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Evaluation of two commercially available chikungunya virus IgM enzyme-linked immunoassays (ELISA) in a setting of concomitant transmission of chikungunya, dengue and Zika viruses

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PII: S1201-9712(19)30440-0

DOI: <https://doi.org/10.1016/j.ijid.2019.11.001>

Reference: IJID 3822

To appear in: *International Journal of Infectious Diseases*

Received Date: 9 July 2019

Revised Date: 31 October 2019

Accepted Date: 1 November 2019

Please cite this article as: Kikuti M, Tauro LB, Moreira PSS, Nascimento LCJ, Portilho MM, Soares GC, Weaver SC, Reis MG, Kitron U, Ribeiro GS, Evaluation of two commercially available chikungunya virus IgM enzyme-linked immunoassays (ELISA) in a setting of concomitant transmission of chikungunya, dengue and Zika viruses, *International Journal of Infectious Diseases* (2019), doi: <https://doi.org/10.1016/j.ijid.2019.11.001>

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Title: Evaluation of two commercially available chikungunya virus IgM enzyme-linked immunoassays (ELISA) in a setting of concomitant transmission of chikungunya, dengue and Zika viruses

Running Title: Accuracy of a chikungunya virus IgM ELISA

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Highlights

- Inbios and Euroimmun CHIKV IgM ELISAs had low sensitivities for acute sera, as expected.
- For convalescent sera, both kits had high sensitivities (>92%).

- Convalescent sera specificity was 91% for the Inbios and 84% for the Euroimmun kit.
- The Euroimmun kit may yield more false-positive cases if CHIKV circulation is low.

Abstract (200 words)

Objective: To evaluate the diagnostic performance of the Inbios (Seattle, US) and Euroimmun (Luebeck, Germany) chikungunya virus (CHIKV) IgM enzyme-linked immunoassays (ELISAs).

Methods: We evaluated the tests' accuracy on sera from 372 patients enrolled in an acute febrile illness surveillance study performed in Salvador, Brazil from Sept/2014 to Jul/2016, a period of simultaneous CHIKV, dengue (DENV), and Zika (ZIKV) virus transmission. We assessed the sensitivity on acute and paired convalescent sera from RT-PCR-confirmed CHIKV cases (collected at median one and 19 days post-onset of symptoms, respectively), and the specificity on sera of RT-PCR-confirmed DENV and ZIKV cases, and on negative patients.

Results: The Inbios and Euroimmun tests' sensitivities for acute samples were 4.0% and 10.3%, while for convalescent samples they were 92.4% and 96.9%, respectively. Overall, Inbios IgM ELISA specificities for acute and convalescent samples were 97.7% and 90.5%, respectively, and Euroimmun specificities were 88.5% and 83.9%, respectively.

Conclusions: Both tests presented high sensitivity for convalescent samples. However, the Euroimmun test returned more equivocal results and presented a slightly lower specificity, which might result in a higher rate of false positives if the test is used in scenarios of low CHIKV transmission, when the chance of CHIKV infection is lower.

Keywords: accuracy, sensitivity and specificity, diagnostic performance, chikungunya virus, enzyme-linked immunosorbent assay, ELISA

Introduction

Chikungunya virus (CHIKV) is an alphavirus transmitted by *Aedes (Stegomyia)* spp. mosquitoes (WHO 2008). Transmission was initially restricted to small outbreaks and sporadic cases in Africa and Asia, but since early 2000s the virus has caused large outbreaks in India and Southeast Asia. In 2013, CHIKV spread for the first time in the modern scientific era to the Americas, including throughout the Caribbean (Zeller et al. 2016) and Latin America (Yactayo et al. 2016). In 2016 alone, ~350,000 cases were reported in Latin America (PAHO 2017), the majority (>260,000) occurring in Brazil, particularly in the northeast (BRASIL 2017).

Acute clinical manifestations associated with CHIKV infections are non-specific, usually including fever, rash, and arthralgia, the latter being the most prominent symptom that may last for months or years, causing chronic disabilities (WHO 2008). In areas where other arboviruses with similar clinical manifestations co-circulate, such as dengue (DENV) and Zika (ZIKV) viruses, laboratory diagnostic tools that distinguish CHIKV infections from them, as well as from other acute febrile illness, are essential for effective surveillance and appropriate clinical management.

