Development of Field-Based Real-Time Reverse Transcription–Polymerase Chain Reaction Assays for Detection of Chikungunya and O’nyong-nyong Viruses in Mosquitoes

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Abstract. Chikungunya (CHIK) and O’nyong-nyong (ONN) are important emerging arthropod-borne diseases. Molecular diagnosis of these two viruses in mosquitoes has not been evaluated, and the effects of extraneous mosquito tissue on assay performance have not been tested. Additionally, no real-time reverse transcription–polymerase chain reaction (RT-PCR) assay exists for detecting ONN virus (ONNV) RNA. We describe the development of sensitive and specific real-time RT-PCR assays for detecting CHIK and ONNV viral RNA in mosquitoes, which have application for field use. In addition, we compared three methods for primer/probe design for assay development by evaluating their sensitivity and specificity. This comparison resulted in development of virus-specific assays that could detect less than one plaque-forming unit equivalent of each of the viruses in mosquitoes. The use of these assays will aid in arthropod-borne disease surveillance and in the control of the associated diseases.

INTRODUCTION

Alphaviruses (family Togaviridae) are an important cause of disease worldwide with geographic distributions that are divided into the Old and New World viruses. Old World alphaviruses generally cause rash, arthritis, and arthralgia in affected humans and are distributed throughout Africa, Asia, and Europe. New World alphaviruses may cause an acute febrile illness and encephalitis in affected humans and are widely distributed throughout the Americas. Two important re-emerging Old World alphaviruses, Chikungunya virus (CHIKV) and O’nyong-nyong virus (ONNV), are responsible for causing millions of recent cases of disease worldwide. CHIKV and ONNV are members of the Semliki Forest antigenic complex and contain a linear, positive-sense, single-stranded RNA genome that is approximately 11.8 kb in length. Four non-structural proteins (NSP1–NSP4) are encoded at the 5′ end of the genome, and the structural proteins are encoded at the 3′ end of the genome and are expressed from a subgenomic 26S RNA. The structural proteins consist of the viral capsid (C), two envelope glycoproteins (E1 and E2), and two peptides (E3 and 6K). Three genotypes of CHIKV are known and include the West African, Asian, and the East/Central/South African genotypes. The Asian strains comprise a distinct group within the southern and East African/Asian genotype. Currently, only one genotype of ONNV is known. In recent years, CHIK has dramatically re-emerged as a disease affecting millions of persons throughout the countries surrounding the southwest Indian Ocean. The virus was first isolated in 1953 from a febrile human during an outbreak in Tanganyika (now Tanzania) and has since been isolated frequently in both Africa and Southeast Asia. Previous large outbreaks of CHIKV have included Thailand in 1958 and India in 1963–1964. After a 30-year gap, the virus re-emerged in the French islands of La Reunion, Mayotte, Mauritius, and Seychelles, which are all located in the Indian Ocean. The normal transmission of CHIKV to humans involves Aedes sp. mosquito vectors. In Africa, CHIKV is maintained through a sylvatic transmission cycle between wild primates and Aedes fuscatus, A. africanus, A. luteocephalus, and A. taylori mosquitoes. In Asia, CHIKV is transmitted from human to human primarily by Aedes aegypti and Aedes albopictus. A second re-emerging alphavirus, ONNV, was first isolated in 1959 and has been associated with relatively few outbreaks. However, several large-scale epidemics have occurred, including one during 1959–1962 in northern Uganda that involved more than two million cases. In 1996, ONNV reappeared after a 35-year hiatus and was responsible for causing another epidemic in Uganda. The most recent outbreak of ONNV was documented in the fall of 2003 and involved refugees from Western Côte d’Ivoire, an area not normally associated with ONNV transmission. In contrast to CHIKV, ONNV is transmitted primarily by anopheline mosquitoes such as Anopheles gambiae and An. funestus and, as observed for Asian CHIKV, it may be maintained in nature by human-to-human transmission by its mosquito vector.

