Chikungunya Virus RNA and Antibody Testing at a National Reference Laboratory since the Emergence of Chikungunya Virus in the Americas

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Since first reported in the Americas in December 2013, chikungunya virus (CHIKV) infections have been documented in travelers returning from the Caribbean, with many cases identified by CHIKV antibody and/or RNA testing at our laboratory. We used our large data set to characterize the relationship between antibody titers and RNA detection and to estimate IgM persistence. CHIKV RNA was measured by nucleic acid amplification and CHIKV IgG/IgM by indirect immunofluorescence. Of the 1,306 samples submitted for RNA testing in January through September 2014, 393 (30%) were positive; for 166 RNA-positive samples, CHIKV antibody testing was also ordered, and 84% were antibody negative. Of the 6,971 sera submitted for antibody testing in January through September 2014, 1,811 (26%) were IgM positive; 1,461 IgM positives (81%) were also IgG positive. The relationship between the CHIKV antibody titers and RNA detection was evaluated using 376 IgM-positive samples (138 with RNA testing ordered and 238 deidentified and tested for RNA). RNA detection showed no significant association with the IgM titer but was inversely related to the IgG titer; 63% of the IgG negative sera were RNA positive, compared to 36% of sera with low IgG titers (1:10 to 1:80) and 16% with IgG titers of ≥1:160. Using second-sample results from 62 seroconverters, we estimated that CHIKV IgM persists for 110 days (95% confidence interval, 78 to 150 days) after the initial antibody-negative sample. These findings indicate that (i) RNA detection is more sensitive than antibody detection early in CHIKV infection, (ii) in the absence of RNA results, the IgG titer of the IgM-positive samples may be a useful surrogate for viremia, and (iii) CHIKV IgM persists for approximately 4 months after symptom onset.

Chikungunya virus (CHIKV) is an alphavirus transmitted from one person to another via mosquitoes of the genus Aedes (1–3). Nearly all individuals infected with CHIKV become symptomatic, typically exhibiting fever, rash, and debilitating arthralgia (1–3). Most infected individuals show complete recovery within a few weeks; however, 15 to 60% of patients develop chronic arthralgia (1–3). Intrapartum mother-to-child transmission has been documented, with serious neurologic and hemorrhagic complications observed in affected infants (8).

Since CHIKV was first identified in 1953 (9), there have been multiple epidemics of CHIKV infections throughout Africa and Asia (2). A particularly large CHIKV outbreak began in eastern Africa in late 2004 and then spread to Indian Ocean islands, India, and southeastern Asia over the next 2 years. Estimates suggest that nearly 2 million people became infected during this outbreak (2, 10–15).

Because the mosquito vectors for CHIKV transmission are present in tropical and temperate regions worldwide and recently infected travelers moving between areas where CHIKV is endemic and not endemic exhibit high levels of viremia (16), epidemiologists have warned that CHIKV could move into new geographic regions, including Australia, Europe, and the Americas (5, 6). This prediction came to fruition on a small scale in 2007, when a local outbreak of CHIKV infection occurred in Italy following the visit of a recently infected individual from India (17). More recently these warnings were realized late in 2013, when the World Health Organization reported local transmission of CHIKV on the Caribbean island of St. Martin (18). Since then CHIKV has spread explosively throughout the Caribbean islands, Central America, and northern countries of South America (19, 20), with nearly 800,000 suspected cases as of October 2014 (21). In conjunction with this outbreak, the number of documented CHIKV infections in the United States has increased dramatically from historic numbers. From 2006 to 2013, the mean annual number of CHIKV cases identified in U.S. residents returning from areas where CHIKV is endemic was 28; in contrast, thus far in 2014 (21 October), 1,455 CHIKV cases in U.S. residents returning from affected areas in the Americas have been reported to the Centers for Disease Control and Prevention (22). Because CHIKV is not a nationally reportable disease, the number of cases is likely higher than the number reported. Related to this surge in travel-related cases of CHIKV, a small number of locally transmitted CHIKV cases have been identified in Florida, raising concerns about further spread throughout areas of the United States where the mosquito vectors are found (20, 22).