Here, we evaluated the diagnostic performance of two commercially available enzyme-linked immunoassays (ELISAs) (Inbios International, Inc, Seattle, USA; and Euroimmun, Luebeck, Germany) for detection of CHIKV-specific IgM antibodies in acute and convalescent paired sera of febrile outpatients from Salvador, Brazil, during a period of simultaneous CHIKV, DENV, and ZIKV transmission (Cardoso et al. 2015, 2017; Silva et al. 2018). The tests' accuracies were estimated using results of reverse-transcription polymerase chain reaction (RT-PCR) as the gold-standard reference test.

Methods

Surveillance for acute febrile illness.

From September 2014 to July 2016, we enrolled patients attending a public emergency health unit of Salvador (São Marcos Emergency Center, SMEC) in an acute febrile illness (AFI) enhanced surveillance study (Silva et al. 2018). Inclusion criteria were ≥ 6 months of age and reported or measured fever ($\geq 37.8^{\circ}\text{C}$) up to 7 days of duration. Demographic and clinical characteristics data of patients who consented to participate were obtained through a structured standardized interview. In addition, acute-phase (at enrollment) and paired convalescent-phase (≥ 15 days after enrollment) blood samples were drawn for arboviral diagnosis. Samples were refrigerated until centrifugation, and obtained sera were stored at -20°C and -70°C for serological and molecular testing, respectively.

RT-PCR testing for arboviral diagnosis.

All acute-phase sera, which had not been previously thawed, were submitted to RNA extraction and tested by RT-PCR for DENV, ZIKV, and CHIKV. Briefly, viral RNA was extracted using the Maxwell® 16 Total RNA Purification kit (Promega, Wisconsin, USA) or QIAmp® Viral RNA mini kit commercial kit (Qiagen, Hilden, Germany) according to manufacturer's specifications. Subsequently, RT-PCR (Access RT-PCR kit- Promega, Wisconsin, USA) was performed separately on the extraction product using specific primers to identify DENV (Lanciotti et al. 1992), ZIKV (Balm et al. 2012) or CHIKV (Edwards et al. 2007). For the IgM ELISA evaluation, we defined confirmed CHIKV, DENV, and ZIKV cases based on a positive result in each arboviral-specific RT-PCR. We defined as non-arboviral AFI patients those presenting negative RT-PCR for all the three tested arboviruses.

Detection of CHIKV-specific IgM antibodies by ELISA

We tested the acute- and paired convalescent-phase sera available from all the CHIKV, DENV, and ZIKV RT-PCR-positive patients enrolled during surveillance with both the CHIKjj Detect IgM-capture ELISA kit (cat no. CHKM-R, Inbios International, Inc, Seattle, USA) and the Anti-Chikungunya virus ELISA (IgM) Test (cat no. EI 293a-9601 M, Euroimmun, Luebeck, Germany). We also applied the Inbios and the Euroimmun CHIKV IgM ELISA tests to the acute- and paired convalescent-phase sera from 175 patients randomly selected from those with negative RT-PCR results (for all 3 arboviruses) and with paired sera available. These random sample of 175 RT-PCR negative patients provided good precision for the specificity estimation (95% confidence for a precision of +/-4% and an anticipated specificity $\geq 90\%$).

Tests were performed according to the manufacturer's instructions. The ELISA reading was performed by automated microplate reader at 450nm (TECAN, Maennedorf, Switzerland). The optical density ratio obtained from the patients' serum and the calibrator was interpreted according to the manufacturer. Samples yielding equivocal/borderline ratio results were repeated once, and the second results were considered final.