Currently, the diagnosis of CHIK and ONN is determined by either virus isolation, detection of virus-specific antibodies by enzyme-linked immunosorbent assay, or by genomic detection using reverse transcription–polymerase chain reaction (RT-PCR). Several groups have developed real-time RT-PCR assays for the molecular diagnosis of CHIK involving human samples. However, these assays have not been evaluated for detecting CHIKV RNA in infected mosquitoes. Additionally, no real-time RT-PCR assay exists for detecting ONNV RNA in human or mosquito samples.

The development of real-time RT-PCR assays for detecting viral RNA in mosquitoes requires primers and probes that are able to specifically detect the virus of interest in the presence of potentially large amounts of mosquito nucleic acids. Because infection rates in field-collected mosquitoes are typically very low, mosquitoes are usually pooled (often in pools of 25 or 50 mosquitoes each) before being homogenized. Depending on the nucleic acid extraction procedure used, varying amounts of mosquito DNA and RNA are co-purified with the targeted viral nucleic acids. We describe the development of real-time RT-PCR assays for detecting CHIKV and ONNV in laboratory-infected mosquitoes. Three approaches were used to design the real-time RT-PCR primers and probes.
These approaches were manual design, use of AlleleID software (Premier Biosoft International, Palo Alto, CA), and the use of the Applied Biosystems (ABI) (Foster City, CA) custom gene expression assay service. After optimizing the real-time RT-PCR assay conditions for each design approach, we evaluated the assays for ease of development (i.e., primer/probe design and optimization), sensitivity, and specificity. Because these assays were designed to detect viral RNA isolated from mosquitoes collected in the field, adding extraneous mosquito tissue was analyzed for its effects, if any, on the performance of the assays.

MATERIALS AND METHODS

Viruses. CHIKV and ONNV were propagated in baby hamster kidney cells, and cell culture supernatants were harvested, distributed to vials, and stored at −70°C for later use. Viral titers were determined by standard plaque assays using Vero cells and were 8.4 and 7.2 log_{10} plaque-forming units (PFU)/mL for CHIKV and ONNV, respectively. Sindbis virus (SINV) (titer = 7.7 log_{10} PFU/mL), Semliki Forest virus (SFV) (titer = 9.7 log_{10} PFU/mL), Mayaro virus (MAYV) (titer = 9.7 log_{10} PFU/mL), Una virus (UNAV) (titer = 7.5 log_{10} PFU/mL), Ockelbo virus (OCKV) (titer = 8.4 log_{10} PFU/mL), Aura virus (AURAV) (titer = 7.4 log_{10} PFU/mL), Ross River virus (RRV) (titer = 6.2 log_{10} PFU/mL), Nduvu virus (NDUV) (titer = 7.2 log_{10}), Venezuelan equine encephalitis virus (VEEV) (Trinidad donkey; titer = 9.7 log_{10} PFU/mL), and eastern equine encephalitis virus (EEEV) (FL-91-4697; titer = 9.3 log_{10} PFU/mL) were used to evaluate the specificity of the real-time RT-PCR assays.

Primer and probe design. CHIKV primers and probes were designed using the consensus sequence created by comparing the S-27 African prototype strain (GenBank accession no. NC_004162) and a strain isolated from the Indian Ocean islands (GenBank accession no. DQ443544). The ONNV primers and probes were designed by using Gulu strain sequences (GenBank accession nos. M20303 and NC_001512).

Three different approaches were used to design the real-time RT-PCR primers and probes for detecting CHIKV, ONNV, or both CHIKV and ONNV RNA in mosquito samples. The first approach involved designing the primers and probes without the aid of specific primer/probe design software (i.e., manual design). This approach used the alignment of genomic sequences followed by the selection of primers with the proper characteristics.24 The second approach used the primer/probe design software AlleleID version 5. In addition to the individual CHIKV and ONNV assays, a dual CHIKV/ONNV assay was designed using this software. The third approach took advantage of the ABI custom gene expression assay service. This approach required the submission of a target sequence to ABI, who then returned a custom-designed gene expression assay that included optimal primers and probes in one tube. The sequences of the different primers and probes determined by the three different approaches are shown in Table 1. The Basic Local Alignment Search Tool (BLASTn)25 was used for a preliminary assessment of oligonucleotide specificity.