The primary laboratory tool for identifying CHIKV infections are assays for viral genomic RNA and antibodies (IgM and IgG) (3, 4).
5, 23). CHIKV genomic RNA is detectable at the time of symptom onset and then declines to undetectable levels within 7 days (5, 8, 11, 13, 17, 23). In contrast, IgM is not uniformly positive until day 5 after illness onset, with IgG appearing 1 to 2 days after IgM (5, 8, 24, 25). To our knowledge, our facility is the only commercial U.S. reference laboratory offering these CHIKV assays. The number of samples submitted to our facility for these tests increased dramatically during the second quarter of 2014, presumably reflecting suspected cases of CHIKV infection in travelers returning from the Caribbean and other areas where CHIKV is endemic (19). Here, we share our experience with CHIKV RNA and antibody testing during the first 9 months of 2014. We also took advantage of the large data set generated to assess the relationship between antibody titers and RNA detection and the persistence of CHIKV IgM and IgG.

MATERIALS AND METHODS

Specimens. The samples included in this analysis were submitted to the Focus Diagnostics Reference Laboratory for CHIKV RNA and/or CHIKV antibody (IgG and IgM) testing between 1 January and 30 September 2014. Neither new clinical findings, the date of symptom onset, nor travel history was supplied with any of the samples. CHIKV RNA-positive patients who also had CHIKV antibody testing ordered on the same collection were identified by searching the laboratory information system (LIS). Similarly, the LIS was searched to identify CHIKV IgM-positive patients who also had CHIKV RNA testing ordered on the same collection and to identify CHIKV IgM-positive patients who submitted an earlier serum sample that was negative for CHIKV antibodies (IgG and IgM). No attempt was made to identify patients with both CHIKV RNA and CHIKV antibody testing ordered on the same collection with negative results for all 3 analytes (RNA, IgG, and IgM). The relationship between antibody titers and RNA results was evaluated using data from 376 IgM-positive samples; of these, 138 had both antibody and RNA testing ordered initially, whereas 238 were initially tested only for antibodies and were subsequently deidentified and tested for RNA. The sera in the deidentified group were selected to span the range of positive CHIKV IgM titers (1:10 to 1:5120) and the range of CHIKV IgG results (<1:10 to ≥1:5120).

CHIKV RNA detection. CHIKV genomic RNA was detected using a laboratory-developed real-time reverse transcriptase PCR (RT-PCR) assay targeting conserved sequences of the CHIKV gene encoding an RNA-dependent RNA polymerase. Primers and probes were found within nucleotide positions 6800 to 7000 of GenBank accession number L37661 (exact primer and probe sequences are proprietary); GenBank searches showed no cross-reactivity with any of the 4 dengue virus serotypes or West Nile virus. The assay’s limit of detection is 900 copies/ml serum. Nucleic acids were extracted from 0.2-ml aliquots of patient serum samples; of these, 138 had both antibody and RNA testing ordered initially, whereas 238 were initially tested only for antibodies and were subsequently deidentified and tested for RNA. The sera in the deidentified group were selected to span the range of positive CHIKV IgM titers (1:10 to 1:5120) and the range of CHIKV IgG results (<1:10 to ≥1:5120).

CHIKV IgM IFA. A laboratory-developed indirect immunofluorescence assay (IFA) based on the Titerplane system (Euroimmun US, Inc., Morris Plains, NJ) was used to measure CHIKV IgG (26, 27). Sera diluted 1:10 in phosphate-buffered saline (PBS) were added to reaction fields of a reagent tray, and a slide containing mounted glass biochips coated with CHIKV (strain EU 14)-infected Vero cells or uninfected Vero cells was placed into the recesses of the reagent tray. After 30 min at room temperature, the slide was washed three times with PBS containing 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO). It was then placed into reagent tray reaction fields containing fluorescein isothiocyanate (FITC-)– conjugated goat anti-human IgG (heavy chain specific) (Life Technologies, Grand Island, NY) diluted to the working concentration in PBS containing 0.0025% Evans blue dye (Sigma-Aldrich). After 30 min at room temperature, the slide was washed as described; mounting fluid and a coverslip were then added, and the slide was examined at 200× using a fluorescence microscope (Olympus, Center Valley, PA). A positive reaction was defined as a homogeneous to granular pattern of fluorescence in the cytoplasm of infected cells, and no fluorescence of uninfected cells. Sera producing positive CHIKV-specific fluorescence were serially diluted, and all dilutions were tested to determine the endpoint CHIKV IgG titer, defined as the highest dilution giving dim but definite (1+–) fluorescence.

