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Mosquito-Borne Viruses and Insect-Specific Viruses Revealed in Field-Collected Mosquitoes by a Monitoring Tool Adapted from a Microbial Detection Array

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ABSTRACT Several mosquito-borne diseases affecting humans are emerging or re-emerging in the United States. The early detection of pathogens in mosquito populations is essential to prevent and control the spread of these diseases. In this study, we tested the potential applicability of the Lawrence Livermore Microbial Detection Array (LLMDA) to enhance biosurveillance by detecting microbes present in *Aedes aegypti*, *Aedes albopictus*, and *Culex* mosquitoes, which are major vector species globally, including in Texas. The sensitivity and reproducibility of the LLMDA were tested in mosquito samples spiked with different concentrations of dengue virus (DENV), revealing a detection limit of >100 but <1,000 PFU/ml. Additionally, field-collected mosquitoes from Chicago, IL, and College Station, TX, of known infection status (West Nile virus [WNV] and *Culex* flavivirus [CxFLAV] positive) were tested on the LLMDA to confirm its efficiency. Mosquito field samples of unknown infection status, collected in San Antonio, TX, and the Lower Rio Grande Valley (LRGV), TX, were run on the LLMDA and further confirmed by PCR or quantitative PCR (qPCR). The analysis of the field samples with the LLMDA revealed the presence of cell-fusing agent virus (CFAV) in *A. aegypti* populations. *Wolbachia* was also detected in several of the field samples (*A. albopictus* and *Culex* spp.) by the LLMDA. Our findings demonstrated that the LLMDA can be used to detect multiple arboviruses of public health importance, including viruses that belong to the *Flavivirus*, *Alphavirus*, and *Orthobunyavirus* genera. Additionally, insect-specific viruses and bacteria were also detected in field-collected mosquitoes. Another strength of this array is its ability to detect multiple viruses in the same mosquito pool, allowing for the detection of cocirculating pathogens in an area and the identification of potential ecological associations between different viruses. This array can aid in the biosurveillance of mosquito-borne viruses circulating in specific geographical areas.

IMPORTANCE Viruses associated with mosquitoes have made a large impact on public and veterinary health. In the United States, several viruses, including WNV, DENV, and chikungunya virus (CHIKV), are responsible for human disease. From 2015 to 2018, imported Zika cases were reported in the United States, and in 2016 to 2017, local Zika transmission occurred in the states of Texas and Florida. With globalization and a changing climate, the frequency of outbreaks linked to arboviruses will increase, revealing a need to better detect viruses in vector populations. With the capacity of the LLMDA to detect viruses, bacteria, and fungi, this study highlights its ability to broadly screen field-collected mosquitoes and contribute to the surveillance and management of arboviral diseases.

KEYWORDS *Aedes aegypti*, *Aedes albopictus*, *Culex*, *Wolbachia*, cell-fusing agent virus, *Culex* flavivirus, insect-specific virus, microarrays

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Mosquito-borne viruses emerge and reemerge at accelerating rates, causing significant morbidity and mortality in humans and animals (1). Due to globalization, mosquito vectors and associated arboviruses have been introduced into new geographic regions (2–5). One noteworthy example was the introduction of West Nile virus (WNV) into the New World. The virus was first detected in New York in 1999 and then spread throughout the United States (6) using several *Culex* species as vectors. The yellow fever mosquito, *Aedes aegypti*, and the Asian tiger mosquito, *Aedes albopictus*, are invasive mosquito species that are widespread in urban environments of tropical, subtropical, and temperate regions and are responsible for the emergence or reemergence of multiple mosquito-borne diseases caused by different viral agents, including dengue virus (DENV) (7–9), chikungunya virus (CHIKV), and, more recently, Zika virus (ZIKV). Since its introduction in Brazil in 2014, ZIKV has spread to the rest of South America and moved north to Central and North America, resulting in the local transmission of the virus in Florida and Texas in 2016 to 2017 (10–12).

These mosquito-borne viruses have proven to be difficult to manage and control despite considerable attention, and the ability to broadly screen mosquitoes for microbes has appeal on many fronts. Microarrays have the ability to detect multiple targets that would be missed by other more-specific or targeted assays and could reveal important components of the mosquito microbiome relevant to the transmission of viruses of public and veterinary health importance. Typically, microbial diversity associated with mosquitoes has been studied using both culture-dependent and -independent approaches (13–16). While culture-dependent approaches are time-consuming, molecular techniques such as reverse transcription PCR (RT-PCR) (17–19) and quantitative real-time PCR (qRT-PCR) (20–22) are typically designed to be specific at the species or family level. More recently, many new forms of next-generation sequencing (NGS) (23, 24) have proven to be effective to characterize the mosquito microbiome, but they require the depletion of host-derived nucleic acid in order to sensitively detect viruses (25, 26). For bacterial discovery, 16S rRNA sequencing is usually performed (27, 28), but it detects only conserved regions of the 16S rRNA gene of bacteria and does not allow for the detection of viruses and other microbes in the sample. Shotgun metagenomic sequencing provides the highest resolution to detect different kinds of microbes in a sample (29) but remains expensive and time-consuming and requires extensive bioinformatic expertise.

Accordingly, this study utilized the Lawrence Livermore Microbial Detection Array (LLMDA), which was designed to screen diverse samples for thousands of bacteria, viruses, fungi, and protozoa (30, 31). The LLMDA version used in this study detects 10,261 species of microbes, including 4,219 viruses, 5,367 bacteria, 293 archaebacteria, 265 fungi, and 117 protozoa (32). The LLMDA has been previously used to detect viral and bacterial pathogens from clinical and archeological samples (30, 33). We conducted a pilot study to evaluate the utility of the LLMDA to screen mosquito pools collected from multiple regions of Texas from 2016 to 2017 for mosquito-borne viruses. The LLMDA was able to detect and identify DENV serotype 2 (DENV-2), Rift Valley fever virus (RVFV), and Mayaro virus (MAYV) in spiked mosquito samples and WNV, *Culex* flavivirus (CxFLAV), and cell-fusing agent virus (CFAV) from field-collected mosquitoes. LLMDA results from field-collected mosquitoes were further confirmed using standard and/or quantitative PCR methods, and coinfection with multiple viruses was detected in spiked and field-collected mosquitoes. Viruses were detected in pools of mosquitoes of various size and in tissues, including midguts (MG) and salivary glands (SG). Additionally, *Wolbachia* was detected in field-collected *Aedes aegypti* and *Culex* mosquitoes.

