



# Evaluation of Euroimmun Anti-Zika Virus IgM and IgG Enzyme-Linked Immunosorbent Assays for Zika Virus Serologic Testing

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**ABSTRACT** With the emerging Zika virus (ZIKV) epidemic, serologic diagnosis relies on a labor-intensive IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) and confirmation by a plaque reduction neutralization test (PRNT). To streamline serologic testing, several commercial assays have been developed. Our aim was to compare the commercial Euroimmun anti-ZIKV IgM and IgG assays to the reference MAC-ELISA and PRNT currently in use. Serum specimens submitted to Public Health Ontario Laboratory, Canada, were tested for IgM and IgG using the Euroimmun assays and the results were compared with those from MAC-ELISA. The PRNT was performed on positive or equivocal specimens using either MAC-ELISA or Euroimmun assays, MAC-ELISA-inconclusive specimens, and a convenience sample of specimens negative by both assays (cohort 1). Another set of specimens selected on the basis of PRNT results was subsequently tested by the Euroimmun assays (cohort 2). MAC-ELISA was positive, equivocal, negative, and inconclusive in 57/223, 15/223, 147/223, and 4/223 specimens, respectively. Among the 76 specimens that were MAC-ELISA positive, equivocal, or inconclusive, 30 (39.5%) were Euroimmun IgM and/or IgG positive or equivocal. Among the 147 MAC-ELISA-negative specimens, 136 (92.5%) were Euroimmun IgM and IgG negative. The sensitivity of the combined Euroimmun IgM/IgG against the PRNT was 83% (cohort 1) and 92% (cohort 2), whereas the specificity was 81% (cohort 1) and 65% (cohort 2). The combined Euroimmun IgM/IgG showed good specificity (92.5%) but suboptimal sensitivity (39.5%) compared with that of the MAC-ELISA. However, the sensitivity of the combined Euroimmun IgM/IgG against the PRNT was significantly higher (83 to 92%). More studies are needed before commercial assays are implemented for routine ZIKV serologic diagnosis.

**KEYWORDS** Zika virus, diagnostic testing, epidemic, flavivirus, serology

Zika virus (ZIKV) is a single-stranded positive-sense RNA virus in the genus *Flavivirus* (1), which, similar to dengue virus (DENV) and chikungunya and yellow fever viruses, is transmitted by the bite of *Aedes* mosquitoes. After its discovery in Uganda in 1947, ZIKV circulated at low levels in Asia and Africa until 2007, when it caused a febrile outbreak with conjunctivitis, rash, and arthralgia in Yap, Micronesia (2). ZIKV continued to spread eastward among the South Pacific Islands (3–5), before reaching South America at the end of 2014 (6). Since then, active ZIKV transmission has been reported

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all over the Americas, especially in South and Central America, with millions of persons likely infected.

ZIKV laboratory diagnosis relies on reverse-transcription PCR (RT-PCR) and serology testing during the acute phase of ZIKV infection. IgM antibodies become detectable by the end of the first week after symptom onset, quickly followed by an increase in neutralizing antibodies, mainly consisting of IgG antibodies (7, 8). For all patients fulfilling ZIKV testing criteria, including symptomatic pregnant women, the U.S. Center for Diseases Control and Prevention (CDC) currently recommends IgM testing on specimens collected 2 to 12 weeks after symptom onset, and within 14 days of symptom onset if RT-PCR is negative (9, 10). For asymptomatic pregnant women, if specimens can be collected within 14 days after a potential exposure, RT-PCR should be performed. If RT-PCR is negative, and in other asymptomatic pregnant women who present more than 14 days after potential exposure, IgM testing should be performed 2 to 12 weeks after a potential exposure (10).

Cross-reactivity between flaviviruses, such as between DENV and ZIKV, is well documented and can lead to misdiagnosis (7, 11, 12). The current reference gold standard is the Food and Drug Administration (FDA) emergency-use-authorized (EUA) CDC-developed IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) (13). The plaque reduction neutralization test (PRNT), which detects virus-specific neutralizing antibodies, is required to confirm a positive MAC-ELISA serology result and to confirm or rule out equivocal or inconclusive results that were not resolved after retesting using MAC-ELISA (14).