CHIKV IgM IFA. An approach similar to that described for CHIKV IgG detection was used to measure CHIKV IgM (26, 27). Sera were diluted 1:10 in PBS containing goat anti-human IgG (Focus Diagnostics Products, Cypress, CA), and after 15 min at room temperature, diluted sera were transferred to reagent tray reaction fields. A CHIKV slide was then placed into the reaction tray and incubated for 1 h at 37°C. Following washing, the slide was incubated with appropriately diluted FITC-conjugated goat anti-human IgM (heavy chain specific) (Life Technologies) for 1 h at 37°C. The slide was then washed and examined for fluorescence as described for the IgG assay; positive samples were diluted 1:10 in the same sample buffer used for the screening assay and then serially diluted in PBS. All dilutions were then tested to determine the endpoint CHIKV IgM titer.

RESULTS

CHIKV RNA testing. During the first 9 months of 2014, 1,306 sera were submitted for CHIKV RNA testing, and 30% of samples were CHIKV RNA positive (Table 1). Figure 1 presents the results segregated by month of testing. The number of samples submitted for RNA testing increased slightly in May, with marked increases in June and July. Similarly, the numbers of CHIKV RNA-positive samples increased in May and June and then increased even further in July. After July, the number of samples tested for CHIKV RNA decreased somewhat, but the number of CHIKV RNA-pos-

### Table 1: CHIKV test results, January through September 2014

| Test ordered       | Result          | No. of samples | % of total |
|--------------------|-----------------|----------------|------------|
| CHIKV RNA          | Negative        | 913            | 69.9       |
|                    | Positive        | 393            | 30.1       |
|                    | Total           | 1,306          | 100.0      |
| CHIKV IgM and IgG  | IgM negative, IgG negative | 5,108 | 73.3 |
|                    | IgM positive, IgG negative | 350 | 5.0 |
|                    | IgM positive, IgG positive | 1,461 | 21.0 |
|                    | IgM negative, IgG positive | 52 | 0.7 |
|                    | Total           | 6,971          | 100.0      |
itive samples remained stable. The proportion of samples positive in the RNA assay increased more than 5-fold in May than in April (50% versus 10%, respectively), even though the number of samples tested increased only 2-fold (28 in May versus 14 in April). Higher proportions of samples testing positive for CHIKV RNA were consistently detected in the months following May, ranging from 25% to 40%.

An LIS search revealed that 166 CHIKV RNA-positive samples also had CHIKV antibody testing ordered. Of these 166 samples, 139 (84%) were negative for antibodies, 24 (14%) were IgM positive, IgG negative, and 3 (2%) were IgM positive, IgG positive; none were IgM negative, IgG positive.

**CHIKV antibody testing.** During the first 9 months of 2014, 6,971 sera were submitted for CHIKV antibody testing; the distribution of samples among the various antibody reactivity patterns is presented in Table 1. Overall, 1,863 (26.7%) samples were antibody positive; nearly all antibody-positive samples were IgM positive (1,811/1,863 = 97.2%), and most IgM-positive sera were also IgG positive (1,461/1,811 = 80.7%). Very few samples (<1%) were positive for IgG but negative for IgM.

The numbers of samples submitted for antibody testing and the numbers of positive samples segregated by month of sample collection are shown in Fig. 2. An increase in the monthly number of submitted samples was evident in May, with further, stepwise, increases in June and July. The number of submitted samples then stabilized during August and September. Monthly numbers of submitted samples were >7-fold higher in July, August, and September than in May. Similarly, an increase in the number of IgM-positive, IgG-positive samples was clearly evident in July through September compared to the numbers in previous months. Although not evident from Fig. 2, there was also a marked increase in the monthly numbers of IgM-positive, IgG-negative samples be-
Beginning in June and continuing through September; in May there were only 3 IgM-positive, IgG-negative samples identified compared to between 54 and 110 per month during June through September.

Figure 3 presents the percentages of samples exhibiting various CHIKV antibody reactivity patterns segregated by the month of the sample collection. The proportion of samples exhibiting the CHIKV IgM-positive, IgG-positive reactivity pattern increased steadily from 3% in April to 26% in September; similarly, the proportion of samples exhibiting the IgM-positive, IgG-negative pattern increased from 1% in May to 6% in June and then remained stable in subsequent months. In contrast, the proportion of samples exhibiting the IgM-negative, IgG-positive pattern decreased from 4% in April to <1% in all subsequent months.