Detection of CHIKV-specific IgG antibodies by ELISA

In order to further investigate whether individuals from the control groups (DENV, ZIKV, and negative patients) presenting IgM positive results by either tests truly represented false positive cases or actually represented individuals who had past CHIKV infections and retained a positive CHIKV IgM response, we used the CHIKjj Detect™ IgG ELISA Kit (cat no. CHKG-C, Inbios International, Inc, Seattle, USA) to test all available acute-phase samples from control patients who had a IgM positive result in either Inbios or Euroimmun IgM-ELISA in the acute- and/or convalescent-phase sera. We also applied the same CHIKV IgG test on the acute- and convalescent-phase sera of CHIKV RT-PCR positive cases that yielded a negative

IgM result in the convalescent-phase sample to investigate possible false positive results in the RT-PCR.

Data analysis.

Patients included in the study were described according to their demographic and clinical characteristics. The Wilcoxon-Mann-Whitney test or the Fisher's exact test were used to compare these characteristics between the patients with positive and negative RT-PCR result for CHIKV. Accuracy measures were calculated for both acute- and convalescent-phase samples using the CHIKV RT-PCR result as the reference test. In addition to the overall specificity of the ELISAs, we also calculated specificities by subgroups, according to the RT-PCR result for the other tested arboviruses: i) DENV-positive cases; ii) ZIKV-positive cases; and iii) negative for the three tested arboviruses. Confidence intervals of 95% (95% CI) were calculated for all accuracy measurements.

Ethic statement.

The Research Ethics Committee at Instituto Gonçalo Moniz, Fundação Oswaldo Cruz approved this study. All adult subjects provided written informed consent and participants <18 years of age who were able to read provided written assent following written consent from their parent or guardian. All study data were anonymized before analysis.

Results

The surveillance study enrolled 948 AFI patients with at least one available sample for laboratory testing. However, due to insufficient volumes of sera, RT-PCR for DENV and

CHIKV was performed for 915 patients (96.5%), while RT-PCR for ZIKV was performed for 914 patients (96.4%). Among the patients who underwent RT-PCR testing, 197 (21.5%) were positive for at least one of the arboviruses and the remaining 718 (78.5%) were negative for all three arboviruses. Specifically, mono-infections were detected for 184 (20.1%) cases: 139 (15.2%) of CHIKV, 32 (3.5%) of DENV, and 13 (1.4%) of ZIKV, whereas co-infections occurred for 13 (1.4%) cases: 12 (1.3%) CHIKV/DENV co-infections, and one (0.1%) DENV/ZIKV co-infection.

The 13 CHIKV/DENV co-infection cases were included among the RT-PCR-confirmed chikungunya cases during the sensitivity analyses, but not among the DENV cases during the specificity analyses. The DENV/ZIKV co-infection case was included among both the DENV and the ZIKV cases for the specificity analyses. Thus, based on the RT-PCR results, the final number of CHIKV-RT-PCR-confirmed cases used for the sensitivity analyses was 151 cases (139 CHIKV mono-infections plus 12 CHIKV co-infections with DENV). The final number of non-CHIKV cases used for the specificity analyses was 221 cases (32 DENV, 13 ZIKV, 1 DENV/ZIKV co-infection, and 175 randomly selected among the 718 AFI cases without RT-PCR evidence for an arboviral infection).

Arthralgia was more frequently reported by CHIKV cases (94.7%) compared to non-CHIKV cases (67.0%) ($p<0.001$), as were myalgia (95.4% vs 84.4%, $p=0.001$), and swollen joints (41.7% vs 24.0%, $p<0.001$) (Table 1). However, rash (25.3% vs 33.9%, $p=0.08$) and pruritus (16.6% vs 32.1%, $p=0.001$) were less frequent among CHIKV cases than non-CHIKV cases. CHIKV cases also sought medical assistance earlier, and thus had their acute-phase blood sample collected sooner than the non-CHIKV patients (median of one vs. three days post-onset of symptoms, respectively) ($p<0.001$).

