Evaluation of a Field-Portable DNA Microarray Platform and Nucleic Acid Amplification Strategies for the Detection of Arboviruses, Arthropods, and Bloodmeals

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Abstract.   Highly multiplexed assays, such as microarrays, can benefit arbovirus surveillance by allowing researchers to screen for hundreds of targets at once. We evaluated amplification strategies and the practicality of a portable DNA microarray platform to analyze virus-infected mosquitoes. The prototype microarray design used here targeted the non-structural protein 5, ribosomal RNA, and cytochrome *b* genes for the detection of flaviviruses, mosquitoes, and bloodmeals, respectively. We identified 13 of 14 flaviviruses from virus inoculated mosquitoes and cultured cells. Additionally, we differentiated between four mosquito genera and eight whole blood samples. The microarray platform was field evaluated in Thailand and successfully identified flaviviruses (*Culex* flavivirus, dengue-3, and Japanese encephalitis viruses), differentiated between mosquito genera (*Aedes*, *Armigeres*, *Culex*, and *Mansonia*), and detected mammalian bloodmeals (human and dog). We showed that the microarray platform and amplification strategies described here can be used to discern specific information on a wide variety of viruses and their vectors.

INTRODUCTION

Arthropod-borne viruses, or arboviruses, are an emerging global public health threat; with dengue alone affecting 50 million people a year.[1] Surveillance of arthropod vectors and arthropod-borne pathogens is critical for early detection and rapid intervention of arboviral diseases. However, with over 300 known or probable arboviruses representing a wide variety of virus families (*Togaviridae*, *Flaviviridae*, *Bunyaviridae*, *Reoviridae*, *Rhabdoviridae*, *Orthomyxoviridae*, and *Asfarviridae*), virus detection and identification alone presents a unique challenge for a comprehensive surveillance program.[2] To gain new insights into virus, vector, and vertebrate relationships, there is a need to accurately identify the arthropod and the bloodmeal.[3] The increase in genomic sequence data available to the public has allowed for the development of effective molecular diagnostic methods for viruses, arthropods, and bloodmeals, and the transition from serological to molecular-based methods has improved the specificity of virus and bloodmeal identification. An examination of the molecular diagnostic tools used for mosquito species identification and virus detection highlighted a trend toward high-throughput, multiplexed assays.[4] Microarray assays, as one such technology, has shown the ability to make a distinction between hundreds and thousands of targets.

To aid in the field detection and confirmation of arthropod-borne pathogens by surveillance programs or preventive medicine units, ruggedized and miniaturized equipment must be developed to hold up to the conditions of overseas travel while adhering to size and weight restrictions. It must also withstand harsh environments, be simple to operate, and be cost-effective. Recent work has demonstrated that some detection platforms are conducive for use in the field.[5–7] The ElectraSense microarray platform, developed by CustomArray, Inc. (CustomArray, Inc., Bothell, WA) was identified to fit our specifications for effective field deployment. This platform relies on electro-chemical detection (ECD) of a reaction that occurs after the capture probe hybridizes to the target sequence and allows for the development of compact microarray readers for potential field use.[8,9] The version of the microarray chip we used with this platform was divided into sectors so that four samples could be tested simultaneously against 2,240 oligonucleotide DNA probes attached to individual microelectrodes. Furthermore, the platform was shown to be a useful tool for Influenza A virus genotyping, providing evidence that the microarray can be used for the detection and genetic characterization of pathogens.[10,11]

Even though some microarrays have been developed to target mosquito-borne viruses, particularly flaviviruses pathogenic to humans, none were evaluated with infected mosquitoes.[12–17] Recently, a microarray was adapted to identify *Culicoides* species belonging to the Obsoletus group, potential bluetongue virus vectors, however it was not tested in the field.[18] Furthermore, microarrays have not been published for the detection of arthropod bloodmeals. These issues present a lack of knowledge for microarrays to be used for arbovirus surveillance. Using the sample preparation methods adapted for field use by O'Guinn and others,[19] we sought to demonstrate proof-of-concept strategies for microarray analysis of field-collected mosquitoes. The microarray prototype reported here, the ArboChip3.0, targets the non-structural protein 5 (NS5) gene of 33 flaviviruses, ribosomal RNA (rRNA) of four genera of culicine mosquitoes, and the cytochrome *b* (*Cytb*) gene of eight vertebrate species to identify bloodmeals. To examine the functionality in the field, the microarray platform and methods were tested at two rural sites in Thailand.

MATERIALS AND METHODS

Microarray probe targets and design. A total of 392 probes were created for the prototype ArboChip3.0 design. The majority of the probes were designed by using a service offered by CustomArray, Inc., whereas some probes were designed by using the Clustal W software (DNASTAR, Inc., Madison, WI) to bind to unique target regions between the amplifying primers. Some probes were also designed by modifying select 70-mer probes published by Wang and others.[12]

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Positive and negative control probes were added to the design as previously reported.[20] The probe lengths were 25–45 basepairs (bp), with a mean of 35 bp. Probes were selected based on having melting temperatures between 70 and 75°C, secondary structure threshold above 70°C, and guanine-cytosine content (GC-content) between 40% and 60%. The final probe sequence list can be found in Supplemental Table 1. The oligonucleotide probes were synthesized directly on the ElectraSense 4x2K sectored microarray slides by CustomArray, Inc.

