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Establishment of a comprehensive and high throughput serological algorithm for Zika virus diagnostic testing

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ABSTRACT

The previous serological algorithm for Zika virus (ZIKV) comprised screening by anti-ZIKV IgM capture ELISA (MAC-ELISA) for samples collected within 3 months postexposure or onset (MPEO). Samples positive by MAC-ELISA and samples collected beyond 3 MPEO were tested by the confirmatory plaque reduction neutralization test (PRNT), which proved laborious and time-consuming during the 2015 outbreak. Thus, we evaluated several ZIKV ELISAs to establish an anti-IgM and anti-IgG combination for use as a screening tool for all samples prior to PRNT confirmation. The MAC-ELISA or InBios-M in combination with the Euroimmun-G demonstrated sensitivities of 99.1% and 97.2%, respectively, and nonflavivirus specificity of 96.0%. Their cross-reactivities were 71.4% and 50.0%, respectively, for sera positive for Dengue virus antibodies. Due to near-perfect interrater agreement with PRNT and excellent detection of samples collected beyond 3 MPEO, these combinations were recommended as a screening protocol in a new high-throughput algorithm with special considerations for ZIKV diagnostics.

1. Introduction

1.1. Zika virus and the 2015–2016 epidemic

Zika virus (ZIKV) is a flavivirus transmitted by mosquitoes which circulate throughout Africa and Asia but have expanded geographically (Dick et al., 1952; Epelboin et al., 2017; Kraemer et al., 2015). ZIKV was not deemed a significant public health threat until 2007, when an outbreak occurred in Yap State, Federated States of Micronesia (Lanciotti et al., 2008). The largest and most widespread ZIKV epidemic since then began in 2015, beginning Brazil and subsequently spreading to over 65 countries throughout the Americas (Campos et al., 2015; Cao-Lormeau et al., 2014; Weaver et al., 2016; Wikan and Smith, 2016). While human ZIKV infections are typically asymptomatic or mild and self-limiting, data from recent outbreaks have shown that ZIKV infections can result in more serious clinical manifestations and long-term effects, such as congenital birth defects, neurologic disorders, and sexual transmission (Grossi-Soyster and LaBeaud, 2017; Weaver et al., 2016). A recent study showed that a single point mutation that arose in the current outbreak strain contributed to increased infectivity towards human neural progenitor cells, which could have contributed to the increased incidence of babies born with microcephaly during the epidemics (Yuan et al., 2017). Since congenital ZIKV syndrome in babies born from infected mothers results in serious clinical consequences, preventing CZS in utero is currently of high priority for diagnostic testing (Erbelding and Cassetti, 2017).

1.2. Diagnostic testing for Zika virus

The majority of diagnostic testing is conducted for travelers returning from regions with documented ZIKV transmission. Serum and urine samples collected within 14 days post–symptom onset or postexposure can be confirmed positive by detecting viral RNA by quantitative real-time PCR (qRT-PCR), the gold-standard molecular assay. All serum samples within 3 months postexposure or postonset (MPEO) are screened using an in-house anti-ZIKV IgM capture ELISA (MAC-ELISA) (Lanciotti et al., 2008). The MAC-ELISA is a high-throughput assay that may be limited in detecting positive samples beyond 3 MPEO when the IgM response has waned. For example, in nonhuman primates, the IgM response against ZIKV peaks at 11 days postinfection, waning by 20 days postinfection (Keasey et al., 2017). Since recent studies have correlated the risk of microcephaly with maternal infection in the first trimester of pregnancy, the ability to establish the timing of infection is crucial for health care providers to provide counseling to pregnant women about the risk for CZS (Mlakar et al., 2016). In addition, due to cross-reactivity of the MAC-ELISA with antibodies to other flaviviruses, such as dengue virus (DENV), its ability to act as a confirmatory assay is limited. The confirmatory serological assay is the plaque reduction neutralization test (PRNT), which was previously conducted for samples collected beyond 3 MPEO and used to confirm the presence of ZIKV specific neutralizing antibodies in samples positive by the

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MAC-ELISA. Due to the time-consuming and laborious nature of the PRNT, a high-throughput serological screening protocol which could detect the presence of ZIKV antibody in acute and convalescent serum samples was required as a response to the 2015 ZIKV outbreak, during which thousands of travelers sought testing (Tataryn et al., 2018). While many high-throughput anti-ZIKV IgM ELISAs (M-ELISAs) and anti-ZIKV IgG ELISAs (G-ELISAs) have been developed by commercial vendors, it remains unknown whether they could provide additional diagnostic efficacy when used in combination.