Mosquito infection. *Aedes aegypti* (Rockefeller strain) mosquitoes were reared in an insectary at 27°C and a relative humidity of 80% using a light:dark cycle of 16:8 hours. Adult female mosquitoes were infected intrathoracically 6–8 days after emergence with 0.3 μL of viral suspension containing

### Table 1

Real-time RT-PCR primers and probes for detecting CHIKV and ONNV

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Identification</th>
<th>Sequence (5′→3′)</th>
<th>Sensitivity (PFU/e)</th>
<th>Genome position</th>
<th>Size (bp)</th>
</tr>
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<tbody>
<tr>
<td><strong>Manual</strong></td>
<td></td>
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<tr>
<td><strong>CHIK</strong></td>
<td>CHIK 1F</td>
<td>CAGTGATCCCCAGACCGTGTT</td>
<td>28</td>
<td>E1 (10011–10264)†</td>
<td>253</td>
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<td></td>
<td>CHIK 1R</td>
<td>CCACATAAATGGGTAGACGCC</td>
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<td></td>
<td>CHIK 1P</td>
<td>6FAM-CCGTCATCCCGTCTCCGTACGTGAA-MGBNFQ</td>
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<tr>
<td><strong>ONNV</strong></td>
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<td>CAGTGATCCCCAGACCGTGTT</td>
<td>1.8</td>
<td>E1 (10108–10361)‡</td>
<td>259</td>
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<tr>
<td></td>
<td>ONN 1R</td>
<td>CCACATAAATGGGTAGACGCC</td>
<td></td>
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<td></td>
<td>ONN 1P</td>
<td>6FAM-CCGTCATCCCGTCTCCGTACGTGAA-MGBNFQ</td>
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<tr>
<td><strong>CHIK</strong></td>
<td>CHIK 2F</td>
<td>TTTGTGATCAAATGACCGGCATC</td>
<td>0.3</td>
<td>nsP1 (1089–1163)‡</td>
<td>74</td>
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<td></td>
<td>CHIK 2R</td>
<td>TCAGCCGCCAGAAGCACTTC</td>
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<td></td>
<td>CHIK 2P</td>
<td>6FAM-TTGCTACAGAATCGCATACG-MGBNFQ</td>
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<td><strong>ONNV</strong></td>
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<td>GTGTATCTGTAACATCTATGCCC</td>
<td>0.02</td>
<td>nsP1 (984–1075)‡</td>
<td>91</td>
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<td></td>
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<td></td>
<td>ONN 2P</td>
<td>6FAM-AGTCTCGAACAGATCAGTAG-MGBNFQ</td>
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<tr>
<td><strong>CHIK/ONNV</strong></td>
<td>CHIK 3F</td>
<td>ACACACAGTTACTACATACAGTTTC</td>
<td>0.3 (CHIK)</td>
<td>5′NTR (14–112)‡</td>
<td>98</td>
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<td></td>
<td>CHIK 3R</td>
<td>GCTGTCACGTCTACTGCCACCC</td>
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<td></td>
<td>ONN 3F</td>
<td>GATACACACCGACGTTAG</td>
<td>1.8 (ONNV)</td>
<td>5′NTR (14–112)‡</td>
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<tr>
<td></td>
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<tr>
<td><strong>CHIK</strong></td>
<td>CHIK 4F</td>
<td>CCGAAAGGAAACTCCAAGGCAACT</td>
<td>0.3</td>
<td>nsP2 (3000–3070)‡</td>
<td>70</td>
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<tr>
<td></td>
<td>CHIK 4R</td>
<td>CAGATGCCGCACATTATTGATG</td>
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<tr>
<td></td>
<td>CHIK 4P</td>
<td>6FAM-GGAGGTGGAGCAGATTG-MGBNFQ</td>
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<tr>
<td><strong>ONNV</strong></td>
<td>ONN 4F</td>
<td>ACCCTGTTGGCAGACTAGC</td>
<td>0.2</td>
<td>nsP2 (2465–2523)‡</td>
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<tr>
<td></td>
<td>ONN 4R</td>
<td>TCGCCACAAGTAACGACCTT</td>
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<tr>
<td></td>
<td>ONN 4P</td>
<td>6FAM-GGTTAGACGCGCTAG-MGBNFQ</td>
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</tbody>
</table>

*RT-PCR = reverse transcription–polymerase chain reaction; CHIKV = chikungunya virus; ONNV = O’nyong-nyong virus; PFU = plaque-forming unit equivalent; bp = basepairs; E = envelope; nsP = nonstructural protein; NTR = nontranslated region.
† Based on NC_001512.
‡ Based on NC_004162.
5.5 and 6.4 log_{10} PFU/mL (2.0 and 2.9 log_{10} PFU/mosquito) of CHIKV and ONNV, respectively, and incubated at 27°C with apple slices provided as a carbohydrate source.