Viruses, mosquitoes, and blood. The viruses used in this study are listed in Table 1. Viruses were passed in cell culture and used to spike samples. In addition, mosquitoes were inoculated intrathoracically (0.3 µL/mosquito) with selected viruses to produce more realistic specimens.[21] Inoculated mosquitoes were held for at least 7 days at 26°C to allow for viral replication. Virus-inoculated mosquitoes were triturated individually in diluent (Eagle's minimal essential media supplemented with 10% heat-inactivated fetal bovine serum, 100 units of penicillin and 100 µg of streptomycin per mL, and 0.075% NaHCO$_3$).

Donkey, sheep, cow, dog, and chicken whole blood in sodium citrate was purchased from Lampire Biological Laboratories (Pipersville, PA). Human and pig whole blood in sodium heparin was purchased from Innovative Research (Novi, MI). Goat and horse whole blood was provided by our Veterinary Medicine Division (USAMRIID, Fort Detrick, MD). Blood spike samples were created by adding 25 µL of whole blood to 25 mosquitoes.

Isolation of RNA, synthesis of cDNA, and PCR amplification of the flavivirus NS5 gene. TRIzol® LS (Invitrogen Inc., Carlsbad, CA) extraction of RNA and complementary DNA (cDNA) synthesis using random hexamers and Superscript II (Invitrogen, Inc.) was completed as previously described, except that the RNA was not subjected to a second round of purification before cDNA synthesis.[19] For a 25 µL polymerase chain reaction (PCR) reaction, 2 µL of cDNA, 1 pmole of forward primer mFU1 (TACAACATGATGGGAAAGC GAGAGAAAAA),[22] 1 pmole of reverse primer Flavi-10296-Rev (CATGTCTTCTGTCGTCATCC) (this publication), and 21 µL of nuclease-free water was added to each illustra puRe Taq Ready-To-Go PCR bead (GE Healthcare, Inc., Piscataway, NJ). The PCR amplification was performed as follows: 95°C for two min, followed by 35 cycles: 94°C for 30 sec, 50°C for 30 sec, and 72°C for 90 sec; followed by 72°C for 7 min, and then a final hold at 4°C. Amplicons were visualized using 2% Agarose E-Gel gels (Invitrogen, Inc.) that contained ethidium bromide.

Reverse transcription, 2nd-strand cDNA synthesis, and random PCR (RT-rPCR) amplification. Two µL of isolated RNA was amplified by RT-rPCR using the methods previously described[23] with the following modifications. Superscript II (Invitrogen, Inc.) was used for reverse transcription of Round A. For Round B rPCR, illustra puRe Taq Ready-To-Go PCR beads (GE Healthcare, Inc.), 50 pmoles of primer-B, and 5 µL of randomly primed cDNA were used in a 25 µL rPCR reaction.

Isolation of DNA from blood and PCR amplification of the Cytb gene. Genomic DNA was isolated from whole blood (25 µL; for laboratory evaluation of bloodmeal probes) or clarified mosquito homogenate (250 µL) using DNAzol BD Reagent (500 µL; Invitrogen, Inc.) according to the manufacturer's instructions. The DNA pellets were rehydrated in 20 µL of nuclease-free water. The PCR amplification was accomplished using the Cytb avian-a (forward, GACTGTGA CAAAATCCCNTTCCA; reverse, GGTCTTCATCTYHGG YTTACAAGAC)[24] and mammalian-a (forward, CGAAGC TTGATATGAAAAACCATCGTTG; reverse, TGTAGTTR TCWGGGTCHCCTA)[25] primers as previously desribed.[26] Amplicons were visualized using 2% Agarose E-Gel gels (Invitrogen Inc.).

Biotin labeling and post-PCR purification. The PCR amplified material (1 µg) was biotin labeled using Label IT µArray Biotin Labeling Kits (Mirus Bio, LLC, Madison, WI) following the vendor's instructions. The labeled DNA was purified using MinElute PCR Purification kits (QIAGEN, Inc., Valencia, CA) following the vendor's instructions and eluted from the purification columns using 14 µL of nuclease-free water.

Microarray hybridization and detection. Microarray analysis of the biotin-labeled DNA samples was accomplished according to the manufacturer's instructions: ElectraSense 4x2K/12K Microarrays: Hybridization and Electrochemical

TABLE 1
ArboChip3.0 microarray detection and correct identification of NS5 PCR amplified laboratory preparations of mosquito-borne flaviviruses

	Cell culture-derived virus	Mosquito-derived virus	
Virus (strain)*	Detected†	Mosquito species	Detected†
CYV (ROK144)	+	NT	NT
DENV-1 (HAW)	+	NT	NT
DENV-2 (S16803)	+	NT	NT
DENV-3 (Thai87)	+	*Aedes aegypti*	+
DENV-4 (H-421)	+	*Aedes albopictus*	+
JEV (Th6-0090)	+	NT	NT
MVEV (Original USAMRIID)	+	NT	NT
ROCV (SP H34675)	+	NT	NT
SLEV (Ft. Washington)	+	NT	NT
TMUV (Th3-0385)	+	*Culex tarsalis*	+
WNV L1 (NY397-99)	+	*Culex pipiens*	+
WNV L2 (KLF 146)	+	NT	NT
YFV (17D)	+‡	NT	NT
ZIKV (Original 30306)	+	NT	NT

*CYV = Chaoyang virus; DENV = dengue virus; JEV = Japanese encephalitis virus; MVEV = Murray Valley encephalitis virus; ROCV = Rocio virus; SLEV = St. Louis encephalitis virus; TMUV = Tembusu virus; WNV L1 = West Nile virus lineage 1; WNV L2 = West Nile virus lineage 2; YFV = yellow fever virus; ZIKV = Zika virus; NT = not tested.
†Successfully detected virus at the genus and species level.
‡Detected virus at the genus, but not at the species level.