In this study, we aimed to establish an M-ELISA and G-ELISA (M/G-ELISA) combination which could be used as a primary screening tool preceding the confirmatory PRNT. We conducted a comprehensive evaluation of various ELISAs, including the Euroimmun anti-ZIKV IgM, IgG, and IgA/IgM ELISAs (Euroimmun-M, Euroimmun-G, Euroimmun-AM, respectively) and the InBios ZIKV Detect IgM Capture ELISA (InBios-M). From these results, we developed a new, comprehensive and high-throughput algorithm for ZIKV testing by combining either the MAC-ELISA or InBios-IgM with the Euroimmun-IgG kit as a primary serological screening protocol.

2. Materials and methods

2.1. Samples

Samples were submitted to the National Microbiology Laboratory of the Public Health Agency of Canada (Winnipeg, Manitoba, Canada) for arbovirus diagnostic testing. A total of 170 serum samples were tested by ZIKV and DENV PRNT based on patient travel history to areas with known ZIKV transmission. Of these samples, 106 were confirmed ZIKV-positive (ZIKV+) by PRNT either after testing positive by MAC-ELISA or because they were collected beyond 1 MPEO as this was the diagnostic algorithm at the time. This subset included 45 samples collected between 0 and 1 MPEO, 22 samples collected between 1 and 2 MPEO, 17 samples collected 2–3 MPEO, and 22 samples collected beyond 3 MPEO. In order to evaluate the cross-reactivity of the commercial kits with samples positive for anti-DENV antibodies, 14 samples confirmed DENV-positive (DENV+) by PRNT were included in the study. To establish the specificity of the kits independently and in combination with another assay, 50 negative patient samples, which were confirmed to be both ZIKV− and DENV− by PRNT, were tested.

2.2. In-house diagnostic assays

2.2.1. ZIKV/DENV PRNT

A 90% serum dilution endpoint PRNT was performed on heat-inactivated serum samples serially diluted 2-fold in Dulbecco's Minimum Essential Medium supplemented with 10% FBS, 100 U/mL of penicillin, and 100 mg/mL of streptomycin (Gibco, Carlsbad, CA). ZIKV (PRVABC59 Puerto Rico strain) or DENV (Type 2, M18428 New Guinea strain) diluted to a concentration of 200 PFU/100 μL was mixed 1:1 with the diluted serum samples and incubated at 37 °C for 1 h. Each serum–virus suspension was transferred onto 12-well cell culture plates (Corning, Corning, NY) containing Vero E6 cells at 100% confluence and incubated at 37 °C for 1 h. Each well was then overlaid with 2% agarose. After incubation at 37 °C for 96 h, a second agarose overlay containing 0.005% neutral red was added to each well. Plaques were counted, and the neutralizing titer of a sample was expressed as the reciprocal of the serum dilution yielding a >90% reduction in the number of plaques compared with the negative control. Samples yielding a titer of ≥20 were considered positive, while a titer of <20 was considered negative. A minimum 8-fold difference in titer was required in order to differentiate between ZIKV+ and DENV+ samples.

2.2.2. MAC-ELISA

The in-house ZIKV CDC MAC-ELISA described by Lanciotti et al. (2008) received Emergency Use Authorization through the Food and Drug Administration in 2016. The ratio of the mean optical density (OD) of the patient sample (P) divided by the mean OD of the negative control (N) reacted with the prepared ZIKV antigen. P/N ratios of <2 were considered negative. For specimens with P/N ratios ≥2, further analysis was conducted by dividing the mean OD value of the sample when reacted with ZIKV antigen with the mean OD value obtained when reacted with the normal control antigen. If the quotient was <2, the result was considered "uninterpretable" due to high background reactivity. For specimens with a background quotient ≥2, the result was considered "positive" if P/N ratios were ≥3 or "equivocal" if P/N ratios were between 2 and <3. For simplification of the multilaboratory analysis, both "positive" and "equivocal" samples were considered "positive".

2.3. Commercial kit evaluation and diagnostic algorithm generation

2.3.1. InBios-M

The InBios ZIKV Detect IgM Capture ELISA (InBios International Inc.; Seattle, WA) was evaluated at the NML and the Laboratoire de Santé Publique du Québec. The assay was performed according to manufacturer instructions. For each specimen, an immune status ratio (ISR) was determined by dividing the OD value reacted with ZIKV recombinant envelope (E) glycoprotein by the OD value reacted with the cross-reactive control antigen (CCA). ISR values ≤1.60 were considered negative, while values ≥1.80 are presumptive positive for anti-ZIKV IgM antibodies. Specimens with ISR values <1.70 were further analyzed, including determination of the ratio of the patient sample reacted with ZIKV Ag or CCA divided by a normal control Ag (NCA). Samples with ZIKV Ag/NCA ratio values ≥1.70, regardless of the CCA/NCA value, were interpreted as possible ZIKV positive. Specimens with a ZIKV Ag/NCA ratio <1.70 and CCA/NCA ratio of ≥1.70 or <1.70 were interpreted as other flavivirus positive or negative, respectively. For simplification of the multilaboratory analysis, both "presumptive ZIKV positive" and "possible ZIKV positive" samples were considered "positive."