Responding to the current emerging ZIKV epidemic requires a streamlined and sensitive serologic testing platform—the MAC-ELISA is labor intensive and is only available at certain reference laboratories, significantly increasing turnaround time. Several commercial assays have been developed, such as the Euroimmun anti-ZIKV ELISA IgM and IgG assays (Euroimmun, Lübeck, Germany). The primary aim of this study was to compare the commercial Euroimmun anti-ZIKV ELISA IgM and IgG assays to the MAC-ELISA and PRNT currently in use. The secondary aim of the study was to evaluate serologic profiles of patients with RT-PCR-confirmed ZIKV infection.

## RESULTS

**Cohort 1. (i) MAC-ELISA, Euroimmun, and PRNT results.** Two hundred twenty-three serum specimens from 213 patients were tested by the MAC-ELISA and Euroimmun IgM and Euroimmun IgG assays during the study period.

The median age of the patients was 34.9 years (interquartile range [IQR], 30.4 to 52.5 years). Seventy-eight percent (166/213) of the patients were female, and 52.5% (77/147) of the female patients for which the information was available were pregnant. Among the 155 symptomatic patients, the date of symptom onset was available for 117, for which the median time between symptom onset and specimen collection was 4 days (IQR, 2.0 to 10.5 days). Among the symptomatic patients with either positive, equivocal, or inconclusive MAC-ELISA specimens, the median time between symptom onset and specimen collection was 6.5 days (IQR, 2.3 to 10.0 days; range, 1.0 to 58.0 days).

Among the 223 specimens selected for this study, the MAC-ELISA was positive in 57 (25.6%), equivocal in 15 (6.7%), inconclusive in 4 (1.8%), and negative in 147 (65.9%) (Table 1).

Among the 57 MAC-ELISA-positive specimens, the Euroimmun IgM assay was positive in 15 (26.3%), equivocal in 5 (8.8%), and negative in 37 (64.9%), whereas the Euroimmun IgG assay was positive in 15 (26.3%), equivocal in 2 (3.5%), and negative in 40 (70.2%) (Table 1). Altogether, 49.1% (28/57) of MAC-ELISA-positive specimens were Euroimmun IgM and/or IgG positive or equivocal. Among the 15 MAC-ELISA-equivocal specimens, the Euroimmun IgM assay was positive in 2 (13.3%) and negative in 13 (86.7%), whereas the Euroimmun IgG assay was positive in 1 (6.7%) and negative in 14 (93.3%) (Table 1). Euroimmun IgM and IgG assays were negative in all 4 MAC-ELISA-inconclusive specimens (Table 1).

**TABLE 1** Distribution of specimens according to MAC-ELISA and Euroimmun IgM and IgG assay results and associated sensitivity and specificity for cohort 1<sup>a</sup>

Assay	Result	No. of specimens with corresponding MAC-ELISA result:				% Sensitivity <sup>b</sup>		% Specificity <sup>c</sup>	
		Positive (n = 57)	Equivocal (n = 15)	Inconclusive (n = 4)	Negative (n = 147)	% (no./total no.)	95% CI	% (no./total no.)	95% CI
Euroimmun IgM	Positive	15	2	0	1	28.9 (22/76)	18.8–39.1	96.6 (142/147)	93.7–99.5
	Equivocal	5	0	0	4				
	Negative	37	13	4	142				
Euroimmun IgG	Positive	15	1	0	3	23.7 (18/76)	14.1–33.2	95.2 (140/147)	91.8–98.7
	Equivocal	2	0	0	4				
	Negative	40	14	4	140				
Combined Euroimmun IgM/IgG	IgM and/or IgG positive or equivocal	28	2	0	11	39.5 (30/76)	28.5–50.5	92.5 (136/147)	88.3–96.8
	IgM and IgG Negative	29	13	4	136				

<sup>a</sup>A total of 223 specimens were tested. Sensitivity and specificity of the Euroimmun IgM, IgG, and combined IgM/IgG assays were evaluated against the reference MAC-ELISA; MAC-ELISA, IgM antibody capture enzyme-linked immunosorbent assay; CI, confidence interval.