Of the 635 samples undergoing the Inbios IgM ELISA testing (369 acute- and 266 convalescent-phase samples), 8 (1.3%) presented equivocal results (three CHIKV-RT-PCR-positive acute-phase samples, four CHIKV RT-PCR negative acute-phase samples, and one CHIKV RT-PCR positive convalescent-phase sample), and when retested produced a valid result. Of the 621 samples tested by the Euroimmun IgM ELISA (360 acute-phase and 261 convalescent-phase samples), 36 (5.8%) presented equivocal results (8 CHIKV RT-PCR positive acute-phase samples, 14 CHIKV RT-PCR negative acute-phase samples, three CHIKV-RT-PCR-positive convalescent-phase samples, and 11 CHIKV-RT-PCR-negative convalescent-phase samples). After retesting, an equivocal result remained for 9 (1.4%) of them (one CHIKV RT-PCR positive acute-phase sample, five CHIKV RT-PCR negative acute-phase sample, one CHIKV RT-PCR positive convalescent-phase sample, and two CHIKV-RT-PCR-negative convalescent-phase samples).

For acute-phase sera, the Inbios IgM-ELISA sensitivity was 4.0% (3.7% for samples obtained ≤ 3 days post-symptoms onset (DPSO) and 7.1% for samples obtained 4-7 DPSO) (Table 2). For convalescent-phase sera, the Inbios IgM-ELISA sensitivity was 92.4%. Considering a positive IgM-ELISA result in either the acute- or convalescent-phase sample, of the 66 patients with available paired samples, the combined Inbios test sensitivity was 92.4%. Considering a positive IgM-ELISA result only in the convalescent-phase samples for the 64 RT-PCR-CHIKV-confirmed patients who had a negative result in the Inbios IgM-ELISA applied to the acute-phase sample, we observed a seroconversion sensitivity of 92.2%. Sensitivity of the Euroimmun IgM-ELISA was 10.3% for acute-phase samples (9.2% and 21.4% for samples obtained within ≤ 3 and 4-7 DPSO, respectively), 96.9% for convalescent-phase samples, 96.9% for combined acute- and convalescent-phase samples, and 96.7% when assessing seroconversions (Table 2).

To ascertain potential reasons for occurrence of false negative results in the CHIKV IgM ELISA tests performed on the convalescent-phase samples of RT-PCR-confirmed CHIKV cases, we evaluated the CHIKV IgG status of the five cases whose convalescent-phase sera were IgM negative by either ELISA tests. We found that three cases presented IgG seroconversion, one was IgG-positive in both the acute- and convalescent-phase sera, and one was negative for both acute- and convalescent-phase sera.

Overall, considering all AFI patients with a negative CHIKV RT-PCR result, specificity for the Inbios IgM-ELISA was above 90% in both acute- (97.7%) and convalescent-phase samples (90.5%), whereas the specificity for the Euroimmun IgM-ELISA was 88.6% in acute- and 83.9% in convalescent-phase samples (Table 3). Among DENV cases, the Inbios and Euroimmun IgM-ELISA specificities were 83.9% and 82.8% for the acute-phase samples, and 88.9% and 83.3% for the convalescent-phase samples, respectively. Among ZIKV cases, specificities of the Inbios and Euroimmun IgM-ELISA were 92.9% and 83.3% for the acute-phase samples, and 87.5% and 87.5% for the convalescent-phase samples. In the RT-PCR-negative control group, the Inbios and Euroimmun specificities were 100.0% and 89.5% for acute-phase samples, and 90.3% and 83.3% for convalescent-phase samples, respectively.

To investigate whether maintenance of CHIKV IgM antibodies after a prior CHIKV infection could explain a positive IgM ELISA result among the CHIKV-RT-PCR-negative cases, we investigated the presence of CHIKV IgG in the sera of the 44 CHIKV RT-PCR negative cases that had a positive IgM ELISA result in the acute- or convalescent-phase sample by either tests. We found that 39 (88.6%) cases had negative results for the CHIKV IgG in the acute-phase sera, and thus did not have a prior CHIKV infection, while five (11.4%) cases had CHIKV IgG in their acute-phase sample.

