Zika 3' NS1 F	CCGCTGCCCAACACAAG	77 ²²
Zika 3' NS1 R	CCACTAACGTTCTTTGCAGACAT	
Zika 3' NS1 P	AGCCTACCTTGACAGCAGTCAGACACTCAA	

MERS-CoV = Middle East Respiratory Syndrome Coronavirus; qRT-PCR = real-time reverse transcription polymerase chain reaction; T.b.g. = *Trypanosoma brucei gambiense*.

genes, respectively.^{19,20} Primer and probe sequences were blasted against the National Center for Biotechnology Information Nucleotide database to confirm specificity. For both MERS-CoV and ZIKAV, previously established primer/probe sets designed by the Centers for Disease Control and Prevention for clinical purposes were used.^{21,22} Run statistics for each set of diluted standards is shown in Supplemental Figure 1. The Reed–Muench method was used to calculate 50% end points for each pathogen.²³ Fifty percentage end points were calculated with titers of pathogen in each serial dilution bloodmeal, opposed to titers of pathogen recovered from M-DBS. This value reflects the amount of pathogen required in a bloodmeal (i.e., bacteremia, parasitemia, and viremia, referred to as “pathogenemia”) to be detectable by xenosurveillance 50% of the time.

RESULTS

Pathogens are detected in mosquito bloodmeals at clinically relevant levels. Xenosurveillance using *An. gambiae* mosquitoes fed blood containing serial 10-fold dilutions of *B. anthracis*, *T. b. gambiense*, MERS-CoV, and ZIKAV to determine the limits of parasite, bacteria, and virus detection from mosquito bloodmeals. In all cases, genetic signatures of pathogens were detectable by xenosurveillance when mosquitoes were fed a bloodmeal containing pathogens at or below clinically reported levels (Figure 2).^{24–27} Transcripts from *B. anthracis* were detected in all mosquitoes at all dilutions (input range: 2.21×10^5 – 2.21×10^1), resulting in 50% end-point titer of 6.98×10^1 transcripts/2 μ L (Figure 2A). Transcripts from *T. b. gambiense* were detected in the first three 10-fold serial dilutions (input range: 3.86×10^6 – 3.86×10^4), resulting in a 50% end-point titer of 1.16×10^5 transcripts/2 μ L (Figure 2B). ZIKAV RNA was detected in all mosquitoes tested in the first three 10-fold serial dilutions, and in 4/5 mosquitoes in the fourth (input range: 2.50×10^7 – 2.50×10^4), resulting in a 50% end-point titer of 5.94×10^4 genome equivalence/2 μ L (Figure 2C). RNA from MERS-CoV was detected in all mosquitoes in the first four dilutions (input range: 1.47×10^7 – 1.47×10^4), resulting in a 50% end-point titer of 4.65×10^4 genome equivalence/2 μ L (Figure 2D).

Pathogens can be detected in mosquito bloodmeals up to 24 hours postfeeding. Mosquitoes were fed a 1:1 mixture of pathogen/blood and held for up to 24 hours postbloodmeal. Mosquitoes were sampled at 6, 12, 18 and 24 hours postbloodfeed. RNA from all of the pathogens examined was

stable in the mosquito and detectable by xenosurveillance for up to 24 hours postbloodmeal (Figure 3). The amount of RNA detected remained similar or unchanged for both *B. anthracis* and MERS-CoV for each time point sampled (Figure 3A and D). RNA from *T. b. gambiense* and ZIKAV dropped compared with the input at each time point, but remains at detectable levels for up to 24 hours postbloodfeed (Figure 3B and C).

DISCUSSION

Xenosurveillance has proven to be effective at detecting viral genomes in both laboratory and field conditions;⁴ however, it remains to be determined how useful xenosurveillance is at detecting genetic signatures of human infecting bacteria and parasites. To address this, we performed dilution and time course experiments using *B. anthracis*, *T. b. gambiense*, MERS-CoV, and ZIKAV representing three different taxa of disease causing agents.

For xenosurveillance to be a useful tool, mosquitoes must feed on a pathogenemic host and transcripts or genomes from these pathogens must be detectable in mosquito bloodmeals. Human pathogenemia can vary widely between pathogen taxa, as well as between similar species of pathogens. We performed a serial dilution bloodfeeding experiment with four microbes representing three broad taxa of infectious agents to determine the efficacy of xenosurveillance at various levels of pathogenemia (Figure 2). Every pathogen assessed was detectable by xenosurveillance when mosquitoes were fed bloodmeals at or below reported clinical values. *Bacillus anthracis* was detected by xenosurveillance in all dilution experiments, subsequently resulting in the lowest 50% end-point of 6.98×10^1 (Figure 2A). More transcripts were detected in M-DBSs compared with both the input and DBS controls at each dilution. This phenomenon may be the result of *B. anthracis* actively replicating while in the mosquito midgut/bloodmeal. *Anopheles*, and mosquitoes in general, have diverse microbiomes containing multiple species of commensal bacteria.²⁸ Nonexposed M-DBSs used as a control were negative by qRT-PCR, ruling out the possibility of cross-reactivity with commensal bacteria. Transcripts from *B. anthracis* could be detected by xenosurveillance below the clinical bacteremia reported for this species.²⁴ Xenosurveillance to detect MERS-CoV and ZIKAV resulted in the next lowest 50% end-points, 4.65×10^4 and 5.94×10^4 , respectively (Figure 2C and D). These end-points fall within the clinically reported viremia for both viruses.^{25,26} *Trypanosoma brucei gambiense* detection by xenosurveillance resulted in