RESULTS

In total, we analyzed 39 mosquito pools representing 512 individual mosquitoes (see Table S1 in the supplemental material). Ten pools were field-collected *A. aegypti* ($n = 116$), eight pools were colony-raised *A. aegypti* Liverpool ($n = 80$), eight pools were field-collected *A. albopictus* ($n = 49$), four pools were field-collected *Culex* spp. ($n = 86$), and six pools were field-collected *Culex quinquefasciatus* ($n = 138$). One pool was

TABLE 1 LLMDA limit of detection and reproducibility in spiked mosquito pools

Virus(es)	PFU/ml	LLMDA detection	Log CL ratio	No. of probes positive/total	Mosquito species
DENV-2	10^2	Negative			*A. aegypti*
	10^2	Negative			*A. aegypti*
	10^3	Positive	56.7	20/27	*A. aegypti*
	10^3	Positive	60.7	23/33	*A. aegypti*
DENV-2 + MAYV	10^4, 10^4	Positive	197.1, 78.5	46/47, 20/25	*A. aegypti*
	10^5, 10^4	Positive	224.6, 122.3	53/54, 25/25	*A. aegypti*
RVFV	10^4	Positive	52.8	16/19	*C. quinquefasciatus*
ZIKV	10^4	Negative	0	3/27	*A. aegypti*
	10^2	Negative	0	3/27	*A. aegypti*

colony-raised *C. quinquefasciatus* ($n = 10$), and one pool was an equal mixture of colony-raised *A. aegypti* and *C. quinquefasciatus* ($n = 20$) to serve as a negative control. To understand the compartmentalization of bacteria within *A. aegypti* and *Culex* sp. mosquitoes, four additional pools were analyzed: one pool of 23 midguts (MG) and one pool of 23 salivary glands (SG) for each mosquito species (*A. aegypti* and *Culex* spp.).

LLMDA sensitivity and reproducibility. In order to test the LLMDA sensitivity and reproducibility, we spiked known amounts of DENV serotype 2 (DENV-2) in *A. aegypti* Liverpool mosquito pools, each containing 10 female mosquitoes. Duplicate pools were spiked with 10^2 PFU/ml or 10^3 PFU/ml of virus, and two other pools were spiked with 10^4 PFU/ml or 10^5 PFU/ml (Table 1). According to our results, the limit of detection, or minimum amount of virus required to determine its presence or absence in the sample, is equal to or less than 10^3 PFU/ml and above 10^2 PFU/ml. The DENV-2 dilutions (10^3 PFU/ml, 10^4 PFU/ml, and 10^5 PFU/ml) were all detected using the array, with positive probes hybridizing to different regions of the DENV-2 genome (Fig. 1A). Because positive signals from more than 20% of the probes for DENV-2 were detected and were in several regions of the genome, these DENV-2-spiked samples are considered DENV positive. As seen in Table 1, the number of positive probes was close to matching the total number of probes present on the array for this target, especially for the samples spiked with the largest amount of virus. Additionally, the log CL ratio (ratio between the likelihood of the observed probe signal when assuming the target is present in the sample and the likelihood when assuming no target is present) was above 0, and therefore the samples were considered DENV positive. An increase in the log CL ratio, ranging from 56.7 to 224.6, was observed, correlating with the increase in amount of spiked virus. The reproducibility of the LLMDA was tested for two of the dilutions in duplicates (10^2 PFU/ml and 10^3 PFU/ml) and showed consistency. For the duplicates with 10^2 PFU/ml, no signal was recovered, and for the duplicates with 10^3 PFU/ml, the log CL ratios were similar, with respective values of 56.7 and 60.7.

Samples spiked with the largest amounts of DENV (10^5 PFU/ml and 10^4 PFU/ml) were coinfected with a known amount of Mayaro virus (MAYV) (10^4 PFU/ml). Both viruses were successfully detected by the LLMDA (Fig. 1A and B), demonstrating the ability of the LLMDA to detect viruses from different families if present in the same mosquito sample pool. Additionally, *C. quinquefasciatus* spiked with a known amount of Rift Valley fever virus (RVFV) (10^4 PFU/ml) also resulted in a positive signal, highlighting the ability of the LLMDA to detect other arboviruses of medical and veterinary importance (Fig. 1C). The *A. aegypti* homogenates spiked with ZIKV tested negative by the LLMDA. First, as seen in Fig. 1D, only 3 probes out of the 27 designed to detect ZIKV had a positive signal (the percentage of positive probes was therefore below the default threshold of 20). Second, the 3 high-intensity probes cover only a specific region of the genome instead of spanning across the genome. Third, the log CL ratio was equal to zero. These spiked samples were confirmed to be ZIKV positive using a quantitative PCR (qPCR) assay, with threshold cycle (C_T) values of 20.63 and 28.96 for