<sup>b</sup>Sensitivity comparing MAC-ELISA-positive, -equivocal, and -inconclusive specimens to Euroimmun IgM- and/or IgG-positive or -equivocal specimens.

<sup>c</sup>Specificity comparing MAC-ELISA-negative specimens to Euroimmun IgM- and IgG-negative specimens.

Among the 76 specimens either positive, equivocal, or inconclusive by the MAC-ELISA, the Euroimmun IgM assay was positive or equivocal in 22 (28.9%) and the Euroimmun IgG assay was positive or equivocal in 18 (23.7%); overall, 30 specimens (39.5%) were Euroimmun IgM and/or IgG positive or equivocal (Table 1).

Among the 147 MAC-ELISA-negative specimens, the Euroimmun IgM assay was positive in 1 (0.7%), equivocal in 4 (2.7%), and negative in 142 (96.6%), whereas the Euroimmun IgG assay was positive in 3 (2.0%), equivocal in 4 (2.7%), and negative in 140 (95.2%) (Table 1). Altogether, 7.5% (11/147) of MAC-ELISA-negative specimens were Euroimmun IgM and/or IgG positive or equivocal.

Of the 223 specimens, 41 (18.4%) were Euroimmun IgM and/or IgG positive or equivocal. The MAC-ELISA was positive or equivocal in 30 (73.2%) and negative in 11 (26.8%) of these 41 specimens (Table 1).

Overall, 117 (52.5%) of the cohort 1 specimens were tested by the PRNT (Table 2). Eighty-five (72.6%) specimens were negative; of those, 16 (18.8%) were Euroimmun IgM and/or IgG positive or equivocal and 69 (81.2%) were Euroimmun IgM and IgG negative (Table 2), whereas 40 (47.1%) were MAC-ELISA negative (Table 2). Twenty-four (20.5%) specimens showed evidence of recent ZIKV ( $n = 12$ ) or flavivirus ( $n = 12$ ) infection by the PRNT, of which 20 (83.3%) were either Euroimmun IgM and/or IgG positive or equivocal, whereas 24 (100%) were MAC-ELISA positive or equivocal (Table 2). Among patients with a PRNT showing recent ZIKV infection, the Euroimmun IgM assay was positive or equivocal in 9/12 (75%) and the IgG assay was positive or equivocal in 6/12 (50%); combined, Euroimmun IgM and/or IgG assays were positive or equivocal in 10/12 (83.3%). The PRNT values are detailed in Table S1 in the supplemental material.

Among the 8 specimens with PRNTs showing recent DENV infection, five were both Euroimmun IgM and IgG negative, two were IgM equivocal, and two were IgG equivocal; none were IgM or IgG positive. In comparison, only 1/8 specimens was MAC-ELISA negative (Table 2).

**(ii) Seropositivity among RT-PCR-positive specimens.** Sixty-six of 223 (29.6%) serum specimens tested positive by any one of the RT-PCR methods (reference PCR, Altona PCR, or NS5 PCR). Among the RT-PCR-positive specimens, the median time between the symptom onset and specimen collection was 3.0 days (IQR, 2.0 to 4.0 days). The Euroimmun IgM assay was positive or equivocal in 13 (19.7%), whereas the IgG assay was positive or equivocal in 8 (12.1%); the combined Euroimmun IgM/IgG assays were positive or equivocal in 20 (30.3%). Among these 66 RT-PCR-positive specimens, MAC-ELISA was positive, equivocal, or inconclusive in 28 (42.4%).

Among the 46 RT-PCR-positive specimens collected between day 0 and 4 after symptom onset, the MAC-ELISA was positive, equivocal, or inconclusive in 18, whereas the Euroimmun IgM, Euroimmun IgG, and combined Euroimmun IgM/IgG assays were positive or equivocal in 8, 5, and 12 specimens, respectively. Among the 8 RT-PCR-positive specimens collected between day 5 and 10 after symptom onset, the MAC-ELISA was positive, equivocal, or inconclusive in 5, whereas the Euroimmun IgM, Euroimmun IgG, and combined Euroimmun IgM/IgG assays were positive or equivocal in 2, 2, and 4 specimens, respectively.