Detection manual (CustomArray, Inc.). All reagent preparations and procedures were followed except for a modification to the hybridization step: 13.5 µL of purified biotinylated DNA was combined with 16.5 µL of the hybridization solution. The hybridization solution was also prepared without the addition of nuclease-free water to account for the additional sample volume. Hybridization was conducted using a UVP HB-500 Minidizer hybridization oven (Ultra-Violet Products, LLC, Upland, CA) that was modified to hold the microarrays in microarray clamps fixed onto a rotisserie wheel. Hybridization was optimized with an incubation time of 2 h at 50°C with rotation. ElectraSense Detection kits were used for post-hybridization blocking, labeling, washing, and ECD according to the manufacturer's instructions (CustomArray, Inc.).

Microarray scanning and analysis. The 4x2K microarray chips were scanned using an ElectraSense Reader (CustomArray, Inc.). Data were extracted using the ElectraSense application software (CustomArray, Inc.). The data were transferred to Microsoft Excel (Microsoft Corp., Redmond, WA) software to analyze the individual oligonucleotide probe ECD signals that were measured in picoamps (pA). Each probe was clustered into a group and subgroup base on their phylogenetic relationships. See Supplemental Data 1 for probe groupings and arrangements. We calculated three times the mean ECD signal of all probes and set that value as the background threshold for each microarray analysis. Average group or subgroup probe ECD signals greater than the background threshold were required for the sample to be considered positive. The highest individual probe signal of a group or subgroup is represented by the maximum signal indicator on each figure.

Direct detection of viral RNA. Direct detection of viral RNA was accomplished as follows. Sample RNA (10 µL) was biotin labeled and purified using the methods outlined previously, except that the samples were eluted in 13.2 µL of nuclease-free water. The samples were then fragmented using 3.3 µL of 5X fragmentation buffer included in the Label IT µArray Biotin Labeling Kits (Mirus Bio, LLC) using the vendor's protocols. The fragmented, labeled RNA samples were hybridized as described except using a hybridization temperature of 45°C.

West Nile virus (WNV) real-time PCR. Real-time PCR was completed as follows: a 20 µL reaction contained 0.4 µL of Platinum *Taq* (Invitrogen, Inc.), 10 µL of 2X Reaction Mix (Invitrogen, Inc.), 1 µL of the primer/probe mix, 1.04 µL of 50 mM $MgSO_4$, 0.25 µL of 20 mg/mL bovine serum albumin, 5.31 µL of nuclease-free water, and 2 µL of cDNA template. The primer/probe mix consisted of 18 µM of the forward primer WNV_NSP_F (GGAAAAGTGATTGACCTTGG ATGTG), 18 µM of the reverse primer WNV_NSP_R (ACC CTCTGACTTCTTGGACTCTTT), and 5 µM of the probe WNV_NSP_M (FAM-AACACCAGCCGCCTCT-NFQ). The cycling conditions were as follows: 95°C for 5 min, and then 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Fluorescence was read at the end of the annealing-extension step at 60°C.

Field-site locations, mosquito collections, flavivirus screening, and sequence confirmation. For 2 weeks during March and April 2011, mosquitoes were collected from locations near Lopburi and Kamphaeng Phet, Thailand. The mosquitoes were identified, processed, and tested using methods previously described[19] with the following exceptions. We used MA forward and cFD2 reverse primers (260 bp amplicon) to screen for flavivirus RNA.[27] The ABI 3100 genetic analyzer

and Big Dye 3.1 (PE Biosystems, Inc., Foster City, CA) were used to sequence the amplicons.

RESULTS

Evaluation of virus amplification methods. Dengue virus type 3 (DENV-3), DENV-4, West Nile virus (WNV) lineage 1(WNV L1), and Tembusu viruses (TMUV) were used to evaluate and optimize the methods for virus amplification and microarray detection. Three methods were used, direct detection of viral RNA, RT-rPCR, and NS5 PCR amplification. Cell culture-derived viruses and virus-infected mosquitoes were tested using each method (Figure 1).

Detection of laboratory prepared viruses. The NS5 PCR amplicons were used to evaluate the 302 flavivirus probes included in the ArboChip3.0 design for the detection and differentiation of 14 cell culture-derived flaviviruses. Each viral preparation was tested at least twice. All of the tested flavivirus amplicons (100%) were detected on the microarray using the genus or species level probes. The array identified 13 (93%) of the 14 viruses at the species level, but only identified yellow fever virus (YFV) at the genus level (Table 1). Probes predicted to be specific to YFV were found to cross-hybridize with many other flaviviruses and were either moved to the generic probe cluster or removed from the design. Examples of microarray analysis using WNV L1 strain NY397-99 (A and B) and DENV-3 strain H-421 (C and D) are shown in Figure 2.