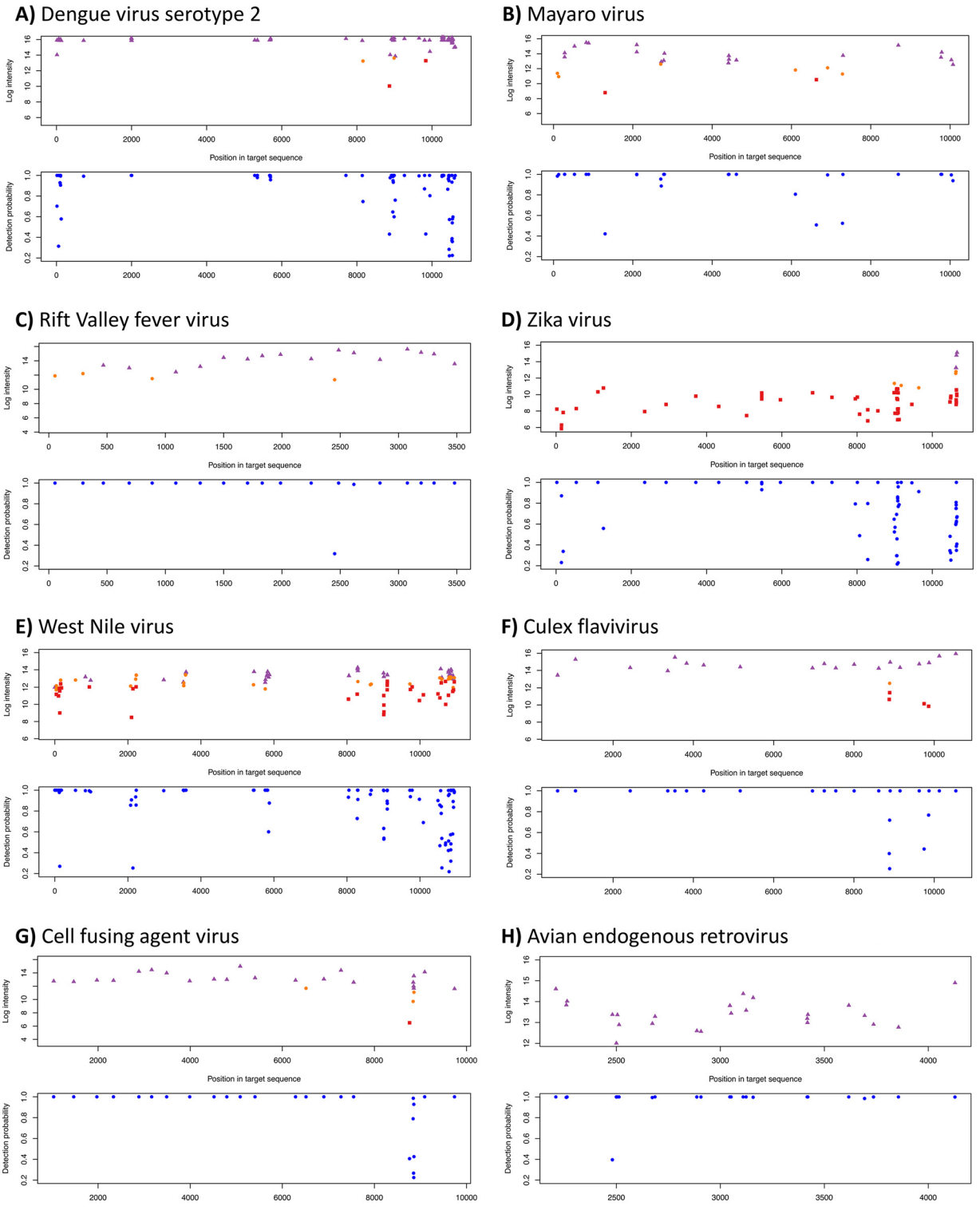

FIG 1 LLMDA probe detection for DENV-2 (A), MAYV (B), RVFV (C), ZIKV (D), WNV (E), CxFLAV (F), CFAV (G), and avian endogenous retrovirus (H). For each virus, two graphs are shown; the upper panel represents the intensity of the probes according to the position of the target in the genome; the lower panel represents the probability of detection according to the genome region. Samples for which intensity was higher than the 99th percentile of that of the control probes are shown in purple, those for which intensity is between the 99th and 95th percentiles are shown in orange, and those for which the intensity was below the 95th percentile are in red.

TABLE 2 Comparison of LLMDA and qPCR results in naturally infected mosquito pools

Virus	qPCR detection	Observed C_T value	LLMDA detection	Log CL ratio	No. of probes positive/total	Mosquito species	Additional LLMDA virus detected	Log CL ratio	No. of probes positive/total
WNV	Positive	15.16	Positive	115.3	58/79	*Culex* spp.	CxFLAV	74.4	19/19
	Positive	19.95	Negative		0/79	*Culex* spp.	CxFLAV		0/19
CxFLAV	Positive	18.24	Negative		0/75	*C. quinquefasciatus*			
	Positive	30.31	Negative		0/75	*C. quinquefasciatus*			

the samples spiked with 10^4 and 10^2 PFU/ml, respectively. In addition, densoviruses were detected in all of the DENV-2- and MAYV-spiked *A. aegypti* samples but were further tested by PCR for confirmation (see Table S4 in the supplemental material).

Application of LLMDA to detection of viruses from field-collected mosquitoes of known infection status. In order to test the ability of the LLMDA to detect natural virus loads within mosquito pools, naturally WNV- and CxFLAV-infected mosquitoes previously collected in Chicago, IL, and College Station, TX, were used (Table 2). Of the two WNV-positive mosquito pools previously detected using qPCR (C_T values, 15.16 and 19.95), only one was successfully identified as WNV by the LLMDA (Fig. 1E). In this particular case, 58 out of the 79 probes that characterized WNV were positive, and a log CL score of 115.3 was observed. Interestingly, of these two pools, one was found positive for *Culex* flavivirus (CxFLAV) by the microarray. In this sample, 19 out of 19 probes were positive (log CL ratio, 74.4), revealing the ability of the microarray to detect coinfections from naturally infected mosquito pools (Fig. 1F). The two CxFLAV-positive controls from College Station (C_T values of 18.24 and 30.31) were not detected using the microarray (Table 2).