**(iii) Analytical sensitivity and specificity.** Compared with those of the MAC-ELISA, the sensitivity and specificity of the Euroimmun IgM assay were 28.9% ([22/76] 95% confidence interval [CI], 18.8% to 39.1%) and 96.6% ([142/147] 95% CI, 93.7% to 99.5%), respectively, whereas the sensitivity and specificity of the Euroimmun IgG assay were 23.7% ([18/76] 95% CI, 14.1% to 33.2%) and 95.2% ([140/147] 95% CI, 91.8% to 98.7%), respectively (Table 1). The combined Euroimmun IgM/IgG assays' sensitivity and specificity against the MAC-ELISA were 39.5% ([30/76] 95% CI, 28.5% to 50.5%) and 92.5% ([136/147] 95% CI, 88.3% to 96.8%), respectively (Table 1).

The sensitivity of the combined Euroimmun IgM/IgG assays against specimens showing recent ZIKV infection by the PRNT was 83.3% ([10/12] 95% CI, 62.2% to 100%). The combined Euroimmun IgM/IgG assays' specificity against the PRNT (PRNT <10 for both ZIKV and DENV) was 81.2% ([69/85] 95% CI, 72.9% to 89.5%) (Table 2). As a

**TABLE 2** Distribution of specimens according to PRNT results and associated sensitivity and specificity for cohort 1<sup>a</sup>

Assay	Results	No. of specimens with corresponding PRNT result for recent infection:					% Sensitivity			% Specificity		
		ZIKV (n = 12)	Flavivirus <sup>b</sup> (n = 12)	DENV (n = 8)	Negative (n = 85)	ZIKV	ZIKV	ZIKV or flavivirus	Negative	ZIKV	ZIKV or flavivirus	Negative
		(n = 12)	(n = 12)	(n = 8)	(n = 85)	% (no./total no.)	% (no./total no.)	% (no./total no.)	% (no./total no.)	% (no./total no.)	% (no./total no.)	% (no./total no.)
Combined Euroimmun IgM/IgG	IgM and/or IgG positive or equivocal	10	10	3	16	83.3 (10/12)	83.3 (20/24)	62.2–100	81.2 (69/85)	68.4–98.2	72.9–89.5	
	IgM and IgG negative	2	2	5	69							
MAC-ELISA	Positive or equivocal or inconclusive	12	12	7	45	100 (12/12)	100 (24/24)	100–100	47.1 (40/85)	100–100	36.4–57.7	
	Negative	0	0	1	40							

<sup>a</sup>A total of 117 specimens for cohort 1 were tested. Sensitivity and specificity of the combined Euroimmun IgM/IgG assays and MAC-ELISA were evaluated against the PRNT. MAC-ELISA, IgM antibody capture enzyme-linked immunosorbent assay; PRNT, plaque reduction neutralization test; ZIKV, Zika virus; DENV, Dengue virus.

<sup>b</sup>A recent flavivirus infection was confirmed when both ZIKV and DENV PRNT were ≥10.

comparison, the sensitivity and specificity of the MAC-ELISA against the PRNT were 100% ([12/12] 95% CI, 100% to 100%) and 47.1% ([40/85] 95% CI, 36.4% to 57.7%), respectively. When the PRNT results showing either recent ZIKV or recent flavivirus infection by the PRNT were taken together, the sensitivity of the combined Euroimmun IgM/IgG assays was similar at 83.3% ([20/24] 95% CI, 68.4% to 98.2%), as was the MAC-ELISA's sensitivity ([24/24] 95% CI, 100% to 100%) (Table 2).

Among the 8 patients with PRNT results showing recent DENV infection, the Euroimmun IgM and IgG assays were negative in 5/8 (62.5%) (Table 2); the three remaining patients had Euroimmun IgM/IgG results that were either equivocal/equivocal ( $n = 1$ ), equivocal/negative ( $n = 1$ ), or negative/equivocal ( $n = 1$ ). As a comparison, the MAC-ELISA was negative in 1/8 (12.5%) patients, the remaining being either positive ( $n = 4$ ), equivocal ( $n = 2$ ), or inconclusive ( $n = 1$ ) (Table 2).