Sensitivity of virus detection and comparison to real-time PCR. The sensitivity of NS5 PCR amplification and microarray detection was evaluated by using serial 10-fold dilutions of RNA extracted from either cell culture-derived WNV strain NY397-99 ($10^{4.6}$ plaque-forming units [PFU]/mL), or from one *Culex pipiens* infected with strain NY397-99 of WNV pooled with 24 uninfected mosquitoes ($10^{5.3}$ PFU/mL). The microarray results were compared with WNV specific real-time PCR. The lower limit of microarray detection was 80 PFU equivalents (PFUe) for cell culture-derived WNV and 400 PFUe for pools that contained one WNV-infected mosquito and 24 uninfected mosquitoes. In comparison, the real-time PCR sensitivities to detect WNV from cell culture and mosquito pools were 0.8 PFUe and 4 PFUe,
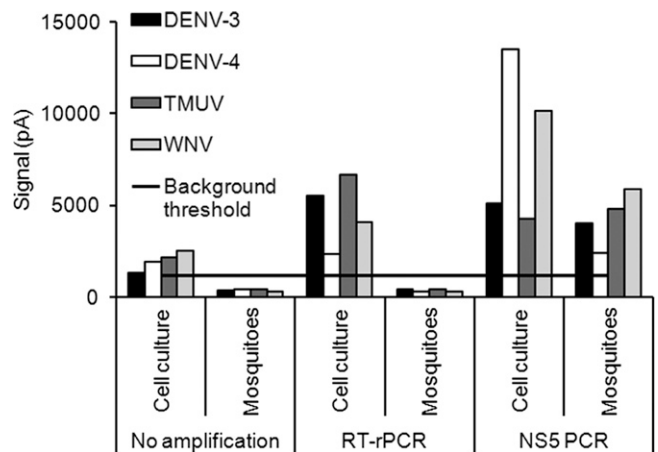

FIGURE 1. Comparison of amplification methods for microarray detection of cell culture and infected mosquito-derived virus. Virus abbreviations: DENV = dengue virus, WNV = West Nile virus, and TMUV = Tembusu virus.
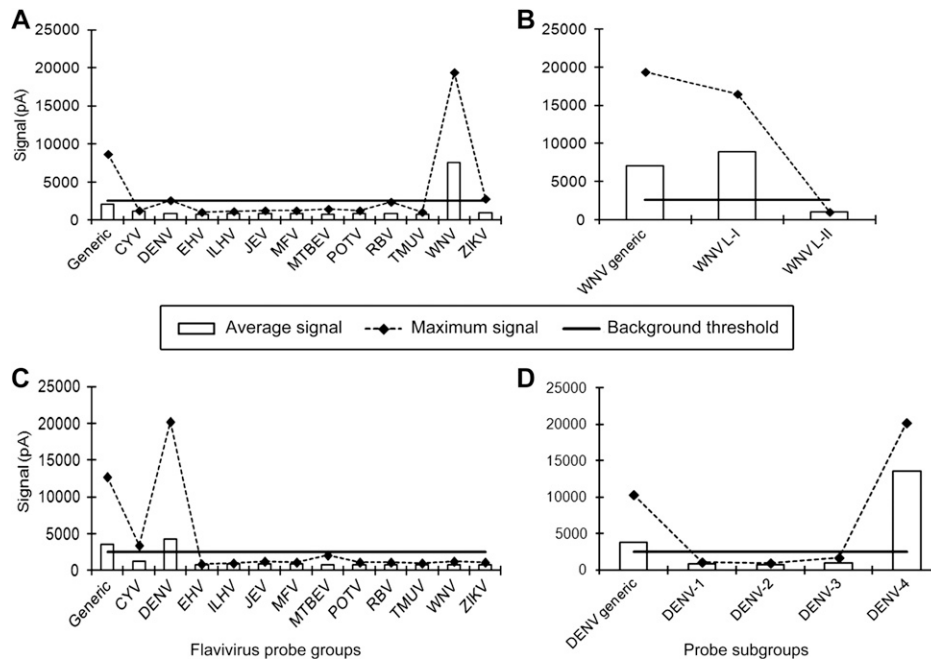
FIGURE 2.   Microarray flavivirus analysis of NS5 PCR amplified (**A**, **B**) cell culture-derived West Nile virus lineage 1 (WNV L-I) and (**C**, **D**) dengue virus type 4 (DENV-4) infected *Aedes albopictus*. (**A**, **C**) Average electrochemical detection (ECD) signals of flavivirus probes clustered into groups are used for first-level identification. Probes from positive groups are further categorized into subgroups. Analysis of the (**B**) WNV and (**D**) DENV subgroups are used to differentiate the viruses from other related species. Virus abbreviations: CYV = Chaoyang virus; DENV = dengue virus, EHV = Edge Hill virus; ILHV = Ilheus virus; JEV = Japanese encephalitis virus; MFV = mosquito-only flavivirus; MTBEV = mammalian tick-borne encephalitis virus; POTV = Potiskum virus; RBV = Rio Bravo virus; TMUV = Tembusu virus; ZIKV = Zika virus.

respectively. Real-time PCR was 100-fold more sensitive than the microarray.