Application of the LLMDA to detection of microbes from field-collected mosquitoes of unknown infection status. (i) LLMDA viral analysis. Several viruses were detected in the field-collected mosquito pools (Fig. 1). *A. aegypti* from Lower Rio Grande Valley (LRGV) ($n = 2$) and San Antonio ($n = 1$) were found to be positive for cell-fusing agent virus (CFAV), an insect-specific flavivirus (Fig. 1G). All 21 probes designed for that virus on the array were positives (log CL ratio = 77). *Aedes aegypti* SG and MG pools were also positive for CFAV (log CL ratio = 77; positive probes/all target probes =21/21). Interestingly, one *A. aegypti* pool from the LRGV was found to be positive for the avian endogenous retrovirus (23 out of 23 expected probes; log CL ratio = 74.9) (Fig. 1H). None of the field-collected *A. albopictus* or *Culex* sp. samples tested positive for viruses, with the exception of the *Culex* population from Chicago (as described in the previous paragraph). To assess the accuracy of the LLMDA to detect the presence of insect-specific viruses, all samples were tested using conventional PCR methods with gene-specific primers designed for CFAV and CxFLAV (Tables 3 and 4). CFAV strain TX AR 11-1022 and CxFLAV strain M23873, obtained from the University of Texas Medical Branch (UTMB) World Reference Center for Emerging Viruses and Arboviruses

TABLE 3 LLMDA and conventional PCR detection of field-collected samples

Locality	Mosquito species	Sample size	Virus	LLMDA detection[a]	PCR detection[a]	% identity by Sanger sequencing (accession no.)
LRGV	*A. aegypti*	96	CFAV	2 (9)	3 (9)	97.7 (GQ165810)
San Antonio	*A. aegypti*	33	CFAV	1 (2)	2 (2)	100 (KJ476731)
Colony	*A. aegypti*	40	CFAV	0 (4)	0 (4)	
LRGV	*A. albopictus*	4	CFAV	0 (3)	0 (3)	
San Antonio	*A. albopictus*	36	CFAV	0 (2)	0 (2)	
College Station	*A. albopictus*	9	CFAV	0 (3)	0 (3)	
LRGV	*C. quinquefasciatus*	25	CxFLAV	0 (2)	0 (2)	
San Antonio	*C. quinquefasciatus*	13	CxFLAV	0 (2)	0 (2)	
College Station	*C. quinquefasciatus*	100	CxFLAV	0 (2)	2 (2)	100 (KX512322)
Chicago	*Culex* spp.	70	CxFLAV	2 (2)	1 (2)	100 (KX512322)
LRGV	*Culex* spp.	16	CxFLAV	0 (2)	0 (2)	

[a]Number of positive pools (total number of pools tested).

TABLE 4 LLMDA and conventional PCR detection of insect-specific viruses in mosquito midguts and salivary glands in 23 samples from the LRGV

Mosquito species	Tissue	*n*	Virus detected	LLMDA detection[a]	PCR detection[a]
A. aegypti	Midguts	23	CFAV	Positive (1/1)	Positive (1/1)
A. aegypti	Salivary glands	23	CFAV	Positive (1/1)	Positive (1/1)
Culex spp.	Midguts	23	CxFLAV	Negative (0/1)	Negative (0/1)
Culex spp.	Salivary glands	23	CxFLAV	Negative (0/1)	Negative (0/1)

[a]Values in parentheses are number of positive pools/total number of pools tested.

(WRCEVA), were used as positive controls for the conventional PCR assay. Samples resulting in an amplicon were Sanger sequenced. The CFAV PCR assay confirmed the 5 microarray CFAV-positive pools and allowed the detection of 3 additional CFAV-positive pools. The CFAV strains detected in the *A. aegypti* pools from the LRGV showed 97.7% identity to CFAV strain from Puerto Rico (accession number GQ165810), while the CFAV strains from the *A. aegypti* population from San Antonio share 100% homology to a CFAV strain from Mexico (accession number KJ476731). *Aedes aegypti* SG and MG were both confirmed positive for CFAV (Table 4). For CxFLAV, only one of the two positive pools from Chicago identified by the microarray was confirmed positive by conventional PCR. While the microarray was not able to detect any CxFLAV as positive in the pools from College Station, these 2 pools were detected as CxFLAV positive by PCR (Table 3). CxFLAV strains from *C. quinquefasciatus* (College Station, TX) and *Culex* spp. (from Chicago) show 100% identity to a CxFLAV strain isolated from *Culex pipiens* in the United States (accession number KX512322).

(ii) LLMDA bacterial analysis. Several *A. albopictus* and *Culex* sp. mosquito pools from Texas and Chicago were found to be naturally infected with *Wolbachia* (Fig. 2). *A. albopictus* mosquitoes from LRGV and San Antonio were infected with the *Wolbachia pipientis* symbiont of *Aedes albopictus* from the supergroup B (wAlbB) (log CL ratio = 199.7; positive probes/all target probes = 55/59) (Fig. 2A). *Culex* sp. mosquitoes from Chicago and Texas (LRGV) were infected with the *Wolbachia pipientis* symbiont of *Culex pipiens* from supergroup B (wPip) (log CL ratio = 95.5; positive probes/all target probes = 42/58) (Fig. 2B). In the San Antonio collection, one pool of *Culex* was found to be infected with wAlbB (log CL ratio = 199.7; probes detected/expected = 55/59), and one pool of *A. albopictus* was infected with the *Wolbachia pipientis* symbiont of *Nasonia vitripennis* from subgroup B (wVitB) (log CL ratio = 169.6; probes detected/expected = 50/56) (Fig. 2C). A few other bacteria, including *Pseudomonas*, *Klebsiella*, and *Erwinia* spp., were detected in various samples (Table S4). All mosquito pools identified as positive for *Wolbachia* using the microarray were subject to a *Wolbachia* surface protein gene (*wsp*) qPCR assay (Table 5). *A. albopictus* from the LRGV and San Antonio were confirmed to be harbor *wspB*. Additionally, these samples were found to be positive for the *wspA* gene. Whereas 2 *A. albopictus* pools from San Antonio were found to be positive with the LLDA, only one was confirmed using the *wsp* qPCR assay. The *Culex* spp. from San Antonio, TX, Chicago, IL, and the LRGV were all confirmed positive for the *wspB* gene, with C_T values of 23.47, 29.77, and 19.99, respectively.