**Cohort 2. (i) Euroimmun and PRNT results.** One hundred twenty-four additional specimens selected on the basis of their PRNT results were retested by the Euroimmun assays. Among those, the PRNT showed recent ZIKV infection in 26 (21.0%), recent flavivirus infection in 45 (36.3%), and recent DENV infection in 13 (10.5%); the PRNT was negative in 40 (32.3%). The PRNT values are detailed in Table S1.

Among the 13 specimens with PRNT results showing recent DENV infection, only two were both Euroimmun IgM and IgG negative; five were reactive in the IgM assay (3 positive and 2 equivocal), whereas eight were reactive in the IgG assay (7 positive and 1 equivocal).

**(ii) Analytical sensitivity and specificity.** The sensitivity of the combined Euroimmun IgM/IgG assays against specimens showing recent ZIKV infection by the PRNT was 92.3% ([24/26] 95% CI, 82.1% to 100%); the combined Euroimmun IgM/IgG assays' specificity against the PRNT (PRNT <10 for both ZIKV and DENV) was 65.0% ([26/40] 95% CI, 50.2% to 79.8%). When PRNT results showing either recent ZIKV or recent flavivirus infection were taken together, the sensitivity of combined the Euroimmun IgM/IgG assays was 97.2% ([69/71] 95% CI, 93.3% to 100%).

## DISCUSSION

The diagnosis of ZIKV infection using serologic assays is particularly challenging because of the cross-reactivity with other flaviviruses (7, 11, 12). This study compares the commercial Euroimmun IgM and IgG assays to the reference MAC-ELISA in 223 clinical serum specimens. The high proportion of female patients in our cohort is related to ZIKV testing selection criteria in Canada, where asymptomatic patients were accepted only if they were pregnant.

The sensitivities of the Euroimmun IgM and IgG assays against the MAC-ELISA were suboptimal at 29.8% and 23.7%, respectively. When combined, the Euroimmun IgM/IgG assays' sensitivity increased to 39.5%. This is significantly lower than previously reported. The only other study to compare these two assays evaluated only the Euroimmun IgM assay and showed 100% sensitivity among 25 MAC-ELISA-positive specimens (15). Similarly, in the manufacturer's kit insert, combined IgM/IgG sensitivity was also reported as 100% among the 29 ZIKV-positive specimens tested, although the assay against which the Euroimmun assay was evaluated was not mentioned (16). When evaluated against a nonreference indirect immunofluorescent assay (IIFA) serology, the Euroimmun IgM assay's sensitivity was 100% in one study (17), whereas the combined IgM/IgG assays' sensitivity was 91.8% in another study (18).

The sensitivity of the combined Euroimmun IgM/IgG assays against the PRNT ranged between 83% (cohort 1) and 92% (cohort 2). As a comparison, the MAC-ELISA's sensitivity against the PRNT was 100%. This difference might be related to our PRNT clinical testing algorithm, as most specimens are usually only tested by the PRNT if the MAC-ELISA is positive, equivocal, or inconclusive. On the other hand, the presumed lower sensitivity of the Euroimmun assays against the MAC-ELISA might be due to MAC-ELISA-false-positive specimens; in this case, the Euroimmun assay's sensitivity against the PRNT would be more reflective of the true sensitivity of the assay. Interestingly, in cohort 2, the Euroimmun assays' sensitivity was better among specimens

showing recent flavivirus infection (100%) than among specimens showing recent ZIKV infection (92%). The role of sequential exposures to different flaviviruses needs to be studied in more detail to better understand the Euroimmun assay performance. On the other hand, the specificity of the Euroimmun assays against the MAC-ELISA was excellent, reaching 96.6% for the IgM assay, 95.2% for the IgG assay, and 92.5% for the combined IgM/IgG assays. This is in line with results from other studies, including the only other study that compared the Euroimmun IgM assay to the reference MAC-ELISA, which showed a specificity of 100% among the 25 tested specimens (15). Similarly, the combined Euroimmun IgM/IgG assays' specificity was 100% among the 100 ZIKV-negative specimens tested in the manufacturer's documentation, albeit the method against which the Euroimmun assay was evaluated was not provided (16). In another cohort of more than 1,000 healthy subjects, the Euroimmun IgM and IgG assays were positive in only 2 (0.2%) and 2 patients (0.2%), respectively, confirming the high specificity of the assays (16, 18).