Detection of laboratory mosquito genera. *Aedes aegypti*, *Aedes albopictus*, *Armigeres subalbatus*, *Mansonia uniformis*, *Culex quinquefasciatus*, *Culex tritaeniorhynchus*, and *Culex tarsalis* RT-rPCR amplicons were used to evaluate the methods and 40 mosquito ArboChip3.0 probes for the detection and differentiation of the genera *Aedes*, *Armigeres*, *Culex*, and *Mansonia*. Both individual mosquitoes and pools up to 25 were used for testing. Each preparation was tested at least twice. All of the mosquito samples tested (100%) were detected and differentiated based on genus. Some hybridization differences were noted between two species within the same genera. For example, *Ae. aegypti* tested positive for seven of the 11 (63.6%) *Aedes* probes, whereas *Ae. albopictus* tested positive for four (36.4%) of the same 11 probes. Microarray analysis of *Ae. albopictus* and *Ma. uniformis* are shown in Figure 3A and B, respectively. The mosquito species listed here, free of virus and blood, were also tested using the arbovirus and bloodmeal methods and did not result in any cross-hybridization to off-target probes.

Detection of laboratory prepared whole blood samples. Chicken, cow, dog, donkey, horse, human, pig, and sheep whole blood were used to evaluate the methods and 50 bloodmeal probes present on the microarray. All of the blood samples were detected by the microarray and were identified by their predicted groups except that the probes specific for cow and sheep blood were found to be cross-reactive and were moved to the mammalian probe cluster. Testing of whole blood samples spiked into mosquito pools showed similar results (data not shown). Microarray identifications of avian (chicken) and mammalian (pig) blood are shown in Figure 4A and B, respectively.

Additionally, we compared using DNAzol BD and Trizol-LS to extract DNA from mosquito pools spiked with whole blood. Approximately 2-fold greater bloodmeal probe ECD signals were observed with DNA samples extracted using DNAzol BD versus DNA extracted from the interphase step of RNA extraction using Trizol-LS (data not shown).

Field evaluation. To evaluate the described methods and to determine the usefulness of the microarray in the field, 11,566 mosquitoes were collected and sorted into 642 pools over the course of 2 weeks in Lopburi and Kamphaeng Phet, Thailand. The microarray was used to identify virus, mosquito genera, and/or bloodmeal source from 10 mosquito pools and one tick collected from a local dog (Table 2). Microarray results are shown in Figure 5 for three of these samples. *Anopheles peditaeniatus* RT-rPCR amplicons were also tested on the microarray and found not to cross-hybridize to any of the mosquito probes (data not shown). Upon returning to a fixed laboratory, RNA and/or DNA were extracted from the field-tested mosquito homogenates and PCR amplified using the ma/cFD2 flavivirus and/or the mammalian-a bloodmeal primers. The field microarray results were confirmed by sequencing the PCR amplicons produced in the laboratory using the samples collected and processed in the field.

DISCUSSION

To our knowledge, the research presented here was the first describing strategies to determine if DNA microarrays can be used in the field to analyze mosquito vectors for pathogens and other bionomic data (e.g., bloodmeals). The prototype ArboChip3.0 DNA microarray successfully detected fourteen flaviviruses, differentiated between four culicine mosquito
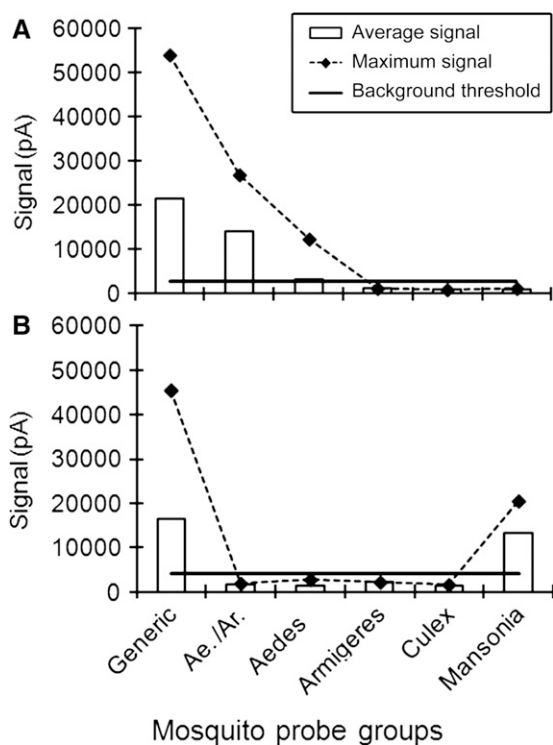
FIGURE 3. Microarray discrimination between the mosquito genera *Aedes*, *Armigeres*, *Culex*, and *Mansonia* of RT-rPCR amplified (**A**) *Aedes albopictus* and (**B**) *Mansonia uniformis*.