These time-related increases in the numbers of samples submitted for CHIKV RNA and/or antibody testing and in the proportion of submitted samples positive for CHIKV RNA and IgM reflect the timeline for increasing numbers of suspected CHIKV infections among residents of the Caribbean basin (21, 22). Information on patient residence locale was available for the first 608 antibody-positive patients identified at our facility; 39 of the 48 contiguous United States were represented (as well as the District of Columbia, Puerto Rico, and U.S. Virgin Islands) with 5 states (New York, Florida, Massachusetts, Pennsylvania, and New Jersey) accounting for 57% of the antibody-positive patients. These demographic results are consistent with the U.S. Centers for Disease Control and Prevention data for CHIKV infections in U.S. residents returning from international travel, particularly from the Caribbean (22).

Relationship of antibody titer to RNA detection. We capitalized on the availability of our large data set to assess the relationship between CHIKV antibody titers and CHIKV RNA detection; a panel of 376 IgM-positive samples was employed for this purpose. For this analysis, positive titers (both IgM and IgG) were grouped into 3 broad categories; titers of 1:10 to 1:80 were considered low positive, those of 1:160 to 1:640 medium positive, and those of ≥1:1,280 high positive. Figure 4 presents the proportion of each titer group that was CHIKV RNA positive. Among the 3 IgM titer groups, there were no statistical differences in the proportions of samples that were CHIKV RNA positive. In contrast, a statistically significant inverse relationship between the IgG titer and the proportion of CHIKV RNA-positive samples was observed; the CHIKV RNA-positive proportion decreased from 63% for the CHIKV IgG-negative group to 36% for the low IgG titer group, with further decreases to <18% for the medium and high IgG titer groups.

Kinetics of antibody production and IgM persistence. Antibody production kinetics was evaluated using second-sample data for 24 seroconverters who had an antibody-positive sample collected within 14 days of an initial antibody-negative sample. As shown in Fig. 5, IgM was detectable by day 4 after the initial antibody-negative sample, with stable titer levels by day 6; similarly, IgG was detectable in most patients by day 4, with stable titers by day 6. Based on these findings, we then assessed the CHIKV IgM persistence using second-sample data for 62 seroconverters who had an IgM-positive sample collected 7 or more days after an initial antibody-negative sample. As shown in Fig. 6, IgM titers in seroconverters were inversely related to days after the negative sample. Based on the extrapolated regression trend line, the cut-point titer distinguishing positive results from negative results (1:10) was associated with a value of 110 days after the negative sample, with a 95% confidence interval of 78 to 150 days. These findings indicate that CHIKV IgM titers are expected to revert to negative (titer <1:10) by around 16 weeks (4 months) after the onset of symptoms. As expected, a plot of IgG titers versus days after the negative sample for these same 62 patients showed a slight increase in IgG titers over time, rather than a decrease (Fig. 7).

DISCUSSION
The timeline we observed for the increasing numbers of samples submitted for CHIKV RNA and/or antibody testing and the proportions of samples positive for these markers occurred concurrently with the timeline for increasing numbers of suspected CHIKV infections in the Caribbean (21) and the number of CHIKV cases among U.S. residents returning from the Caribbean...
We thus presume that our observed increase in testing volume represented cases of suspected CHIKV infection contracted in the Caribbean basin. The high rates of CHIKV RNA and CHIKV IgM detection indicate that we identified many patients who were recently infected with CHIKV (3, 5, 8, 13, 23). Consistent with published data (3, 5, 23), our findings indicate that CHIKV RNA testing is more sensitive than CHIKV antibody testing for identifying patients with very recent CHIKV infections. The vast majority (84%) of CHIKV RNA-positive samples with antibody testing also ordered were antibody negative, reflecting the time lag in antibody production following infection (5, 8, 24, 25). An analysis of CHIKV antibody production in seroconverters showed that CHIKV IgM was detectable in all patients by 4 days after the antibody-negative sample, whereas IgG was detectable in all patients by 6 days after the antibody-negative sample. These findings are consistent with published data showing that CHIKV IgM becomes detectable in nearly 100% of cases by day 5 after symptom onset, with CHIKV IgG detectable 1 or 2 days later (8, 20, 23, 24, 25). A limitation to these seroconversion data is that none of the initial (antibody-negative) samples were tested for CHIKV RNA; thus, we have no direct proof that these samples were CHIKV RNA positive. However, the documented seroconversion, along with the assumption that the patients were symptomatic when the antibody-negative sample was collected, supports the view that these patients were in the very early stages of CHIKV infection.