2.3.2. Euroimmun-M

The Euroimmun conventional anti-ZIKV IgM ELISA (EI 2668-9601M; Euroimmun AG, Luebeck, Germany) was performed according to manufacturer instructions. The OD of each well was measured by spectrophotometry at 450 nm, with a 620-nm reference wavelength. A sample with a ratio <0.8 was considered "negative," ≥0.8 but less than 1.1 was considered "borderline," and ≥1.1 was considered "positive" for anti-ZIKV IgM antibodies. For simplification of the analysis, both "positive" and "borderline" samples were considered "positive."

2.3.3. Euroimmun-A/M

The Euroimmun conventional anti-ZIKV IgA/IgM ELISA (EI 2668-9601Q; Euroimmun AG, Luebeck, Germany) was performed according to manufacturer instructions. The OD of each well was measured by spectrophotometry at 450 nm, with a 620-nm reference wavelength. A sample with a ratio <0.8 was considered "negative," ≥0.8 but less than 1.1 was considered "borderline," and ≥1.1 was considered "positive" for anti-ZIKV IgA and/or IgM antibodies. For simplification of the analysis, both "positive" and "borderline" samples were considered "positive."

2.3.4. Euroimmun-G

Euroimmun conventional anti-ZIKV IgG ELISA (EI 2668-9601G; Euroimmun AG, Luebeck, Germany) was performed according to manufacturer instructions. The OD of each well was measured by spectrophotometry at 450 nm, with a 620-nm reference wavelength. Point-to-point plotting of the adjusted OD values of 3 calibrators was used to generate a standard curve to calculate the concentration of antibodies in patient samples in relative units (RU) per mL. If the mean adjusted OD for the specimen was equal to or greater than the OD of calibrator 2, the linear equation between calibrator 1 (200 RU/mL) and calibrator 2 (20 RU/mL) was used to determine the concentration of antibodies in the specimen. If the mean adjusted OD for the specimen was less than

the OD of calibrator 2, the linear equation between calibrator 2 (20 RU/mL) and calibrator 1 (2 RU/mL) was used to determine the concentration of antibodies in the specimen. A sample with a concentration of <16 RU/mL was considered "negative," ≥16 but less than 22 RU/mL was considered "borderline," and ≥22 RU/mL was considered "positive" anti-ZIKV IgG antibodies. For simplification of the multilaboratory analysis, both "positive" and "borderline" samples were considered "positive."

2.4. Statistical analysis

Sensitivity was defined as the proportion of ZIKV+ (PRNT confirmed) samples which also tested positive by the ELISA being assessed. A 2-tailed McNemar's test (continuity corrected) was conducted to compare the sensitivity of individual M-ELISAs compared with its sensitivity when combined with Euroimmun-G and vice versa for ZIKV+ samples collected at different time points. Specificity was defined as the percentage of ZIKV− samples which also tested negative. These values, along with the 95% confidence intervals (95% CIs), were calculated with Wilson score interval continuity corrected using GraphPad Prism Software (Newcombe, 1998; Wilson, 1927) (GraphPad, La Jolla, CA). Kappa values (κ values) as a measure of interrater agreement between each ELISA and ZIKV PRNT were also calculated. Assays yielding κ values between 0 and 0.20 indicate no agreement, while a κ value of 1 indicates perfect agreement. κ values 0.21–0.39, 0.40–0.59, 0.60–0.79, 0.80–0.90, and greater than 0.90 indicate minimal, weak, moderate, strong, and near-perfect agreement, respectively (McHugh, 2012). Discordance from the confirmatory assay was assessed using a 2-tailed McNemar's test (continuity corrected) comparing results of each ELISA with those of the ZIKV PRNT. An assay or ELISA combination was considered unsuitable for use as a diagnostic screening tool for ZIKV if results were significantly discordant from ZIKV PRNT. Cross-reactivity was defined as the percentage of samples confirmed DENV+ and ZIKV− by PRNT which tested positive in the ELISA being assessed. The combined sensitivity, specificity, and cross-reactivity of a proposed screening protocol were defined as above, combining the results of an M-ELISA (or A/M-ELISA) with the results of a G-ELISA.