Of these five cases with evidence of a prior CHIKV infection, the Inbios test returned a positive IgM result for three cases (one in both the acute- and convalescent-phase sera and

two in the acute-phase sera of cases that did not have paired sample). Excluding these three cases from the Inbios test evaluation, the overall Inbios specificities did not substantially differ (99.1% (214/216) for acute-phase sera and 91.0% (181/199) for convalescent-phase sera). All five cases with evidence of a prior CHIKV infection were IgM-positive by the Euroimmun test (two in both the acute- and convalescent-phase sera, one only in the acute-phase sample with a negative result in the convalescent-phase serum, and two in the acute-phase samples of cases that did not have paired sample). Excluding these 5 cases from the test evaluation, the overall Euroimmun specificities also did not substantially differ (90.7% (185/204) and 84.7% (161/190) for acute- and convalescent-phase sera, respectively).

Discussion

Our evaluation of the Inbios and the Euroimmun IgM ELISAs shows that, despite low sensitivities when applied to sera obtained within the first week of chikungunya symptoms onset, they performed well when convalescent-phase samples were used (sensitivity >92%). Overall, specificities were also high for Inbios (91-98%) and slightly lower for Euroimmun (84-89%).

The low sensitivity for both tests on acute-phase samples was expected, because all the confirmed chikungunya cases included in this evaluation were detected by RT-PCR and, thus, were still in the viremic phase of the illness, when IgM antibody presence is unlikely. Although the Euroimmun IgM-ELISA presented a higher sensitivity for acute-phase samples than the Inbios test (10% vs. 4%, respectively), it was still sub-optimal, reaching a maximum of 21.4% for samples obtained 4-7 days after symptoms' onset.

Despite the good specificities, positive IgM ELISA results for both tests occurred in 10-15% of the samples of CHIKV RT-PCR-negative cases, which can be explained in two ways. First, the reference diagnostic test (the CHIKV RT-PCR) may have failed to confirm

CHIKV infections in some of the study patients presenting low viremia; thus, it is possible that a fraction of the positive IgM ELISA results among CHIKV RT-PCR-negative patients actually represent true acute infections. Second, as Salvador experienced an outbreak of CHIKV during 2015 (Cardoso et al. 2015, 2017; Silva et al. 2018), it is also possible that some of the CHIKV-RT-PCR-negative patients included in our study experienced a recent CHIKV infection (1-3 months before study enrollment), and that CHIKV IgM antibodies persisted during our study. In that regard, our IgG detection analysis showed that prior CHIKV infection could only explain a small fraction of the false positive IgM ELISA results. However, in settings where CHIKV transmission is epidemic, a positive IgM result may represent a recent, rather than an acute infection. In such a context, efforts for early diagnosis by molecular methods should be encouraged to ensure accurate CHIKV detection. It is also important to highlight that cross-reactions with DENV or ZIKV infections are unlikely, because CHIKV is an alphavirus, while DENV and ZIKV are antigenically unrelated flaviviruses.

Previous evaluations demonstrated 100% sensitivity and 93-100% specificity for the Inbios IgM-ELISA, and 94-100% sensitivity and 96-100% specificity for the Euroimmun IgM-ELISA in a panel of serum samples from laboratory-confirmed CHIKV cases and a diverse set of controls (Johnson et al. 2016). Sensitivity and specificity of the Euroimmun IgM-ELISA were also reported to be 85% and 82%, respectively, in a panel of samples from reference laboratories, with cross-reactivity reported with o'nyong-nyong virus (Prat et al. 2014). Here, we evaluated the tests performance in a panel of samples obtained from febrile outpatients recruited in the context of intense arboviral co-circulation, reflecting a challenging but realistic scenario of test use. However, we did not include patients with other alphaviruses (such as Venezuelan equine encephalitis, Madariaga, and Mayaro viruses) among the control group, because there is no evidence of their transmission in this region of the country. A few sporadic or outbreak-associated human cases of these arboviral infections have occurred in other parts

(North and Central-West) of Brazil (Lopes et al. 2014; Vieira et al. 2015) and further studies should be performed to determine the specificity of CHIKV IgM ELISA tests in such settings where different alphaviruses co-circulate.