DISCUSSION

Viruses. The LLMDA version used in the study (v7) was developed in 2014 and can detect 4,219 viruses, 5,367 bacteria, 293 archaebacteria, 265 fungi, and 117 protozoa. We utilized this platform to evaluate its ability to screen mosquito pools for viruses and other microbes. Our study demonstrates that the LLMDA is a broad screening tool that can be used to detect introduced or emerging pathogens in mosquito populations as well as the presence of other insect-specific viruses and bacteria. The LLMDA is able to generate a comprehensive analysis of microbes circulating in mosquito populations of a specific area that could be used to implement future vector control programs. Because it is highly multiplexed and is based on random amplification, the LLMDA presents advantages over singleplex and multiplex PCR assays and cost and time

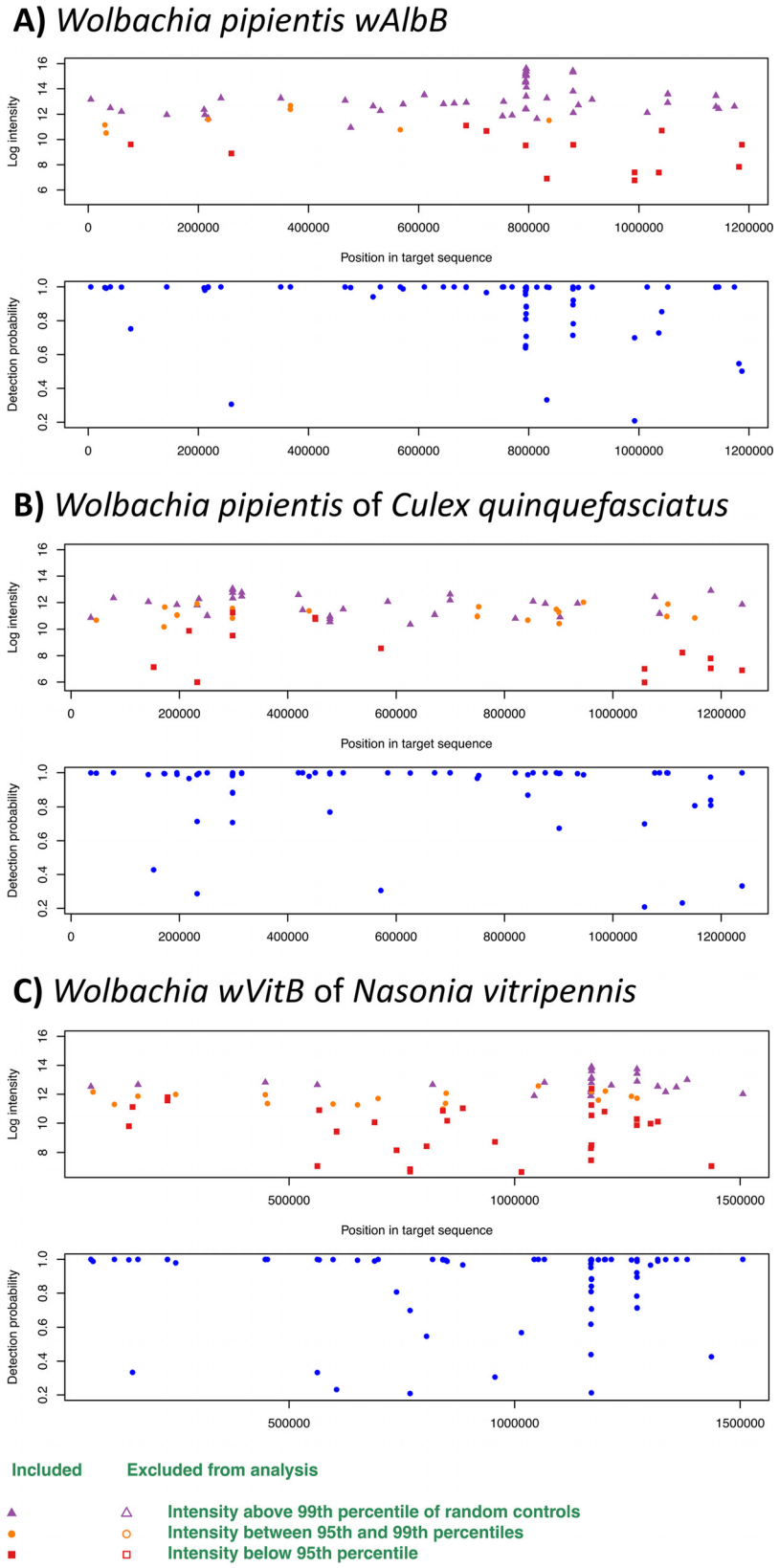

FIG 2 LLMDA probe detection of *Wolbachia* strains. (A) *Wolbachia pipientis* wAlbB; (B) *Wolbachia* endosymbiont of *Culex quinquefasciatus*; (C) *Wolbachia* endosymbiont wVitB. For each bacterium, the upper panel represents the intensity of the probes according to the position of the target in the genome (>99th percentile of control in purple, 95th to 99th, in orange, and <95th in red). The lower panel represents the probability of detection according to the genome region.

TABLE 5 *Wolbachia* detection in field mosquito sample from Texas and Chicago using LLMDA and qPCR with the *wsp* gene

			LLMDA		qPCR		
Locality	**Mosquito species**	***n***	**Detection[a]**	**Strain**	**Detection[a]**	**Wsp**	**C_T value(s)**
LRGV	*A. albopictus*	4	1 (3)	wAlbB	1 (3)	A + B	25.0, 24.34
San Antonio	*A. albopictus*	36	2 (2)	wAlbB	1 (1)	A + B	19.37, 21.70
				wVitB	1 (1)	B	19.99
San Antonio	*C. quinquefasciatus*	13	1 (2)	wAlbB	1 (2)	B	23.47
Chicago	*Culex* spp.	70	1 (2)	wPip	1 (2)	B	29.77
LRGV	*Culex* spp.	41	1 (4)	wPip	1 (4)	B	19.99

[a]Number of positive pools (total number of pools tested).