3. Results

3.1. Detection of ZIKV+ samples by in-house and commercial ZIKV ELISAs at different time points

The MAC-ELISA and InBios-M both detected 100% of ZIKV+ samples collected at 0–1 MPEO, while Euroimmun-M, Euroimmun-AM, and Euroimmun-G detected 57.8%, 66.7%, and 62.2%, respectively (Table 1). Combined with Euroimmun-G, the MAC-ELISA and InBios-M assays detected 100% of samples collected at 0–1 MPEO, while the Euroimmun-M and Euroimmun-AM assays detected 82.2% when combined with Euroimmun-G. For samples collected at 1–2 MPEO, InBios-M assay demonstrated the highest detection followed by MAC-ELISA, Euroimmun-G, Euroimmun-M, and Euroimmun-AM (100%, 95.5%, 81.8%, 50.0%, and 40.9%, respectively). Combined with Euroimmun-G, the MAC-ELISA and InBios-M assays each detected 100% of samples collected at 1–2 MPEO, while the Euroimmun-M and Euroimmun-AM assays detected 90.9% and 86.4%, respectively. Individually, the MAC-ELISA, InBios-M, and Euroimmun-G assays each detected 100% of samples collected at 2–3 MPEO, while Euroimmun-M and Euroimmun-AM detected 5.88% and 11.8%, respectively. All of the M/G-ELISA combinations each detected 100% of samples collected at 2–3 MPEO. For samples collected >3 MPEO, Euroimmun-G demonstrated the highest detection (88.4%) followed by MAC-ELISA and InBios-M, which each detected 72.7% of samples. Both the Euroimmun-M and Euroimmun-AM assays were unable to detect any samples collected >3 MPEO. Combined with Euroimmun-G, the MAC-ELISA detected 95.5% of samples collected >3 MPEO. When combined with Euroimmun-G, the InBios-M, Euroimmun-M, and Euroimmun-AM assays each detected 86.4% of samples collected at this time point.

95% CIs are indicated in parentheses. Two-tailed McNemar tests were conducted to analyze the significance of the increase in detection conferred by M-ELISA compared with G-ELISA and vice versa for samples collected at each time point. Increased detection of samples by M-ELISA compared with G-ELISA * = $P < 0.05$, ** = $P < 0.001$, *** = $P < 0.0001$. Increased detection of samples by G-ELISA compared with M-ELISA † = $P < 0.05$, †† = $P < 0.001$, ††† = $P < 0.0001$.

Compared with Euroimmun-G, the MAC-ELISA (+$P = 0.0001$) and InBios-M ($P = 0.0001$) demonstrated increased detection of samples collected 0–1 MPEO. There was no significant difference in detection between Euroimmun-G and Euroimmun-M ($P = 0.8231$) and Euroimmun-AM ($P = 0.8026$). Euroimmun-G demonstrated increased detection of samples collected at 1–2 MPEO compared with Euroimmun-AM ($P = 0.0159$). However, there was no significant difference in detection between Euroimmun-G and MAC-ELISA ($P = 0.3711$), InBios-M ($P = 0.1336$), or Euroimmun-M ($P = 0.0704$). Euroimmun-G demonstrated increased detection of samples collected at 2–3 MPEO compared with Euroimmun-M ($P = 0.0002$) and Euroimmun-AM ($P = 0.0003$); MAC-ELISA and InBios-M detected the same number of samples collected at 2–3 MPEO as Euroimmun-G. Euroimmun-G demonstrated increased detection of samples collected beyond 3 MPEO compared with Euroimmun-M ($P < 0.0001$) and Euroimmun-AM ($P < 0.0001$); there was no significant difference in detection of these samples by MAC-ELISA ($P = 0.4497$) and InBios-M ($P = 0.2482$) compared with Euroimmun-G. Total detection of samples by MAC-ELISA and InBios-M was significantly higher compared with Euroimmun-G ($P = 0.003$ and 0.0005, respectively). Total detection of samples by Euroimmun-G was significantly higher compared with Euroimmun-M ($P < 0.0001$) and Euroimmun-AM ($P < 0.0001$).

Table 1
Detection of ZIKV+ samples collected at different time points by various ZIKV immunoassays, individually and in an M/G-ELISA combination.