FIGURE 4. Microarray bloodmeal analysis of *Cytb* polymerase chain reaction (PCR) amplified (**A**) chicken (*Gallus gallus*) whole blood and (**B**) pig (*Sus scrofa domesticus*) whole blood.

genera, and identified a small number of mosquito bloodmeals (probes for targets not tested are to be considered as investigational). To complete the study, we assessed the microarray system as a surveillance platform using field-adapted sample processing and screening methods described by O'Guinn and others.[19] The microarray equipment proved to be rugged enough to be transported into a rural field environment and endured temperatures and humidity varying between 17.8 and 35.6°C and 64% and 92%, respectively. Field-collected mosquito pools are more complex than laboratory prepared samples. They consist of a wide variety of mosquito species, and the mosquitoes in the pool may contain a variety of different blood meals. The described system displayed its potential value for vector surveillance by identifying medically important flaviviruses, bloodmeals associated with human activity, and discerning the genus of six species of mosquitoes. Additionally, the microarray identified CxFV from a single human blood-fed *Cx. quinquefasciatus* mosquito (Figure 5A–C). The significance of discovering CxFV is that it has not been previously described to be circulating in any species of mosquito in Thailand, and without the microarray, we would not have identified it in the field. Using the strategies discussed here and by further development of the microarray in critical areas (e.g., probe design for additional pathogens and targeted pathogen nucleic acid enrichment), field-adapted microarrays can be used to discern specific information on a wide variety of viruses and their vectors.

Virus obtained from cell culture supernatant is usually high-titered and relatively pure, allowing for the use of many different microarray detection methods. Mosquito homogenate, on the other hand, is a complex matrix of proteins and nucleic acid from the host and potentially even from environmental con-
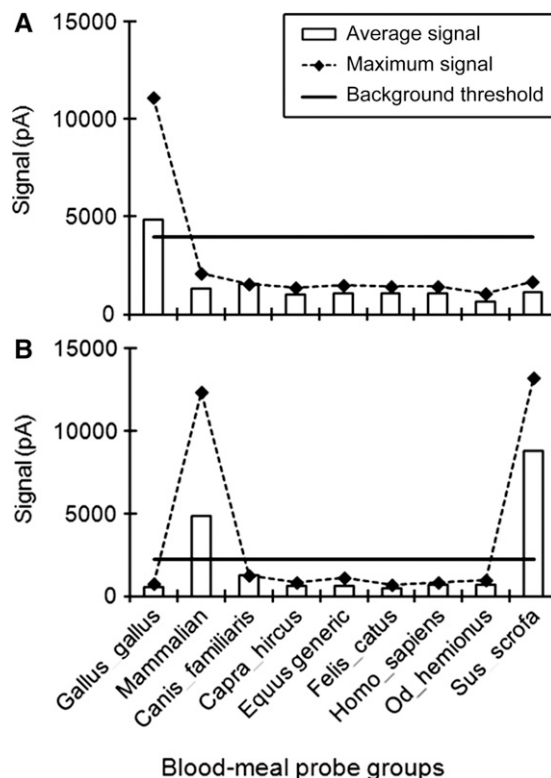
taminants or co-infecting parasites. To aid in the ability to detect viruses, a random, sequence-independent RT-rPCR protocol was evaluated to amplify viral RNA.[23] Other groups have reported using a similar method to randomly amplify virus for microarray detection from other sources such as nasal lavages,[12] endotracheal aspirates,[28] and homogenized mouse brains.[11] Using RT-rPCR amplicons, we were able to detect virus derived from cell culture but not from infected mosquitoes (Figure 1). Perhaps the ratio of viral RNA compared with non-viral RNA in samples from previous studies was greater than that found in infected arthropods, making the amplification of viral RNA less efficient in the presence of excessive amounts of arthropod RNA and DNA. Attempts were made to improve the ratio of viral RNA to non-targeted nucleic acids in the mosquito samples but without avail (results not shown). We investigated passing WNV-infected *Cx. pipiens* homogenates through a 0.22 μm filter and treating the clarified homogenates with nucleases (DNase 1, RNase A, RNase T1, and RNase I) before RNA extraction. The thought was that the encapsidated viral nucleic acid would be protected, whereas the enzymes would degrade the unprotected mosquito nucleic acids, as was shown to be a plausible treatment because no significant reduction in the WNV probe ECD signals was noted after cell culture-derived WNV was treated with nucleases. After nuclease treatment of homogenate containing WNV-infected mosquitoes, we observed a modest but not complete reduction in *Culex* probe ECD signals, although we were not able to detect WNV. Using the same extracted RNA, we also found that the detection of WNV by real-time RT-PCR was greatly reduced (2- to 3-fold increase in threshold cycle values). This

Sample number	Arthropod species (pool size)	Collection method*	location	Microarray identification†		
				Virus	Blood-meal	Mosquito genus
Th9-0011	*Cx. quinquefasciatus* (1)	BA	House	CxFV	Human	*Culex*
Th9-0024	*Ae. aegypti* (1)	BA	House	NA	NA	*Aedes*
Th9-0042	*Armigeres subalbatus* (1)	BA	House	NA	NA	*Armigeres*
Th9-0047	*Cx. quinquefasciatus* (10)	BA	House	NA	Human	*Culex*
Th9-0122	*Ae. aegypti* (1)	BA	House	DENV-3	DNP	*Aedes*
Th9-0164	*Cx. tritaeniorhynchus* (24)	LT	Farm	JEV	NA	*Culex*
Th9-0167	*Ae. albopictus* (1)	LT	Farm	NA	NA	*Aedes*
Th9-0175	*Cx. tritaeniorhynchus* (25)	LT	Farm	JEV	NA	*Culex*
Th9-0235	*Cx. tritaeniorhynchus* (25)	BA	Cow pen	NA	Mammal§	*Culex*
Th9-0278	*Mansonia uniformis* (6)	LT	Farm	NA	NA	*Mansonia*
Th9-0643	*Dermacentor* species (1)	NA	Lab	NA	Dog	NA