Mosquitoes were killed in triplicate and a single, positive mosquito body was homogenized with 0, 4, 14, or 24 negative mosquitoes on days 2, 4, and 7 after inoculation. In addition, two legs from each of the above virus-inoculated mosquitoes were added to specimens of uninfected mosquitoes to create pools containing 0, 4, 14, or 24 negative mosquitoes on days 2, 4, and 7 after inoculation. Mosquito samples were triturated in 2 mL of diluent (Medium 199 with Earle’s salts containing 10% heat-inactivated fetal bovine serum, 5 μg of amphotericin B, 50 μg of gentamicin, 100 units of penicillin, 100 μg of streptomycin/mL, and 0.075% NaHCO₃), split into two 1-mL aliquots, and then stored at −70°C until used. A plaque assay was conducted with the mosquito homogenates and used to measure the virus present in a subset of the samples.

**Extraction of RNA.** Mosquito samples were thawed and clarified by centrifugation (12,000 × g for 10 minutes at 4°C), and then total RNA was extracted and purified using a QIAamp viral RNA mini-kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. Viral RNA from cell culture–derived virus was also extracted and purified using the QIAamp viral RNA mini-kit. RNA was eluted from the kit columns in 60 μL of nuclease-free water and stored at −70°C until used.

**Real-time RT-PCR assay.** One-step RT-PCRs were performed using the SuperScript III one-step RT-PCR system with platinum taq (Invitrogen, Carlsbad, CA) in a final volume of 20 μL containing 5 μL of RNA, 10 μL of 2x reaction mixture, an additional 3 mM MgSO₄, 5 μg of bovine serum albumin (Roche, Pleasanton, CA), 0.9 μM of forward and reverse primers, and 0.2 μM of probe for the manual design, and AlleleID design assays. The above concentrations of Mg SO₄, and primers were determined to be optimal for these assays. For the ABI custom assay, 2 μL of 20x primer/probe stock (18 μM of forward and reverse primers and 8 μM of probe) was substituted for the forward and reverse primers and the probe. Real-time RT-PCR assays were carried out using the Ruggedized Advanced Pathogen Identification Device (RAPID) (Idaho Technology, Salt Lake City, UT) using the following cycling conditions: reverse transcription at 50°C for 20 minutes, denaturation at 95°C for 5 minutes, 40 cycles at 95°C for 1 second (15 second for the ABI custom assay only) and 60°C for 20 seconds (60 seconds for the ABI custom assay only).

**Statistical analysis.** SAS version 9.1.3 (SAS Institute Inc., Cary, NC) was used to compare the mean values between groups (virus-positive bodies or legs) using a t-test with stepdown Bonferroni adjustment.

**RESULTS**

**Sensitivity and specificity evaluation of real-time RT-PCR assays.** The sensitivities of the real-time RT-PCR assays for CHIKV and ONNV were evaluated by determining the detection threshold of each assay using 10-fold serial dilutions of RNA extracted from viral stocks measured by plaque assay. Assay results were then used to determine a real-time RT-PCR cycle threshold (the time at which a sample is determined positive by real-time RT-PCR) to PFU equivalents (PFUe), which enables a qualitative determination of the presence of infectious virus. Not surprisingly, the primers and probes designed manually (i.e., without the aid of specific primer/probe design computer software) proved to be the least sensitive of the three assays with a sensitivity of 28 PFUe for CHIKV RNA and 1.8 PFUe for ONNV RNA. Primers and probes designed using the AlleleID software program yielded sensitivities of 0.3 PFUe for the individual CHIKV assay and the dual CHIKV/ONNV assay. Similarly, these primers and probes were able to detect 0.02 PFUe for the individual ONNV assay and 1.8 PFUe for the dual CHIKV/ONNV assay. The ABI custom assay service produced primers and probes with similar sensitivities as those designed using the AlleleID software program, and resulted in primers and probes with a sensitivity of 0.3 PFUe for CHIKV RNA and 0.02 PFUe for ONNV RNA (Table 1). The intra-assay variation was < 5% and 7% for the CHIKV and ONNV assays, respectively, and the inter-assay variation was 2% and 6% for the CHIKV and ONNV assays, respectively.