MPEO	Individual immunoassays					M/G-ELISA combinations			
	MAC-ELISA	InBios-M	Euroimmun-M	Euroimmun-AM	Euroimmun-G	MAC-ELISA + Euroimmun-G	InBios-M + Euroimmun-G	Euroimmun-M + Euroimmun-G	Euroimmun-AM + Euroimmun-G
0–1	100%**	100%**	57.8%	66.7%	62.2%	100%	100%	82.2%	82.2%
$n = 45$	(92.1–100%)	(92.1–100%)	(42.2–72.3%)	(51.1–80.0%)	(46.5–76.2%)	(92.1–100%)	(92.1–100%)	(68.0–92.0%)	(68.0–92.0%)
1–2	95.5%	100%	50.0%	40.9%†	81.8%	100%	100%	90.9%	86.4%
$n = 22$	(77.2–99.9%)	(84.6–100%)	(28.2–71.8%)	(20.7–63.7%)	(59.7–94.8%)	(84.6–100%)	(84.6–100%)	(70.8–.98.9%)	(65.1–97.1%)
2–3	100%	100%	5.88%††	11.8%††	100%	100%	100%	100%	100%
$n = 17$	(80.5–100%)	(80.5–100%)	(0.01–28.7%)	(1.45–36.4%)	(80.5–100%)	(80.5–100%)	(80.5–100%)	(80.5–100%)	(80.5–100%)
>3	72.7%	72.7%	0.00%†††	0.00%†††	86.4%	95.5%	86.4%	86.4%	86.4%
$n = 22$	(49.8–89.3%)	(49.8–89.3%)	(0.00–15.4%)	(0.00–15.4%)	(65.1–97.0%)	(77.2–99.9%)	(65.1–97.0%)	(65.1–97.0%)	(65.1–97.0%)
Total	93.4%*	94.3%*	35.9%†††	38.7%†††	85.9%	99.1%	97.2%	87.7%	86.8%
***n* = 106**	(86.9–97.3%)	(88.0–97.8%)	(26.8–45.8%)	(29.4–48.6%)	(77.7–91.9%)	(94.9–100%)	(92.0–99.4%)	(80.0–93.3%)	(78.9–92.6%)

Table 2
Sensitivity, specificity, and accuracy of ZIKV immunoassays, individually and in an M/G-ELISA combination.

	Individual immunoassays					M/G-ELISA combinations			
	MAC-ELISA	InBios-M	Euroimmun-M	Euroimmun-AM	Euroimmun-G	MAC-ELISA + Euroimmun-G	InBios-M + Euroimmun-G	Euroimmun-M + Euroimmun-G	Euroimmun-AM + Euroimmun-G
Sensitivity	93.4% (86.9–97.3%)	94.3% (88.0–97.8%)	35.9% (26.8–45.8%)	38.7% (29.4–48.6%)	85.9% (77.7–91.9%)	99.1%* (94.9–100%)	97.2% (92.0–99.4%)	87.7%*** (80.0–93.3%)	86.8%*** (78.9–92.6%)
Specificity	96.0% (86.3–99.5%)	96.0% (86.3–99.5%)	98.0% (89.4–100%)	100% (92.9–100%)	100% (92.9–100%)	96.0% (86.9–97.3%)	96.0% (86.9–97.3%)	98.0% (89.4–100%)	100% (92.9–100%)
κ value	0.871 (0.789–0.953)	0.885 (0.807–0.962)	0.250 (0.158–0.342)	0.288 (0.195–0.381)	0.705 (0.699–0.892)	0.956 (0.906–1.00)	0.927 (0.864–0.990)	0.806 (0.711–0.902)	0.808 (0.714–0.902)
Discordance with PRNT	ns	ns	***	***	***	ns	ns	*	**

Sensitivity: percentage of serological confirmatory (PRNT) ZIKV+ samples which also tested positive in the immunoassay. **Specificity:** percentage of reference standard ZIKV− samples which also tested negative in the immunoassay. **κ value:** measure of interrater agreement with the confirmatory assay. κ values 0.21–0.39, 0.40–0.59, 0.60–0.79, 0.80–0.90, and greater than 0.90 indicate minimal, weak, moderate, strong, and near-perfect agreement, respectively. 95% CIs are indicated in parentheses. * = $P < 0.05$, ** = $P < 0.001$, *** = $P < 0.0001$.

3.2. Combining MAC-ELISA, Euroimmun-M, and Euroimmun-AM with Euroimmun-G significantly improves overall sensitivity

InBios-M demonstrated the highest individual sensitivity of 94.3%, followed by MAC-ELISA (93.4%), Euroimmun-G (85.9%), Euroimmun-AM (38.7%), and Euroimmun-M (35.9%). The MAC-ELISA/Euroimmun-G combination demonstrated the highest sensitivity (99.1%), followed by InBios-M/Euroimmun-G (97.2%), Euroimmun-M/Euroimmun-G (87.7%), and Euroimmun-AM/Euroimmun-G (86.8%) (Table 2). Sensitivities of the MAC-ELISA/Euroimmun-G ($P = 0.0412$), Euroimmun-M/Euroimmun-G ($P < 0.0001$), and Euroimmun-AM/Euroimmun-G ($P < 0.0001$) were significantly higher than the sensitivities of their respective M-ELISA conducted independently. However, the sensitivity of the InBios-M/Euroimmun-G combination was not statistically significantly different compared with that of InBios-M alone.