*BA = backpack aspiration; LT = Centers for Disease Control and Prevention CO_2 baited light trap.
†DNP = did not prime; NA = not applicable; CxFV, *Culex* flavivirus; DENV-3, dengue virus type 3; JEV = Japanese encephalitis virus. Mosquito pools that were not suspected of being infected with flaviviruses by polymerase chain reaction (PCR) screening or not containing any bloodfed mosquitoes were not analyzed on the microarray for flavivirus or bloodmeal identification.
‡Mosquito contained a bloodmeal but did not amplify with mammalian or avian primers.
§Bloodmeal PCR amplicon was identified as *Bos indicus* (brahman cow) *Cytb* by sequencing, cow-specific probes were not present on this microarray design.

argues that the RNases may be degrading non-encapsidated viral genomes released from disrupted cells.

Virus genus-specific PCR was determined to be the most applicable strategy and is recommended for the amplification of viral nucleic acids from mosquito homogenates for microarray detection. Even using cell culture-derived viruses, PCR amplicons specific for the NS5 gene produced greater ECD signals than those from RT-rPCR and direct virus detection (Figure 1). Our results compliment a previous study[29] comparing target-specific PCR and random amplification strategies for microarray-based pathogen detection. We found that NS5 PCR amplification and microarray detection was sensitive enough to detect WNV present in a single mosquito with a disseminated virus infection in a pool of 24 uninfected mosquitoes, the
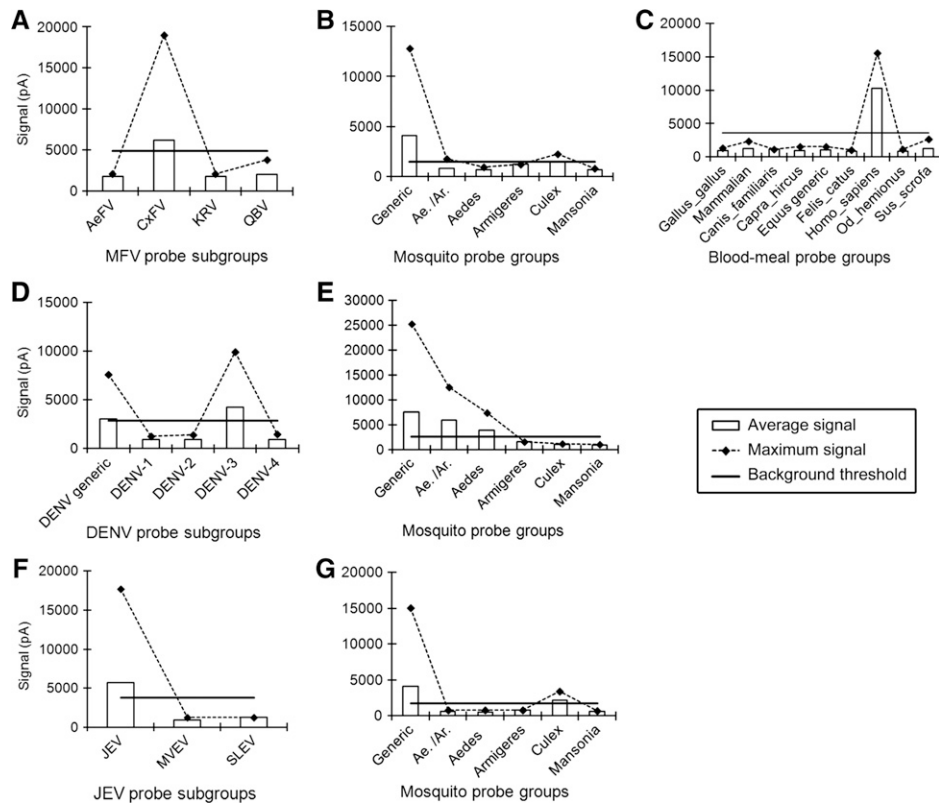

FIGURE 5. Field evaluations of the ArboChip3.0 DNA microarray platform using flavivirus-positive mosquito pools trapped in Lopburi (LB) and Kamphaeng Phet (KPP), Thailand, in March 2011. (**A**–**C**) Sample Th9-0011 contained a single blood-fed *Culex quinquefasciatus* mosquito. (**D**, **E**) Sample Th9-0122 contained a single blood-fed (bloodmeal not identified) *Aedes aegypti* mosquito. (**F**, **G**) Pool Th9-0164 contained 24 *Culex tritaeniorhynchus* mosquitoes. (**A**, **D**, **F**) Subgroup analysis of flavivirus non-structural protein 5 (NS5) reverse transcription-polymerase chain reaction (RT-PCR) amplicons positive for the groups mosquito-only flavivirus (MFV), dengue virus (DENV), and Japanese encephalitis virus (JEV), respectively. (**B**, **G**, **E**) Discrimination between the mosquito genera *Aedes*, *Armigeres*, *Mansonia*, and *Culex* of RT-rPCR amplicons. (**C**) Bloodmeal group analysis of mammalian *Cytb* PCR amplicons.