advantages over next-generation sequencing. First, the sensitivity of the array was determined to be above 10^2 and below 10^3 PFU/ml using serial dilutions of DENV-2, a virus of major public health importance around the world. The array probes were designed to detect both conserved and unique regions of DENV using whole-genome sequences from 3,097 DENV genomes from all four serotypes, of which 403 were specific to DENV-2. The limit of detection of this virus in our array is within the range of viral detection from previous studies using the LLMDA (31, 34) and of other microarrays (35, 36). An interesting feature of the LLMDA is its ability to detect multiple infections from a single sample pool that would normally be missed if a gene-specific PCR approach is used. For example, the LLMDA detected both MAYV and DENV in mosquito pools coinfected with known amounts of both viruses. The LLMDA also successfully detected several viruses in field-collected mosquitoes of known (Table 2) and unknown (Table 3) infection status. For instance, in our study, one *Culex* sp. pool from Chicago, IL, was found to be dually infected with WNV and CxFLAV, which confirms prior studies documenting the cocirculation of these two viruses (37, 38). The presence of several viruses in a mosquito pool does not necessarily mean coinfection in a single mosquito, but coinfection of these two viruses has been previously reported (38, 39). Additionally, CxFLAV has been shown to interact with WNV transmission in *Culex* mosquitoes (40). This highlights the ability of the LLMDA to detect and identify two closely related viruses and viruses from different families within a sample if present.

LLMDA and PCR assays both detected the presence of CFAV and CxFLAV in several mosquito pools. When the LLMDA v7 array was designed in 2014, 22 CxFLAV sequences and one CFAV genome were publicly available. CxFLAV was detected in *Culex* sp. mosquito pools collected in Chicago, IL, but not in *C. quinquefasciatus* pools from College Station, TX. The inconsistency of the microarray to detect CxFLAV could be due to the variation in sequence between CxFLAV strains from different geographic origins or from different host species. Here the portion of the NS5 gene sequenced shows 100% homology to that of *C. pipiens* strain KX512322, but CxFLAV strains from different localities and different mosquito species have been shown by full genome analysis to cluster in two different clades (clade 1 and 2), with all the *C. quinquefasciatus*-related strains clustering together in clade 2 (41).

Additionally, the inconsistency of the results could be due to the difference in sensitivity between the two techniques and the fact that while the conventional PCR relies on the use of gene-specific primers, the microarray relies on the use of random primers during the amplification process. All *Aedes* sp. pools were found to be negative for CxFLAV.

CFAV was detected in *A. aegypti* from San Antonio, TX, and the LRGV, TX. Once again, the conventional PCR allowed the detection of CFAV in two additional samples, probably due to the difference in sensitivity between the two techniques. The tissue dissection revealed the presence of CFAV in both the MG and the SG, the two main barriers of arbovirus replication within the mosquito. This tropism suggests its potential for interaction with other viruses present within the mosquito. The ability of CFAV to transmit from one generation to the next (42), as well as its ability to interact with DENV in an *A. aegypti* cell line (43), makes it a promising candidate for paratransgenesis. *Culex*

sp. pools were found to be negative for CFAV. The ability of the LLMDA to detect insect-specific viruses is of interest because it allows the characterization of ecological associations between insect-specific viruses and human pathogens that occur in nature. These could in turn be investigated for the impact of the insect-specific virus on the transmission of the human pathogen and serve as potential future vector control strategies.

The ZIKV strain PRVABC59 used in this study belongs to the Asian lineage and was not detected using the LLMDA. The LLMDA was designed in 2014, when the only ZIKV sequence available was that of the MR-766 African-lineage strain (accession number NC_012532.1). The two viral strains share only 87 to 90% homology (44, 45). Thus, it is likely that the genetic diversity of the PRVABC59 ZIKV strain compared to the MR-766 African strain did not allow for an efficient detection by the Zika probes present on the LLMDA. This result specifically highlights the need to design additional probes capable of recognizing the more-contemporary Asian lineage of ZIKV and, more broadly, the perpetual need to update the microarray as new viruses or viral strains are discovered or emerge.

Overall, this study was able to detect several viral symbionts. In the *A. aegypti* samples spiked with DENV-2 and/or MAYV, densoviruses were detected, but they were not detected in the nonspiked sample. This reflects the presence of the densoviruses in the C6/36 cells used to grow the different viruses (46–49). Surprisingly, endogenous avian retrovirus (EAV) was found in one pool of female *A. aegypti* collected from an autocidal gravid ovitrap (AGO) from the LRGV. EAVs are noninfectious ancient elements of virus that integrated into their host genome and are found in all species of the genus *Gallus* (50, 51). Many homeowners in the communities where mosquito trapping was done have chickens, and this result suggests that *A. aegypti* had previously fed on chickens or that chicken DNA had contaminated the mosquitoes. However, no human pathogen was detected using the LLMDA, presumably due to our limited set of field samples. In Texas, there was a total of 381 imported human Zika cases and 10 locally acquired ZIKV cases in the LRGV, with 6 cases in 2016 and 4 cases in 2017 (11, 12). In this context, the probability of detecting ZIKV-infected mosquitoes was low, especially because these mosquitoes were not being collected from or around the homes of human ZIKV cases. The use of the LLMDA for virus detection should be further tested using mosquitoes collected from regions with active arbovirus transmission areas and, if possible, from confirmed or probable human case households.

Although the number of viral species detected in our field samples is low, our results are comparable to those from other studies using microarrays to determine the virome of field-collected mosquitoes. For example, a study of 10 mosquito pools collected in Thailand revealed the presence of three different viruses: CxFLAV in *Culex quinquefasciatus* ($n = 1$), DENV-3 in *Aedes aegypti* ($n = 1$), and Japanese encephalitis virus (JEV) in two pools of *Culex tritaeniorhynchus* containing, respectively, 24 and 25 mosquitoes (35). Authors using pan-viral family primers coupled with conventional PCR also report low numbers of virus-positive pools. For example, in a study performed in Puerto Rico, 528 pools representing 1,584 mosquitoes lead to the identification of one insect-specific virus: CFAV in 67 pools (52). Other authors, using cell culture (observation of cytopathic effect [CPE]) followed by conventional PCR using pan-viral family primers to detect viruses in mosquito samples, have rarely detected extensive numbers of viral species. For example, in a study done in Brazil, researchers collected 950 adult female mosquitoes representing 16 species. From these, only two pools tested positive for flavivirus, and these were later identified as Nhumirim virus and Ilheus virus (53, 54).