In summary, our findings indicate an overall good sensitivity of both tests for convalescent-phase serum samples. Conversely, sensitivity in acute-phase samples was low for both tests, as expected. These results reinforce the notion that sera collected during the first week after symptoms onset are better suited for testing by CHIKV RT-PCR, rather than by CHIKV serological assays. In addition, for subjects suspected of CHIKV infection who present a negative CHIKV IgM result from an acute-phase sample, clinical suspicion should not be discarded. Rather, a second serological test should be performed on a convalescent-phase sample. We also found that the Euroimmun IgM ELISA returned more equivocal results and presented slightly lower specificity than that found for the Inbios IgM ELISA, which may result in a higher rate of false-positive cases if the Euroimmun test is applied in low chikungunya prevalence scenarios.

Acknowledgements

We thank health professionals from the Centro de Emergência São Marcos, in Pau da Lima, Salvador, Brazil and all subjects of this study.

Conflict of interests

SCW holds a patent for antigen production used in the Inbios ELISA kits. Other authors have no conflict of interest.

Funding

This study was supported by the Brazilian National Council for Scientific and Technological Development (grants 400830/2013-2 and 440891/2016-7 to GSR; and scholarships to UK, MGR, and GSR); the Bahia Foundation for Research Support (grants PET0026/2013, APP0044/2016, and PET0022/2016 to GSR); the Coordination for the Improvement of Higher Education Personnel, Brazilian Ministry of Education (grant 88887.130746/2016-00 to GSR and scholarship to MK), the Oswaldo Cruz Foundation; the Federal University of Bahia; and the Department of Science and Technology, Secretariat of Science, Technology and Strategic Inputs, Brazilian Ministry of Health. The funding sources had no role in study design; in the collection, analysis and interpretation of data; in the writing of the paper; and in the decision to submit the article for publication.

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Table 1. Demographics, clinical characteristics, and serum sample availability for 372 acute febrile illness (AFI) patients included in the CHIKV IgM ELISA evaluation study, and according to RT-PCR result, Salvador, September 2014 to July 2016.

Characteristics		AFI patients according to RT-PCR result for CHIKV		CHIKV-negative patients according to RT-PCR result for DENV and ZIKV		
		CHIKV-Pos. (n: 151) ^a	CHIKV-Neg. (n=221)	DENV-Pos. ^b (n=33)	ZIKV-Pos. ^b (n=14)	Neg. for the three arboviruses (n=175)
Number with the finding/total with available data (%)						
Demographic						
	Age ^c	34 (22 – 45) ^d	28 (19 – 40) ^d	31 (15 – 40)	22.5 (15 – 41)	28 (20 – 40)
	Males	81/151 (53.6)	98/220 (44.6)	16/33 (48.5)	6/14 (42.9)	76/174 (43.7)
Clinical Manifestations						
	Myalgia	144/151 (95.4) ^d	184/218 (84.4) ^d	26/32 (81.3)	12/14 (85.7)	147/173 (85.0)
	Arthralgia	143/151 (94.7) ^d	148/221 (67.0) ^d	21/33 (63.6)	8/14 (57.1)	120/175 (68.6)
	Polyarthralgia	136/143 (95.1)	145/148 (98.0)	21/21 (100)	8/8 (100)	117/120 (97.5)
	Symmetric	128/143 (89.5)	142/145 (97.9)	21/21 (100)	8/8 (100)	114/117 (97.4)
	Headache	141/151 (93.4)	203/219 (92.7)	30/32 (93.8)	13/14 (92.9)	161/174 (92.5)
	Retro-orbital pain	107/150 (71.3)	155/218 (71.1)	22/32 (68.8)	10/14 (71.4)	124/173 (71.7)
	Swollen joints	63/151 (41.7) ^d	53/221 (24.0) ^d	12/33 (36.4)	5/14 (35.7)	37/175 (21.1)
	Vomit	35/151 (23.2)	59/221 (26.7)	10/33 (30.3)	2/14 (14.3)	48/175 (27.4)
	Rash	38/150 (25.3)	75/221 (33.9)	14/33 (42.4)	10/14 (71.4)	52/175 (29.7)
	Pruritus	25/151 (16.6) ^d	71/221 (32.1) ^d	12/33 (36.4)	10/14 (71.4)	50/175 (28.6)
Blood sample collection						
	Acute-phase sample	151/151 (100)	221/221 (100)	33/33 (100)	14/14 (100)	175/175 (100.0)
	Time (days) between symptoms onset and sample collection ^c	1 (1 – 2) ^d	3 (2 – 4) ^d	4 (3 – 6)	2.5 (2 – 4)	3 (1 - 4)
	Convalescent-phase sample	67/151 (44.4) ^d	200/221 (90.5) ^d	18/33 (54.6)	8/14 (57.1)	175/175 (100.0)
	Time (days) between symptoms onset and sample collection ^c	19 (14 – 33)	24 (16 – 38)	21 (16 – 42)	27 (18.5 – 43.5)	23.5 (16 – 38)