FIGURE 2. Detection of genes and genomes from human bacteria, parasites, and viruses at low levels in mosquito bloodmeals. Input $N = 1$, dried blood spots $N = 3$, mosquito DBS $N = 5$ for each pathogen. Vertical dashed lines demarcate each pathogen:blood dilution. Horizontal dashed lines indicate the lower limit of the real-time reverse transcription polymerase chain reaction (qRT-PCR) assay. Shaded areas show reported clinical ranges of parasitemia, bacteremia, and viremia. Data points on the y axis at 10^0 indicate samples that were tested by qRT-PCR but were negative. Error bars represent the standard error of the mean.

the highest 50% endpoint of 1.16×10^5 (Figure 2B), which falls within the reported clinical parasitemia. However, the amount of *T. b. gambiense* parasites in the blood of an infected individual at any one time can highly vary, from more than 1×10^8

to virtually none.²⁷ Relapsing parasitemia, the result of antigenic variation, is common in *T. b. gambiense*,²⁹ as well as other blood-borne protozoan parasites. Nevertheless, these data demonstrate that xenosurveillance can reliably detect

FIGURE 3. Genes and genomes can be detected from four major human pathogens in mosquito bloodmeals up to 24 hours postbloodfeed. $N = 5$ for each pathogen. Horizontal bars represent the mean of each timepoint. Error bars represent the standard error of the mean.

genetic signatures from three separate taxa of pathogens at or below clinically reported pathogenemia.

The amount of time *An. gambiae* mosquitoes require to process bloodmeals, as well as the amount of time they rest indoors postbloodfeeding, are critical factors to the success of xenosurveillance. The rate of bloodmeal digestion varies between genus and species of mosquito,^{30,31} as well as within species due to environmental conditions.³² A study conducted in The Gambia showed *An. gambiae* mosquitoes can have a gonotrophic cycle as often as every 2 days.³³ Multiple studies show the success of blood source identification using DNA extracted from the bloodmeal of *Anopheles* mosquitoes significantly decreased after 30 hours.^{34,35} These data, along with previous field experience,⁴ demonstrate the amount of time to capture a bloodfed *An. gambiae* mosquito indoors that can be used for xenosurveillance is about 1 day. We experimentally demonstrated that RNA can be reliably detected up to 24 hours postbloodmeal for all four species (Figure 3). Similar to the dilution experiments, *B. anthracis* transcripts were more abundant in M-DBSs than the input bloodmeal (Figure 3A). For the remaining pathogens, *T. b. gambiense*, ZIKAV, and MERS-CoV, transcripts/GEs were less in M-DBSs compared with input; however, transcripts/GE were detected in all mosquitoes at each time point (Figure 3B–D). It is important to note that these pathogens are not vectored by mosquitoes, therefore RNA detected in M-DBS arose from the bloodmeal, although it does appear there is slight replication of *B. anthracis* in the mosquito midgut.

Anopheles gambiae mosquitoes were used in this experiment to best replicate field conditions in sub-Saharan Africa, where we believe xenosurveillance could have the biggest impact. Because of their close association with humans, engorged *An. gambiae* mosquitoes are relatively easy to collect inside homes opposed to other common mosquito species. Pathogens used in this study were selected because their taxonomic diversity, representing different taxa of disease causing organisms, as well their availability, culturability, select-agent status (*B. anthracis*), recent emergence, and epidemic potential. Currently, we have demonstrated that xenosurveillance can be used to detect parasites and bacteria in a laboratory setting; however, this remains to be assessed in the field. Although Grubaugh and others⁴ demonstrated the utility of xenosurveillance to detect viruses in the field, future field-based xenosurveillance studies that use Next-Generation Sequencing techniques need to be conducted. This will determine if xenosurveillance can detect broad groups of pathogens, as well as determine the sensitivity of xenosurveillance compared with more traditional surveillance strategies.

Recent outbreaks of infectious diseases have demonstrated the need for improved surveillance and pathogen detection strategies, especially in resource-limited areas where a majority of pathogens have emerged/reemerged.³ Currently, there are numerous systems developed by local and national governments,^{36–38} research groups,³⁹ and nonprofit organizations^{40,41} aimed at predicting/detecting the next disease outbreak. The majority of these systems are dependent on a clinician or health-care provider to report into a larger network. This is problematic in areas like sub-Saharan Africa where it is estimated that less than 20% of febrile episodes come to the attention of any formal health-care system.¹ The practicality of xenosurveillance allows for crucial data collection in low-tech

environments. This novel data stream can potentially help inform public health officials about specific etiological agents circulating in these environments.

Received January 25, 2017. Accepted for publication March 28, 2017.

Note: Supplemental figure appears at www.ajtmh.org.

Published online May 30, 2017.

Acknowledgments: We would like to thank Jasmine Donkoh, Taylor Clarkson, and Soleil Foy for their assistance in rearing mosquito colonies, and Jason Richardson and Richard Jarman for thoughtful comments on the manuscript.

Financial support: The project was supported in part by the CSU Infectious Disease Supercluster Grant “Xenosurveillance: A novel approach for interrogating the human-pathogen landscape in sub-Saharan Africa” awarded to DEB, BDF, and GDE. This project was also supported in part by an Armed Forces Health Surveillance Center grant awarded to the Walter Reed Army Institute (subcontract DEB).

Disclaimer: The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' addresses: Joseph R. Fauver, Alex Gendernalik, James Weger-Lucarelli, Brian D. Foy, and Gregory D. Ebel, Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, CO, E-mails: joseph.fauver@colostate.edu, alghobbes@gmail.com, james.weger@colostate.edu, brian.foy@colostate.edu, and gregory.ebel@colostate.edu. Nathan D. Grubaugh, Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, CA, E-mail: nathan.grubaugh@yahoo.com. Doug E. Brackney, Center for Vector Biology and Zoonotic Diseases, Connecticut Agricultural Experiment Station, New Haven, CT, E-mail: doug.brackney@ct.gov.

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