The LLMDA is able to detect a wide variety of viruses, including mosquito-borne RNA viruses and insect-specific RNA viruses, and is able to detect coinfection in mosquito pools, making it an efficient tool for surveillance of known pathogens in understudied areas such as the LRGV. Given the recent interest in using bacteria or insect-specific viruses as a biocontrol tool and the role of coinfection in pathogen transmission, this tool can contribute to better understanding of disease dynamics in a particular region. However, periodic updates of probe sequences using genome data

from more-contemporary strains is necessary to enable detection of emergent RNA virus genomes due to their high mutation rates.

Bacteria. The LLMDA results show the presence of *Wolbachia* in several mosquito pools, which was confirmed with a qRT-PCR assay targeting the surface protein gene *wsp*. *A. aegypti*, the primary vector of dengue, Zika, and chikungunya viruses, was found to be negative for the presence of *Wolbachia*, which confirms previous observations (55). The secondary vector of these viruses, *A. albopictus*, was found to be infected with *Wolbachia* in 60% of the pools tested. The presence of *Wolbachia* in natural populations of *A. albopictus* has been previously reported (56), and *A. albopictus* is often found infected with group A (wAlbA) and B (wAlbB) strains, as suggested by our results. Additionally, a report of superinfection with the two strains has been published (55). *Wolbachia* has been shown to limit DENV transmission (57) and modulate CHIKV replication (58) in *A. albopictus*. The current study also detected *Wolbachia* in *Culex* populations from Chicago, San Antonio, and the LRGV, confirming previous studies in *C. quinquefasciatus* from Australia (83) and Brazil and Argentina (59) and in other *Culex* spp. in the United States, such as *Culex pipiens* (56, 60, 61). The presence of these endosymbionts in field populations in Texas is significant, since wPip (group B) has been reported to induce resistance to WNV in *C. quinquefasciatus* mosquitoes (62, 63). Because of its impact on transmission of human pathogens and on the mosquito reproduction, life span, and resistance to insecticides, knowledge of *Wolbachia* strains circulating in specific areas is needed if *Wolbachia*-based vector control strategies are to be implemented.

Overall, the number of bacterial hits in the mosquito pools was lower than expected, which might be explained by the lack of sufficient genomic sequences specific to insect-related bacterial species available during the array probe design, the low concentration of bacterial species in the samples, or the genetic divergence of the bacterial strains present in our samples compared to bacterial genomes used to develop the microarray. Additionally, the LLMDA was designed using only full genome sequences, and if at that time only partial bacterial sequences related to the mosquito microbiome were available, they would not have been included on the microarray. Since the development of this array, many studies have shown the importance of bacteria (64–66), viruses (67–69), and fungi (70, 71) in the epidemiology of mosquito-borne diseases, demonstrating the need to better characterize the mosquito microbiome. Updating the microarray with probes designed to detect the major components of insects' microbiome could help alleviate the low number of bacterial hits detected in this study. In this study, we wanted to test the LLMDA's ability to detect microbes present in mosquito samples without the need for a targeted enrichment. The LLMDA was successful at identifying viral pathogens without a baited approach, but it is not adequate to detect the whole bacterial community. Instead, the LLMDA seems to be efficient at detecting dominant bacterial species. *Wolbachia* has been reported to be the dominant member of *A. albopictus* and *Culex* mosquitoes (56) and has been successfully detected with the LLMDA. Other bacteria, including *Pseudomonas*, *Klebsiella*, and *Erwinia* spp., have been detected in *Culex* spp. and *A. aegypti* in our samples (see Table S4 in the supplemental material) and have already been reported in mosquitoes and their breeding sites (16, 29, 72–75). We encountered issues related to nonspecific probe binding in our samples, mostly to conserved regions of bacteria such as 23S or 16S rRNA genes, which might also explain the low number of bacterial species. Because we used a stringent threshold of determining a positive signal, i.e., at least 20% of probes being detected for a target sequence, and the criterion that probes should cover various regions of the genome, these nonspecific hits were not reported. In our case, after removal of nonspecific bacterial hits, *Wolbachia* was the most significant bacterial species confirmed to be present in the mosquito pools. Such challenges have been reported previously in low-biomass samples (76). Other approaches, such as shotgun metagenomic sequencing, would be alternative methods to characterize the microbiome.

In summary, to explore the potential usefulness of the LLMDA for biosurveillance, we took advantage of an ongoing mosquito surveillance program along the Texas-

Mexico border in the LRGV where ZIKV circulated in 2016 to 2017, resulting in 10 cases of local transmission involving *A. aegypti* as the vector (12). A subset of the mosquito collections was tested using the LLMDA, and although no pools tested positive for ZIKV, the microarray was able to detect CFAV in *A. aegypti* populations from the LRGV and San Antonio, which could have an impact on the epidemiology of *Aedes*-vectored viral diseases. Similarly, CxFLAV was observed in several *Culex* populations. *Wolbachia* was detected at a high frequency in *A. albopictus* and *Culex* sp. mosquitoes but was not found in *A. aegypti*. Further characterization of the presence and strain types of locally occurring insect-specific viruses and *Wolbachia* is important (77, 78) for possible biologically based control interventions (66, 79, 80). This study presents the broad detection capability, sensitivity, and ease of use of the LLMDA approach for surveillance of mosquito-borne diseases of medical importance. This detection array could also aid in the surveillance of pathogens transmitted by other arthropod vectors, such as ticks. The study also demonstrated some limitations of the LLMDA and the need to develop an improved array including updated viral and bacterial full genomic sequences deposited in GenBank since 2014 for more-up-to-date biosurveillance studies.