Abbreviations: AFI = acute febrile illness; RT-PCR: reverse transcription polymerase chain reaction; CHIKV = chikungunya virus; Pos. = positive; Neg. = Negative.

^a Of the 151 CHIKV positive patients, 139 had a CHIKV mono-infection and 12 had a CHIKV and DENV co-infection. These 12 CHIKV and DENV co-infected patients were only included in the group of CHIKV positive patients (and not in the group of DENV positive patients).

^b One patient was simultaneously positive for DENV and ZIKV and was included in both groups.

^c Median (interquartile range)

^d P -value < 0.05 by Wilcoxon-Mann-Whitney or Fisher exact for comparisons between CHIKV positive and CHIKV negative patients.

Table 2. Sensitivity of the Inbios and Euroimmun chikungunya virus IgM-ELISAs in the acute- and convalescent-phase sera, and according to seroconversions.

Sensitivity	Of the Inbios CHIKV IgM-ELISA			Of the Euroimmun CHIKV IgM-ELISA		
	CHIKV IgM ELISA-pos.	CHIKV RT-PCR-pos.	Sensitivity % (95% CI)	CHIKV IgM ELISA-pos.	CHIKV RT-PCR-pos.	Sensitivity % (95% CI)
In acute sample ^a	6	150	4.0% (1.5 - 8.5)	15	145	10.3% (5.9 – 16.5)
≤3 DPOS	5	136	3.7% (1.2 - 8.4)	12	131	9.2% (4.8 – 15.5)
0-1 DPOS	1	80	1.3% (0.0 – 6.8)	4	78	5.1% (1.4 – 12.6)
2-3 DPOS	4	56	7.1% (2.0 – 17.3)	8	53	15.1% (6.8 – 27.6)
4-7 DPOS	1	14	7.1% (0.2 – 33.9)	3	14	21.4% (4.7 – 50.8)
In convalescent sample ^b	61	66	92.4% (83.2 - 97.5)	63	65	96.9% (89.3 – 99.6)
<30 DPOS ^c	44	46	95.7% (85.2 – 99.5)	44	46	95.7% (85.2 – 99.5)
≤14 DPOS	20	20	100.0% (83.2 – 100.0)	20	20	100.0% (83.2 – 100.0)
15-29 DPOS	24	26	92.3%(74.9 – 99.0)	24	26	92.3% (74.9 – 99.1)
≥30 DPOS ^d	17	20	85.0% (62.1 – 96.8)	19	19	100.0% (82.4 – 100.0)
Combined						
Positivity in acute or in convalescent paired samples ^b	61	66	92.4% (83.2 – 97.5)	63	65	96.9% (89.3 – 99.6)
Seroconversion between paired samples ^e	59	64	92.2% (82.7 - 97.4)	58	60	96.7% (88.5 – 99.6)

Abbreviations: CHIKV = chikungunya virus; RT-PCR: reverse transcription polymerase chain reaction; Pos. = positive; CI= confidence interval; DPOS = days post onset of symptoms.

^a Of the 151 CHIKV RT-PCR positive cases enrolled, sera samples were available for testing by the Inbios IgM-ELISA for 150 cases (138 mono-infected patients and 12 CHIKV and DENV co-infected patients), and for testing by the Euroimmun IgM-ELISA for 146

cases (138 mono-infected patients and 8 CHIKV and DENV co-infected patients), of which one mono-infected yielded an equivocal result and was excluded from the analysis.