All of the assays demonstrated high specificities of 96.0% for MAC-ELISA and InBios-M, 98.0% for Euroimmun-M, and 100% for each Euroimmun-AM and Euroimmun-G based on reactivity with samples confirmed negative for ZIKV and DENV. The M-ELISAs demonstrated the same specificity when combined with Euroimmun-G (Table 2). The difference in specificity between Euroimmun-M/G combination (98%) and the assay combinations that demonstrated a specificity of 100% was not statistically significant ($P = 1.000$). The difference in specificities between the MAC-ELISA/Euroimmun-G and InBios-M/Euroimmun-G combinations, which each had specificities of 96%, was also not statistically significant from the assay combinations that demonstrated a specificity of 100% ($P = 0.4795$).

Individually, MAC-ELISA and InBios-M assays had κ values of 0.871 and 0.885, indicating strong interrater agreement with PRNT; Euroimmun-G had a κ value of 0.705, indicating moderate agreement. Euroimmun-M and Euroimmun-AM had κ values of 0.250 and 0.288, respectively, indicating minimal agreement with PRNT. The MAC-ELISA/Euroimmun-G and InBios-M/Euroimmun-G combinations demonstrated near-perfect agreement with PRNT, with κ values of 0.956 and 0.927, respectively. The Euroimmun-M/G and Euroimmun-AM/G combinations demonstrated κ values of 0.806 and 0.808, respectively, indicating strong interrater agreement with PRNT.

Discordance between results of an assay with those of PRNT were significant for Euroimmun-M ($P < 0.0001$), Euroimmun-AM ($P < 0.0001$), Euroimmun-G ($P = 0.0003$), as well as the Euroimmun-M/G combination ($P = 0.0033$) and Euroimmun-AM/G combination ($P = 0.0005$). Discordance from PRNT was not significant for MAC-ELISA ($P = 0.1824$), InBios-M ($P = 0.2888$), as well as the MAC-ELISA/Euroimmun-G combination ($P = 0.617$) and the InBios-M/Euroimmun-G combination ($P = 1.000$).

3.3. Cross-reactivity of in-house and commercial ZIKV ELISAs with DENV+ samples

MAC-ELISA and Euroimmun-AM each demonstrated the highest cross-reactivity with DENV+ samples (71.4%), followed by InBios-M (42.9%). Euroimmun-M and Euroimmun-G each demonstrated the least cross-reactivity with DENV+ samples (14.3%) (Table 3). The MAC-ELISA/Euroimmun-G and Euroimmun-AM/Euroimmun-G combinations each demonstrated the highest cross-reactivity with DENV+ samples (71.4%), followed by InBios-M/Euroimmun-G (50.0%), and Euroimmun-M/Euroimmun-G (21.4%).

4. Discussion

In this study, we identified a new serological screening protocol for ZIKV using an M/G-ELISA combination and developed a high-throughput diagnostic algorithm as depicted in Fig. 1. The MAC-ELISA and InBios-M assays were suitable M-ELISAs for the serological screening protocol due to their strong interrater agreement and concordance with the confirmatory PRNT. These assays only had specificities of 96% compared with Euroimmun-AM and Euroimmun-G, which had specificities of 100%. While this difference was not statistically significant,

Table 3
Cross-reactivity of individual and combined ZIKV immunoassays with DENV+ samples.

	Individual immunoassays					M/G-ELISA combinations			
	MAC-ELISA	InBios-M	Euroimmun-M	Euroimmun-AM	Euroimmun-G	MAC-ELISA + Euroimmun-G	InBios-M + Euroimmun-G	Euroimmun-M + Euroimmun-G	Euroimmun-AM + Euroimmun-G
DENV+ (*n* = 14)	71.4% (41.9–91.6%)	42.9% (17.7–71.1%)	14.3% (1.78–42.8%)	71.4% (41.9–91.6%)	14.3% (1.78–42.8%)	71.4% (41.9–91.6%)	50.0% (23.0–77.0%)	21.4% (46.7–50.8%)	71.4% (41.9–91.6%)

Cross-reactivity: percentage of samples confirmed DENV+ and ZIKV− by PRNT which tested positive in the ZIKV immunoassay. 95% CIs are indicated in parentheses.