**Detection of CHIKV RNA isolated from infected mosquitoes.** The ABI custom assay service designed CHIKV primers and probe, CHIK 4F/4R/4P, were used to evaluate mosquitoes that were infected with CHIKV. Detection of viral RNA from infected mosquito bodies was compared with that observed for the legs from the same mosquitoes, and was also compared with that detected on different days after infection (Figure 1). This comparison was conducted to test the sensitivity of the real-time RT-PCR assay and to determine if virus-positive mosquito legs that were accidentally introduced into a mosquito pool during pooling of the mosquitoes would result in a virus-positive mosquito pool. As expected, the mean amount of viral RNA detected in the legs was less than the bodies for all pools (4.2 versus 5.2 log_{10} PFUe, respectively). The mean amount of viral RNA detected in bodies and legs of infected mosquitoes peaked on day 4 for most of the mosquitoes tested and were 5.5 and 4.5 log_{10} PFUe, respectively.

We also tested if adding virus-negative mosquitoes could interfere with detection of viral RNA isolated from an individual infected mosquito. Although the presence of larger numbers of uninfected mosquitoes appeared to interfere with detection of CHIKV RNA isolated from infected mosquitoes held for 2 days (5.2 log_{10} PFUe for an individual infected mosquito body compared with 4.2 log_{10} PFUe for a pool that...
contained one infected mosquito body plus 24 uninfected mosquitoes), no consistent effect was observed for infected mosquitoes held for 4 and 7 days. All pairwise comparisons between mean values resulted in no statistically significant differences between amounts of viral RNA detected from individual infected mosquitoes or from pools containing an additional 24 negative mosquitoes ($P > 0.05$).

**Detection of ONNV RNA isolated from infected mosquitoes.** The amount of viral RNA detected at all time points in the bodies and legs of ONNV-infected mosquitoes was less overall compared with the CHIKV-infected mosquito bodies and legs (Figure 2). The amount of ONNV RNA detected in the bodies (infected legs were not tested) immediately after infection was above the limit of detection for the assay (mean titer 1.8 log$_{10}$ PFUe), which is in contrast to the CHIKV-infected mosquitoes, in which no viral RNA was detected immediately after infection. The amount of viral RNA detected in the mosquito bodies increased steadily up to 7 days after inoculation (mean titer 4.4 log$_{10}$ PFUe). The viral RNA in the legs peaked on day 4 after inoculation (mean titer 3.0 log$_{10}$ PFUe) and remained relatively constant to day 7 after inoculation. Similar to the results with the CHIKV assay, the addition of negative mosquitoes did not interfere with the detection of ONNV RNA by real-time RT-PCR ($P ≥ 0.07$ for all pairwise comparisons).

A subset of mosquitoes representing all cohorts was titrated by standard plaque assay. Results of the plaque assays were consistent with those of the real-time RT-PCR assay.

**DISCUSSION**

The molecular diagnosis of CHIKV has only been evaluated for human samples and no real-time RT-PCR assay exists for detecting ONNV present in human or mosquito...
samples. Because these are two important re-emerging arboviruses that can cause human disease, we developed and tested real-time RT-PCR assays to detect the RNA of these viruses in mosquitoes.