MATERIALS AND METHODS

Mosquito samples. Mosquitoes were collected in several locations in Texas (San Antonio and the LRGV) using three trapping methods. Autocidal gravid ovitraps (AGOs) (SpringStar Inc.), BG sentinel traps (Biogents), and Prokopack aspirators (John W. Hock Co.) were used (see Table S1 in the supplemental material). Whole female mosquitoes were pooled by trap and species, with a maximum size of 50 individuals per pool. Additionally, MG and SG of *A. aegypti* and *Culex* spp. were obtained by dissection of a subset of mosquitoes from the LRGV and pooled. These specimens were first surface sterilized (5 min in 70% ethanol) and rinsed twice in a sterile phosphate-buffered saline (PBS) solution, and then individual MG and SG were dissected under a dissecting microscope and rinsed in PBS.

LLMDA sensitivity and reproducibility. Four different viruses were used in this assay: one alphavirus (Mayaro virus [MAYV] strain INHRR11a-10), two flaviviruses (DENV-2 strain INH125271 and ZIKV strain PRVABC59), and one bunyavirus (Rift Valley fever virus [RVFV] strain MP-12). For dengue virus, 100 μl of a 10-fold serial dilution (10^5 PFU/ml to 10^2 PFU/ml) of the virus was spiked into an *A. aegypti* Liverpool strain mosquito homogenate. The dilutions corresponding to 10^2 PFU/ml and 10^3 PFU/ml were done in duplicate to assess reproducibility. Additionally, 100 μl of MAYV virus at 10^4 PFU/ml was spiked into the mosquito homogenates containing 100 μl of DENV-2 at 10^4 PFU/ml and 100 μl of 10^5 PFU/ml. One hundred microliters of RVFV at 10^4 PFU/ml was spiked into a *C. quinquefasciatus* pool. One pool of *A. aegypti* and *C. quinquefasciatus* was used as a negative control. For ZIKV, two dilutions were tested, 10^4 PFU/ml and 10^2 PFU/ml. The ZIKV-spiked mosquito pools were tested by the ZIKV reverse transcription quantitative real-time PCR assay targeting the nonstructural protein 5 (NS5) gene (81, 82) to verify the presence/absence of infection (see Table S2 in the supplemental material).

LLMDA validation using field-collected sample of known status. WNV-positive field-collected mosquitoes from Chicago, IL (2010), and CxFLAV-positive field-collected mosquitoes from College Station, TX (2013), were assessed on the LLMDA. These pools had previously tested positive in other studies using qRT-PCR targeting the envelope genes of WNV and CxFLAV (20, 39).

Mosquito sample preparation and nucleic acid extraction. Three sample preparation methods were tested to evaluate different processing protocols that would optimize recovery of nucleic acid, retain the ability to isolate viruses, and remove surface exogenous nucleic acid. In method 1, mosquitoes were directly homogenized in TRIzol. In method 2, mosquitoes were homogenized in Hanks' balanced salt solution (HBSS) (Thermo). In method 3, mosquitoes were washed in 70% ethanol for 5 min, followed by 2 PBS washes. Each mosquito pool was homogenized in a 2-ml microcentrifuge tube containing a single 2.8-mm stainless steel bead. Mosquitoes used for the MG and SG dissection were prepared following the procedure from method 3. Tubes were then centrifuged for 5 min at 15,000 $\times$ *g*. Nucleic acids were extracted from 100 μl of the homogenate supernatant using an RNA and DNA TRIzol extraction method.

LLMDA analysis. The LLMDA v7 4x180K microarray consists of probes that targets both conserved and unique genomic regions of sequenced microbial species and has multiple probes per microbial genomic sequence to serve as an internal validation mechanism (34). All samples were analyzed using the LLMDA as described previously (30, 32). Briefly, RNA was reverse transcribed to cDNA using the phosphorylated random hexamer/SuperScriptIII (P-N6/SSIII) method, which uses the Superscript III reverse transcription kit (Invitrogen) and 5′-phosphorylated random hexamers (P-N6) (Eurofins MWG Operon) followed by the Qiagen QuantiTech whole transcriptome kit (30, 32). Each sample was loaded onto the LLMDA and allowed to hybridize for 40 h at 55°C in a rotator oven. After hybridization, the microarray was washed following standard manufacturer's protocols with CGH wash buffers (Agilent) and further cleaned using a nitrogen gas stream to remove any particulates from the array surface. The microarray was then scanned and the data analyzed using a statistical method described previously (34). Briefly, the intensity of each probe is transformed into a positive or negative signal. A positive signal is obtained when the intensity of the probe exceeds an intensity threshold set to the 95th percentile of that for the negative controls (33). In other words, if the probe intensity is above the 95th percentile of the sum of the intensity of the random control probes on the array, then that probe is considered to have

a positive signal. Given the different parameters used to validate our results, there is still a 5% chance for a false-positive probe signal (100% to 95%). A sample was assigned to a species when at least 20% of all the probes present for this particular species had a positive signal. Since we set a 20% threshold of all probes to assign a species as positive, there is still a certain probability that even with 20% of the probes lighting up, the sample would have a false-positive detection.

We then used a likelihood maximization algorithm to identify the target that explains the largest portion of the observed positive probe signals while minimizing the number of negative probe signals. The log likelihood for each of the possible targets was estimated from the BLAST similarity scores of the array feature and target sequences, together with the feature sequence complexity and other covariates derived from the BLAST results as described previously (34).

PCR assay to confirm microarray results. Confirmation of the viral species detected in the field samples from San Antonio and the LRGV was performed by conventional PCR using gene-specific primers amplifying a 206-bp region of NS5 of CxFLAV (39) and a 340-bp fragment of the CFAV E gene (42). Additionally, the presence of *Wolbachia* in the mosquito samples was confirmed using quantitative PCR targeting the *Wolbachia* outer surface protein genes *wspA* and *wspB* (58) (see Table S3 in the supplemental material).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01202-19.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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We have no conflict of interest to declare.

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