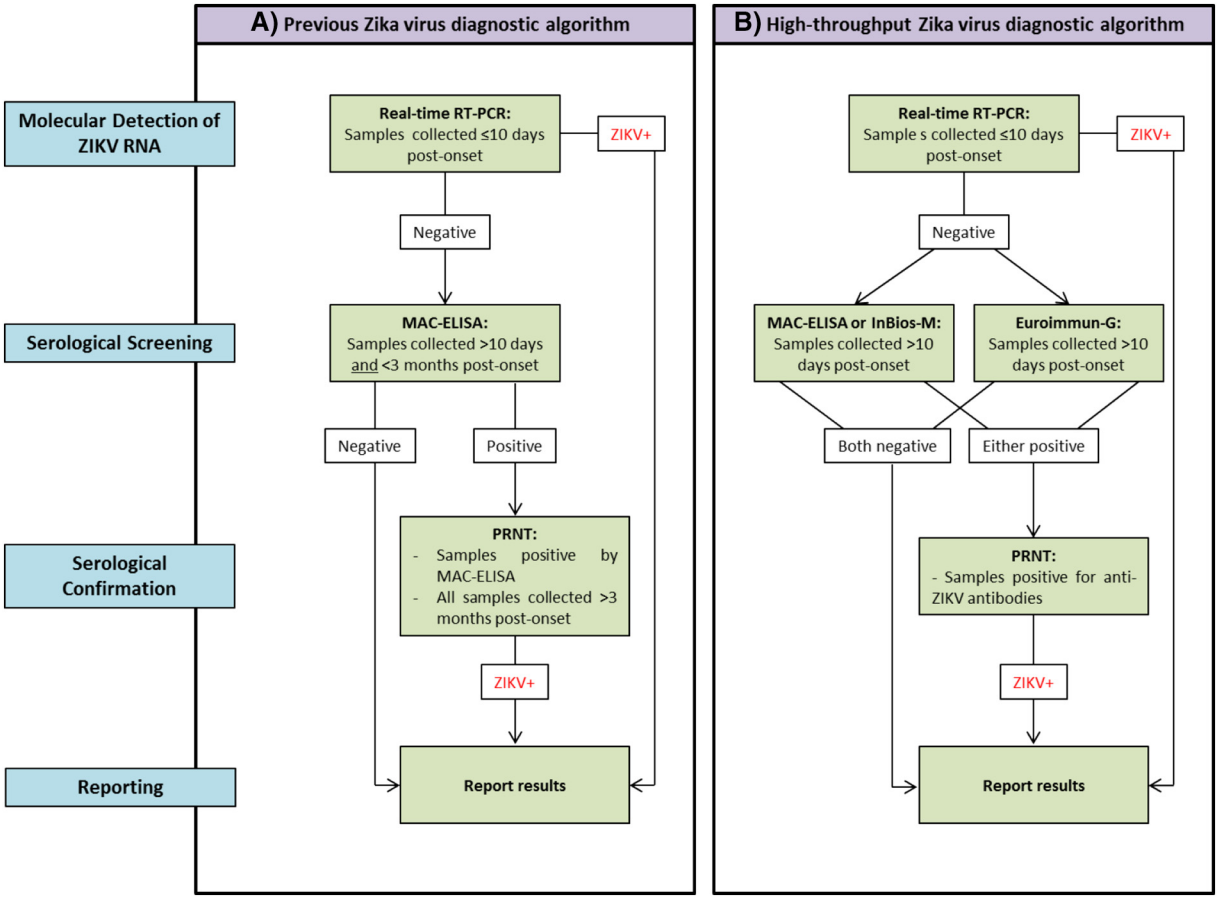

Fig. 1. Two diagnostic algorithms for Zika virus testing. The previous algorithm (A) required that in addition to samples giving a positive result by MAC-ELISA, samples beyond 3 MPEO were sent directly for PRNT, the laborious confirmatory serological assay. The newly proposed high-throughput algorithm (B) enables samples collected beyond 3 MPEO to be sent for serological screening using an M/G-ELISA combination, reducing the number of samples required for PRNT confirmation.

samples giving a positive or equivocal result by either ELISA in the initial serological screen are confirmed via ZIKV and DENV PRNT to minimize the false-positive rate of the algorithm. Due to their significant discordance with PRNT both independently and in an M/G-ELISA combination, Euroimmun-M and Euroimmun-AM were deemed unsuitable for use as serological screening assays for ZIKV. Although Euroimmun-G was deemed unsuitable for use as an independent serological screening assay, it was added to the serological screening procedure given that overall sensitivity was improved when it was conducted alongside most of the M-ELISAs, including the MAC-ELISA.

The 2 combinations proposed for the new serological screening protocol were MAC-ELISA/Euroimmun-G and InBios-M/Euroimmun-G. Addition of the MAC-ELISA and InBios-M significantly expanded diagnostic coverage of samples collected at 0–1 MPEO compared with Euroimmun-G alone. A limitation of the MAC-ELISA analysis is that the samples collected at 0–1 MPEO were originally tested by MAC-ELISA followed by PRNT confirmation as part of the diagnostic algorithm used at the time of the recent outbreak. Thus, inherent selection bias may interfere with our ability to estimate the sensitivity of the MAC-ELISA in this study. Additional coverage conferred by combining M- and G-ELISAs was not significant for samples collected at 1–2 MPEO and 2–3 MPEO. Although Euroimmun-G detected 5 more samples than MAC-ELISA and 3 more samples than InBios-M collected >3 MPEO, this additional coverage was not statistically significant. Overall, the MAC-ELISA and InBios-M assays conferred additional coverage in the M/G-ELISA combination compared with Euroimmun-G alone. However, this observation may be due to the higher number of samples used to evaluate early time points than later time points. Combining M- and G-ELISAs achieved "near-perfect" interrater agreement with the confirmatory serological test, an improvement over the "strong" (MAC-ELISA and InBios-M) and "moderate" (Euroimmun-G) interrater agreements achieved by the assays conducted individually. However, due to the small size of each sample subset, especially beyond 0–1 MPEO ($n = 17–22$), future studies using a larger number of these samples should be performed to establish sensitivity and interrater agreements with narrower CIs. Until the generalizability of this study is established, precaution should be taken when considering the implementation of the described diagnostic algorithms.