![Graph](https://cvi.asm.org/)

**FIG 4** CHIKV RNA detection in relation to CHIKV IgM and IgG titers for a panel of 376 IgM-positive serum samples. For both IgM and IgG, low pos indicates samples with low-positive titers of 1:10 to 1:80, med pos indicates samples with medium-positive titers of 1:160 to 1:640, and high pos indicates samples with high-positive titers of ≥ 1:1,280. The numbers in parentheses indicate the numbers of samples in the indicated groups. *, proportion is significantly different (P < 0.05) from that for the other 3 IgG groups.

**FIG 5** CHIKV antibody production kinetics as assessed using second-sample results from 24 seroconverters.
In addition to investigating CHIKV antibody results in CHIKV RNA-positive samples, we also studied the RNA detection/antibody detection relationship by investigating RNA results in IgM-positive sera. We found that CHIKV RNA detection was not related to IgM titers but rather was inversely related to IgG titers. The rate of CHIKV RNA detection was highest among IgM-positive samples that were IgG negative (titer of 1:10) and then decreased stepwise with increasing IgG titer. Since CHIKV RNA-positive (viremic) patients are the source of CHIKV transmission to another individual via transfer by a mosquito bite (16), these findings suggest that, in the absence of RNA testing, the IgG titer of an IgM-positive patient can serve as a surrogate marker for viremia and thus infectivity potential. Thus, an IgM-positive, IgG-negative patient, with a 63% chance of being viremic, is more likely to transmit the infection if bitten by a mosquito than an IgM-positive patient with an IgG titer of 1:280, who has only a 15% chance of being viremic. In addition, identification of an inverse relationship between the CHIKV IgG titer and CHIKV RNA detection sheds light on the mechanism of viral clearance; it appears that IgG plays a more important role than IgM in clearing infectious virus from the circulation. Our results differ somewhat from those of Chusri et al. (24), who quantified CHIKV RNA and found that a decrease in viral load corresponded with rising IgM titers. These two findings are not necessarily inconsistent; however, a plausible hypothesis is that CHIKV IgM is important in reducing the viral load early in the immune response, but it is CHIKV IgG that mediates the complete clearance of CHIKV from the circulation.

Studies of two mosquito-borne flaviviruses, West Nile virus (WNV) and dengue virus, have demonstrated that virus-specific IgM can persist for 6 months or longer, thus complicating the clinical utility of IgM detection as a marker for recent infection (29, 30). To determine if the same pattern of IgM persistence characterizes the mosquito-borne alphavirus CHIKV, we estimated CHIKV IgM persistence using second-sample results from patients with documented seroconversion. A plot of IgM titer versus days after the initial antibody-negative sample yielded an extrapolated regression line, indicating that an IgM titer of 1:10, the cut-point for discriminating positive from negative IgM results, was reached at 110 days (95% confidence interval, 78 to 150 days) after the antibody-negative sample. Although the small number of samples collected >60 days after the negative sample is a limitation to these findings, they suggest that CHIKV IgM persists for approximately 4 months after the onset of symptoms and is thus a reliable marker of recent infection. These findings are in general agreement with those of Panning et al. (25), who found that CHIKV IgM persisted between 50 and 170 days after symptom onset. In contrast, they conflict with results from two other publications (4, 31) reporting CHIKV IgM persistence for at least 18 months in 40 to 50% of patients. The reasons for this discrepancy.

FIG 6 Persistence of CHIKV IgM as assessed using second-sample results from 62 seroconverters. The second sample was collected ≥1 week after the antibody-negative sample. The solid black line represents the trend line from the linear regression analysis. The legend indicates the point where the extrapolated trend line intersects the x-axis (and also the 95% confidence interval [CI]); this value is associated with an IgM titer of 1:10, the titer discriminating positive from negative IgM results.

FIG 7 Persistence of CHIKV IgG as assessed using second-sample results from 62 seroconverters. The second sample was collected ≥1 week after the antibody-negative sample. The solid black line represents the trend line from linear regression analysis.
remain unclear. Although a case report documented CHIKV IgM persistence in a patient with chronic destructive arthritis (32), a larger study of 88 patients found no association between IgM persistence and chronic arthralgia (4). A more likely explanation for the discordance is the method used to measure CHIKV IgM; both our study and the study reporting ChIKV IgM persistence for 4 to 5 months (25) utilized an IFA procedure, whereas both studies reporting CHIKV IgM persistence for 18 months utilized the same laboratory-developed IgM-capture enzyme immunoassay (4, 31). Further studies are needed to understand the relationship between the CHIKV IgM results obtained by these two different methods.

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