^b Of the 67 CHIKV RT-PCR positive cases enrolled for whom a convalescent sera was collected, convalescent sera samples were available for testing by the Inbios IgM-ELISA for 66 cases (64 mono-infected patients and 2 CHIKV and DENV co-infected patients), and for testing by the Euroimmun IgM-ELISA for 66 cases (64 mono-infected patients and 2 CHIKV and DENV co-infected patients), of which one mono-infected yielded an equivocal result and was excluded from the analysis.

^c Minimum of 9 days post onset of symptoms.

^d Maximum of 70 days post onset of symptoms.

^e Seroconversion was evaluated only for the RT-PCR-positive CHIKV patients with available paired sample whose acute-phase sample was negative on the IgM-ELISA.

Table 3. Specificity of the Inbios and Euroimmun chikungunya virus IgM-ELISAs in the acute- and convalescent-phase sera, and according to other arboviral diagnosis.

Specificity	Of the Inbios CHIKV IgM-ELISA			Of the Euroimmun CHIKV IgM-ELISA		
	CHIKV IgM ELISA-neg.	CHIKV RT-PCR neg.	Specificity % (95% CI)	CHIKV IgM ELISA-neg.	CHIKV RT-PCR neg.	Specificity % (95% CI)
In acute sample ^a						
Non-CHIKV AFI patients	214	219	97.7% (94.8 – 99.3)	185	209	88.5% (83.4 – 92.5)
DENV cases	26	31	83.9% (66.3 – 94.6)	24	29	82.8% (64.2 – 94.2)
ZIKV cases	13	14	92.9% (66.1 – 99.8)	10	12	83.3% (51.6 – 97.9)
AFI patients negative for the 3 arboviruses	175	175	100.0% (97.9 – 100.0)	153	171	89.5% (83.9 – 93.6)
In convalescent sample ^b						
Non-CHIKV AFI patients	181	200	90.5% (85.6 – 94.2)	162	193	83.9% (78.0 – 88.8)
DENV cases	16	18	88.9% (65.3 – 98.6)	15	18	83.3% (58.6 – 96.4)
ZIKV cases	7	8	87.5% (43.4 – 99.7)	7	8	87.5% (47.4 – 99.7)
AFI patients negative for the 3 arboviruses	158	175	90.3% (84.9 – 94.2)	140	168	83.3% (76.8 – 88.6)

AFI = CHIKV = chikungunya virus; RT-PCR: reverse transcription polymerase chain reaction; Neg. = negative; CI= confidence interval; acute febrile illness.

^a Of the 221 CHIKV RT-PCR negative cases included in this evaluation study, acute-phase serum samples were available for testing by the Inbios IgM-ELISA for 219 cases, including 30 patients with a DENV mono-infection, 13 patients with a ZIKV mono-infection, 1 patient with a DENV and ZIKV co-infection (included in both DENV and ZIKV groups), and 175 patients with negative RT-PCR results for CHIKV, DENV, and ZIKV. Acute-phase sera were available for testing by the Euroimmun IgM-ELISA for 214 cases, including 28 patients with a DENV mono-infection, 11 patients with a ZIKV mono-infection, 1 patient with a DENV and ZIKV co-infection (included

in both DENV and ZIKV groups), and 174 patients with negative RT-PCR results for CHIKV, DENV, and ZIKV (5 of which yielded an equivocal result and were excluded from the analysis).

^b Of the 221 CHIKV RT-PCR-negative cases included in this evaluation study, convalescent-phase samples were available for testing by the Inbios IgM-ELISA for 200 cases, including 17 patients with a DENV mono-infection, 7 patients with a ZIKV mono-infection, 1 patient with a DENV and ZIKV co-infection (included in both DENV and ZIKV groups), and 175 patients with negative RT-PCR results for CHIKV, DENV, and ZIKV. Convalescent-phase serum samples were available for testing by the Euroimmun IgM-ELISA for 195 cases, including 17 patients with a DENV mono-infection, 7 patients with a ZIKV mono-infection, 1 patient with a DENV and ZIKV co-infection, and 170 patients with negative RT-PCR results for CHIKV, DENV, and ZIKV (2 of which yielded an equivocal result and were excluded from the analysis).