largest pool size that we use in field studies. However, it was not as sensitive as a real-time PCR assay specifically designed to detect WNV. More efficient primers and PCR reagents may help improve the sensitivity of the system, and biochemical amplification following target hybridization may also boost the sensitivity of DNA microarrays. Two such techniques include secondary enzymatic enhancement and the super avidin-biotin system, both designed to increase electrochemical signal generation by adding layers of horseradish peroxidase onto biotinylated target DNA.[20] Furthermore, microarray detection of target-specific amplicons maybe improved and streamlined by making the amplification asymmetric and incorporating biotin labeling into the PCR assay. Asymmetric amplification benefits microarray detection by synthesizing a greater concentration of single-stranded antisense sequences complimentary to the microarray probes. This can be accomplished by adding more reverse primer than forward primer into the PCR assay or by using reverse primers with melting temperatures at least 10°C greater than the forward primers.[30]

Arthropods are traditionally identified based on morphological characteristics discerned by a trained expert; however, some species are morphologically similar, making accurate identification difficult. In response, molecular detection assays have been developed to characterize and identify vector species.[31–33] Most molecular diagnostic assays have been developed to distinguish between closely related species and would not be applicable for the broad-range identification of mosquito species encountered in field surveillance.[4] For example, using target-specific PCR amplification of mosquito acetylcholinesterase or cytochrome *c* oxidase I (*COI*) genes would require excessive primer pairs to amplify the 23 species of mosquitoes that we encountered during our Thailand field evaluation. Instead, we used random amplification of mosquito RNA with the intent to differentiate between medically important mosquito species by using rRNA targets. The majority of the probes we evaluated were found to cross-hybridize with other species of the same genus. Even so, we were able to make accurate genus level predictions from all laboratory colony and field-collected culicine mosquitoes tested, providing supporting evidence that microarrays can be used for broad-range detection. Expanded bioinformatics of mosquito rRNA sequences for probe development would help improve microarray discrimination of related species.

With over 7.7 million predicted animal species on earth and ~1 million catalogued, creating an all-inclusive microarray for bloodmeal identification would be inconceivable.[34] Choosing bloodmeal targets based on the ecology of the field sites, mosquitoes' feeding habits, and collection methods are a necessity. Because our field studies were conducted at rural sites in Thailand using sentinel animal traps and backpack aspirations of homes, we targeted nine animals associated with agriculture and human activities for inclusion on the prototype microarray. There are several diagnostic markers available for molecular bloodmeal identification, most notably *Cytb*, *COI*, and rRNA genes.[35] In an attempt to streamline our methods, we first investigated targeting eukaryotic *18S*, *5.8S*, and *28S* rRNA genes. Our bioinformatic analysis revealed that there was too little sequence diversity among the targeted sequences for microarray differentiation. As a substitute, we chose to model our approach after a study by Molei and others[26] using target-specific PCR of the *Cytb* gene for identification. The benefit to using this model was the

significant amount of avian and mammalian *Cytb* gene sequence data available. We were successful in using the microarray to distinguish between *Cytb* targets from diverse animal types. However, we were not able to differentiate between *Equus caballus* (horse) and *Equus asinus* (donkey). This study did not determine if the targeted region of the *Cytb* gene contained enough sequence diversity to distinguish between closely related species. For our research objectives, genus-level bloodmeal identification was sufficient to gain useful scientific data concerning vector-feeding activities. Furthermore, using the amplification strategies reported here, new *Cytb* microarray probes for additional bloodmeal targets could be synthesized on the microarray to satisfy a diverse set of specific research objectives.

We demonstrated the proof of concept for using a DNA microarray to detect arboviruses, arthropods, and bloodmeals from field-collected samples. Even though we were not able to determine a universal strategy, we identified effective molecular techniques to amplify each target for microarray detection. Further research into additional molecular targets for probe design and methods to streamline the processes will add to the applicability of portable microarrays. Even in its current developmental stage with multiple target amplification strategies, the system described here has its benefits. Using a single microarray chip design, PCR amplicons from multiple targets can be analyzed together, which can reduce the time and effort of using multiple designs and evaluation methods. Traditional methods for identifying arboviruses and bloodmeals can also include using multiple PCR-based methods. To gain the level of detail equivalent to the microarray, each amplified target would have to be sequenced, requiring a fixed laboratory, additional time, and perhaps, additional cost. Although, a DNA microarray for identifying arboviruses from multiple genera is readily achievable, one for mosquito and bloodmeal identification is only in the developmental stages. A field-portable microarray is able to rapidly identify medically important mosquito-borne viruses during routine surveillance and outbreak investigations. The strategies reported here for detecting RNA viruses from infected mosquitoes will aid us in creating a microarray to identify flaviviruses, alphaviruses, phleboviruses, and orthobunyaviruses.

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