Compared to the PRNT, the specificity of the combined Euroimmun IgM/IgG assays ranged between 65% (cohort 2) and 81% (cohort 1). The MAC-ELISA's specificity against the PRNT was significantly lower at 47.1%. These data show that the MAC-ELISA results in an excessive amount of confirmatory testing by the PRNT. Additional PRNTs on specimens regardless of their MAC-ELISA result are needed to more thoroughly evaluate the test performance of the MAC-ELISA against the PRNT.

Cross-reactivity of serologic assays for the diagnosis of flavivirus infection is well documented (7, 11, 12) and explains why the CDC recommends the PRNT to confirm MAC-ELISA-positive, -equivocal, and -inconclusive results (14). As a 4-fold higher titer by the PRNT might not discriminate between anti-ZIKV antibodies and cross-reacting antibodies in patients previously exposed to flavivirus, the CDC changed the PRNT interpretation criteria in May 2016 to be more conservative; therefore, patients with PRNTs positive for both ZIKV and DENV are labeled as "recent flavivirus exposure," regardless of the PRNT values (14). As per the Euroimmun manufacturer's kit insert, there can be some cross-reactivity with other flaviviruses, including DENV (16). Indeed, among our 2 cohorts, only 7/21 specimens with recent DENV infection documented by the PRNT were Euroimmun IgM and IgG negative. As a comparison, the MAC-ELISA was negative in only 2/19 specimens with confirmed recent DENV infection using the PRNT, suggesting a slightly better specificity of the Euroimmun assays compared with that of the MAC-ELISA. Granger et al. recently reported false-positive Euroimmun IgM and IgG assay results in 6.2% and 30% of DENV-positive specimens, respectively (15). On the other hand, Huzly et al. evaluated the Euroimmun IgM and IgG assays among patients with documented exposure to various flaviviruses and did not show any cross-reactivity, including among the 26 DENV-positive specimens (17). Another group evaluated the Euroimmun assays in 252 patients with various infections, including flaviviruses, and the Euroimmun IgM and IgG assays were positive in only 0.8% and 0.4%, respectively; none of the 93 DENV-confirmed cases had positive Euroimmun ZIKV IgM or IgG (18). The differences in cross-reactivity among the studies are probably multifactorial. First, none of these studies diagnosed DENV infection using the gold standard PRNT, but used commercial assays instead (17, 18). Moreover, in the last study, the Euroimmun IgM- and IgG-equivocal specimens were removed from analysis, and this would likely overestimate specificity (18). One of the limitations of our study is that other flaviviruses were not systematically evaluated; this would have provided a better understanding of the Euroimmun assays' potential cross-reactivity.

In conclusion, this is the first study comparing the Euroimmun IgM and IgG assays to the reference MAC-ELISA in a large number of clinical specimens. Our data show that the combined Euroimmun IgM/IgG assays' specificity is very good, and better than that of the MAC-ELISA, which seems to be frequently falsely positive. However, cross-reactivity of the Euroimmun IgM and IgG assays seems to be more frequent than previously reported, especially with DENV. On the other hand, the sensitivity of the Euroimmun assays, including the combined IgM/IgG assays, appears to be lower than that of the MAC-ELISA to detect PRNT-positive cases showing either recent ZIKV or

recent flavivirus infection. Interestingly, the Euroimmun assays seem to perform better among specimens showing recent flavivirus infection than for specimens showing recent ZIKV infection.

For optimal utility, screening tests should have high sensitivity and, in the current setting of ZIKV testing, should be used for the detection of specimens requiring PRNT confirmation. This study does not currently provide sufficient data to recommend routine use of the Euroimmun IgM and IgG assays instead of MAC-ELISA screening followed by PRNT confirmation. However, it could be considered in nonpregnant patients if those data can be reproduced. In the future, a well-performing IgG assay could potentially replace the PRNT. More studies are needed to better evaluate these commercial assays against MAC-ELISA and PRNT together in a large set of clinical specimens. Additionally, longitudinal serologic studies with ZIKV PCR-confirmed cases would allow for a better understanding of IgM and IgG kinetics over time.