The development of real-time PCR assays requires primers and probes to detect a gene or nucleic acid of interest, followed by assay optimization. Many methods exist for primer and probe design and assay optimization requires hands-on laboratory work. This process can be time-consuming, and a direct comparison of different primer and probe design methods has not been completed. We compared three approaches for designing primers and probes for real-time RT-PCR assays. The first approach involved designing primers and probes without the aid of computer software (i.e., manual design). This method was inexpensive, but required knowledge of what constituted good primer and probe sequences and binding sites. The second approach involved using a computer software program (AlleleID) specifically intended for the design of optimal primers and probes for a targeted gene. This program has proved successful for the development of primers and probes for other genes of interest, but requires an understanding of the computer program. The third approach took advantage of a company’s (ABI custom assay service) solicitation to design the primers and probes for the customer. Although this method was slightly more expensive in regard to the initial cost of the primers and probe (compared with the manual design method), it was the simplest of the three methods and did not require purchasing primer/probe design software. Because ABI provides the sequence of the primers and probes with each order, the customer has the ability to obtain additional primer and probe material from a potentially less expensive supplier.

We directly compared the three real-time RT-PCR primer and probe-design approaches described above using serially diluted viral RNA to determine the sensitivity of each assay. The least sensitive assay for detecting both CHIKV and ONNV RNA was the manually designed primer and probe approach. The primers and probes designed using the AlleleID computer software and the ABI custom assay service proved to be the most sensitive. The AlleleID computer software did add the advantage of designing a pan assay. However, we were more interested in pursuing individual assays for CHIKV and ONNV. We chose the ABI custom assay services as the lead approach because of its ease of development and optimization of the primers and probes, and on the basis of the discussion presented above. As part of the ABI custom assay design approach, ABI returns a 20× solution containing concentration optimized primers and probes. Compared with the ABI approach, the manual and the AlleleID approach required optimization of the primer and probe concentrations for each real-time RT-PCR assay.

The primers and probes were designed using sequence information that represents the CHIKV and ONNV genotype(s) with the widest geographic distribution. However, these viruses contain a significant amount of genetic diversity among strains. Designing degenerate primers and probes would enable greater assay flexibility to detect multiple virus strains, but would be more difficult to design using the methods presented above.

The amount of viral RNA detected at all time points in ONNV-infected mosquitoes was less overall compared with CHIKV-infected mosquitoes. This is most likely because ONNV is primarily transmitted by anopheline mosquitoes and not Ae. aegypti, which were used in this study. We used this mosquito species simply as an assessment tool for these real-time RT-PCR assays and not to evaluate their vector competence for ONNV.

To complete the development of real-time RT-PCR assays for detecting CHIKV and ONNV present in infected mosquitoes, we evaluated the potential inhibitory effects from the addition of uninfected mosquitoes to a pool containing one infected mosquito body or two infected mosquito legs. This approach is consistent with the testing of field-collected mosquitoes because they are generally pooled (often 25 or more per pool) before testing. Adding up to 24 uninfected mosquitoes to either one positive mosquito body or two positive legs resulted in no significant inhibition on the detection of CHIKV or ONNV RNA present in an infected mosquito.

It is interesting that virus from the addition of just two legs from a virus-infected mosquito to a pool of 24 uninfected mosquitoes was readily detected by the CHIKV and ONNV assays. In a field setting, mosquito legs often break off during the trapping, taxonomic sorting, and pooling process and may accidentally be included in a pool other than where that mosquito was placed. Our results indicate that if a leg from a CHIKV-infected or ONNV-infected mosquito were accidentally included in a pool of uninfected mosquitoes, that pool would then be positive for the virus, although it did not contain an infected mosquito. Therefore, care should be taken when interpreting field-detection data and the implication of a mosquito species as a potential vector for the field-detected virus. Laboratory studies to confirm the sensitivity of that species to infection and their ability to transmit the virus.
in question need to be conducted before that species can be implicated as a vector of the virus in question.

In conclusion, CHIK and ONN are important re-emerging arboviral diseases that are currently affecting persons worldwide. The rapid and specific detection of CHIKV and ONNV RNA in mosquitoes will enable healthcare and public health officials to better respond to outbreaks of these viral diseases and to implement better control measures for the vectors. We described the development of sensitive and specific real-time RT-PCR assays for use on the RAPID instrument for detection of CHIKV and ONNV in mosquitoes that can be used in a field or deployed setting. Further testing of these assays in the field with field-collected mosquitoes in CHIKV and ONNV endemic/outbreak areas will determine the robustness and utility of these newly developed assays.

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