## MATERIALS AND METHODS

**Definitions.** The following definitions were used for analysis: reference PCR, dual target ZIKV RT-PCR reference assay designed by the CDC; Altona PCR, Altona Diagnostics RealStar ZIKV RT-PCR test kit 1.0 (Altona Diagnostics GmbH, Hamburg, Germany); NS5 PCR, in-house ZIKV RT-PCR targeting a 191-bp internal region of the NS5 gene; MAC-ELISA, FDA emergency-use-authorized CDC-designed IgM antibody capture ELISA; Euroimmun assays, Euroimmun anti-ZIKV ELISA IgM and IgG assays (Euroimmun, Lübeck, Germany).

**Study setting and specimen selection.** The study was conducted at Public Health Ontario Laboratory (PHOL), Ontario's reference microbiology laboratory, and Canada's National Microbiology Laboratory (NML) in Winnipeg, Manitoba. In Ontario, all specimens meeting testing guidelines for ZIKV serology testing are received at PHOL. Clinical criteria for serology testing are publicly available on the PHOL website (19). Euroimmun assays were performed at PHOL, whereas MAC-ELISAs and PRNTs were performed at NML; RT-PCR was performed at PHOL and/or NML. All specimens submitted to PHOL and NML for testing were handled according to strict standard operating procedures. In brief, specimens were shipped to the NML on cold packs and upon receipt, sample information was entered into the NML laboratory information management system. Serum specimens were stored at 4°C prior to testing and then stored at -20°C upon the completion of testing to avoid repeated freeze-thaw cycles.

For cohort 1, 223 specimens were selected on the basis of their MAC-ELISA results and were retested by the Euroimmun IgM and IgG assays. The PRNT was performed on all specimens that were positive, equivocal, or inconclusive using the MAC-ELISA, regardless of the Euroimmun IgM and IgG results. The PRNT was also performed on all specimens either positive or equivocal by the Euroimmun IgM or IgG assay, regardless of the MAC-ELISA results. Moreover, the PRNT was performed in a convenience sample of specimens that were negative using both the MAC-ELISA and Euroimmun assays. There was no intention to perform discrepant analysis.

For cohort 2, 124 specimens were selected on the basis of their PRNT results and were retested by the Euroimmun IgM and IgG assays. This analysis was performed to provide additional data on Euroimmun assay sensitivity against the PRNT given the low number of ZIKV PRNT-confirmed cases in cohort 1.

**MAC-ELISA.** The CDC ZIKV MAC-ELISA was essentially performed as previously described (12, 13). The following reagents were used at the indicated dilutions: goat anti-human coating antibody, 1:2,000 (AH10601; Invitrogen); patient/test sera, 1:400; CDC conjugated monoclonal flavivirus antibody (6B6C-1), 1:1,500; and Vero cell ZIKV antigen was used at a 1:500 dilution. Results were reported based on the P/N ratio, where P is the mean optical density (OD) of the test specimen reacted on ZIKV antigen and N is the mean OD of the normal human serum/negative-control serum reacted with ZIKV antigen (13). Specimens with a P/N of  $\geq 3$  were reported as presumptive positive, those with a P/N of  $\geq 2$  but  $< 3$  are reported as equivocal, and those with a P/N of  $< 2$  are documented as negative. Specimens that generated a high-background optical density were reported as inconclusive (13).

**Euroimmun IgM and IgG assays.** The Euroimmun anti-ZIKV ELISA IgM and IgG assays (Euroimmun, Lübeck, Germany), targeting the NS1 antigen of ZIKV, were performed according to the manufacturer's kit inserts. The IgM results were reported semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of the calibrator. A specimen was considered IgM positive if the IgM ratio was  $\geq 1.1$ , equivocal if the ratio was  $\geq 0.8$  to  $< 1.1$ , and negative if the ratio was  $< 0.8$ . The IgG results were reported quantitatively using the standard curve obtained by point-to-point plotting of the extinction values measured for the 3 calibration sera against the corresponding units (linear/linear). A specimen was considered IgG positive if the result was  $\geq 22$  relative units (RU)/ml, equivocal if  $\geq 16$  and  $< 22$  RU/ml, and negative if  $< 16$  RU/ml.

**PRNT.** The PRNTs were carried out essentially as previously described (7, 20). The viral strains used in the neutralization assay were ZIKV Puerto Rico and DENV-2 New Guinea C.

The target PFU used for incubating with various dilutions of test sera were 100 PFU per well. After a 1-h incubation at 37°C, the mixtures of virus and patient sera were added to six-well Nalgene plates (Thermo Fisher Scientific, Waltham, MA, USA) containing monolayers of Vero cells. Double overlays of nutrient agar with neutral red were added to the plates to visualize plaque formation over a 3-day period. The dilutions of sera were started at a screening dilution of 20-fold and



progressed in a 2-fold pattern. A 90% or greater inhibition of plaque formation was documented as the endpoint dilution/titer.

PRNT interpretation was performed following the CDC's recommendations (14). Briefly, a recent ZIKV infection was confirmed if ZIKV PRNT was  $\geq 10$  and DENV PRNT was  $< 10$ , a recent DENV infection was confirmed if ZIKV PRNT was  $< 10$  and DENV PRNT was  $\geq 10$ . As per the CDC's recommendations, a greater than 4-fold difference between the two flavivirus titers was not sufficient to confirm infection with one virus over the other. Therefore, if both ZIKV and DENV PRNT were  $\geq 10$ , only a recent flavivirus infection could be confirmed, but the specific virus could not be identified (14).

**RT-PCR.** Reference PCR and Altona PCR, as well as NS5 gene PCR and sequencing, were performed on serum specimens as previously described (21).

**Analytical sensitivity and specificity.** The sensitivity and specificity of the Euroimmun IgM and IgG assays were determined in comparison to the CDC-designed MAC-ELISA, the current reference ZIKV IgM assay. Since the CDC recommends performing ZIKV and DENV PRNTs on MAC-ELISA-positive, -equivocal, and -inconclusive specimens, our aim was to compare the Euroimmun IgM- and/or IgG-positive or -equivocal specimens to MAC-ELISA-positive, -equivocal, or -inconclusive specimens to evaluate whether the Euroimmun IgM and IgG assays when used alone or in combination would detect specimens requiring testing by the PRNT. The sensitivity and specificity of the Euroimmun IgM and IgG assays, as well as of the MAC-ELISA, were evaluated against that of the PRNT. For the purpose of sensitivity and specificity analyses, specimens with a PRNT result interpretation showing either recent ZIKV infection or recent flavivirus infection were considered PRNT positive. Given the low number of PRNT-positive specimens in our initial cohort (cohort 1), we performed additional sensitivity and specificity analyses of the Euroimmun assays against the PRNT in another set of specimens selected by their PRNT results (cohort 2). Cross-reactivity of the Euroimmun IgM and IgG assays with other flaviviruses was also determined by performing DENV PRNT.

**Statistical analyses.** Statistics were performed using SPSS software (version 23.0; IBM Corp., Armonk, NY).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JCM.00442-17>.

**SUPPLEMENTAL FILE 1**, XLSX file, 0.1 MB.

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A. Hamid-Allie, L. Papageorgiou, S. Hung, C. F. Wong, and J. B. Gubbay designed the study. A. Hamid-Allie, L. Papageorgiou, S. Hung, C. F. Wong, K. Dimitrova, and D. R. Stein performed the laboratory analyses. A. Hamid-Allie, L. Papageorgiou, S. Hung, and C. F. Wong contributed to the initial verification of the assay. A. G. L'Huillier, R. Olsha, L. W. Goneau, E. Kristjanson, and J. B. Gubbay analyzed the data. A. G. L'Huillier, L. Papageorgiou, L. W. Goneau, D. Safronetz, M. Drebot, and J. B. Gubbay wrote the manuscript. All authors reviewed and agreed to the final version of the manuscript.

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