All of the immunoassays evaluated in this study demonstrated cross-reactivity with DENV+ samples. In particular, the MAC-ELISA/Euroimmun-G and InBios-M/Euroimmun-G screening combinations demonstrated 71.4% and 50.0% reactivity with DENV+ samples, which may raise concerns that a true DENV+ sample could be mistakenly categorized as ZIKV+. However, as mentioned previously, all samples giving a positive or equivocal result by either ELISA in the screening procedure would subsequently be tested by ZIKV and DENV PRNT in the described algorithm, allowing differentiation between true ZIKV+ samples and true DENV+ samples. Differential diagnosis between ZIKV and DENV is crucial to ensure proper prenatal counseling to those infected with ZIKV, as well as prompt treatment to those infected with DENV, which causes a more severe and potentially fatal disease (Mlakar et al., 2016). While the positive results from serological screening are subsequently differentiated by ZIKV and DENV PRNT, this may only identify a fraction of DENV+ samples as the PRNT only tests for neutralizing antibodies against DENV-2, 1 of 4 circulating DENV serotypes. To reduce the possibility that a true DENV+ sample is mistakenly categorized as ZIKV+, future studies could evaluate whether the addition of urea to the wash buffers of the MAC-ELISA or commercial ZIKV

ELISAs could reduce their cross-reactivity with DENV+ samples. While we focused on DENV cross-reactivity, it may be of interest to determine the cross-reactivity of the described serological screening protocols with samples positive for antibodies targeting other emerging/reemerging pathogens within the flavivirus family, such as Yellow Fever virus, and other mosquito-borne viruses with similar geographic distribution, such as Chikungunya virus and Mayaro virus (Hotez and Murray, 2017). This will be important for differential diagnosis and adequate epidemiological monitoring.

The diagnostic algorithm for ZIKV developed in this study offers benefits over the previous algorithm in which samples collected beyond 10 days postexposure or symptom onset were tested by MAC-ELISA, with the exception of those collected beyond 3 MPEO, which were tested directly by PRNT. Practically, a single technician can process up to 35 samples by ZIKV/DENV PRNT at a time. It then takes up to 7 days from cell preparation to plaque reading in order to obtain results. In contrast, the new algorithm includes samples collected beyond 3 MPEO in the serological screening stage. A technician can process up to 270 samples with ease for both the M-ELISAs and G-ELISA. In addition, negative results are ready to report within 2 days for InBios-M and Euroimmun-G and 3 days for the MAC-ELISA. A potential limitation of the new algorithm is that 1 ZIKV PRNT+ sample was not detected by both the MAC-ELISA/Euroimmun-G and InBios-M/Euroimmun-G combinations. The sample was collected beyond 3 MPEO and demonstrated a ZIKV neutralizing titer >40. Two additional PRNT+ samples collected beyond 3 MPEO were not detected by the InBios-M/Euroimmun-G combination, which were detected by MAC-ELISA/Euroimmun-G combination. Thus, there may be a possibility that the serological screening protocol could fail to identify some convalescent infections. Since the CDC recommends that men testing positive for ZIKV infection should defer pregnancy 3 MPEO, patients providing samples for testing beyond 3 MPEO should be at less risk of giving birth to a baby with CZS (Dubaut et al., 2017; Mead et al., 2018). In addition, asymptomatic women with a risk of travel-related exposure who are planning to conceive are advised to defer pregnancy by 2 months after traveling to regions with ZIKV in order to minimize the risk of CZS occurring in the baby. While asymptomatic men are not tested, they are advised to defer pregnancy by 3 months posttravel (Polen et al., 2018). Thus, as long as these demographics follow the guidelines recommended by the CDC, the risk of CZS should be minimal regardless of the test result for a sample collected beyond 3 MPEO. Since ZIKV infection during the first trimester of pregnancy is associated with the highest risk of CZS occurring in the newborn, the limitation of the algorithm has the greatest impact on pregnant women who have traveled to areas with ZIKV transmission during their first trimester and request diagnostic testing beyond 3 MPEO (Johansson et al., 2016). Thus, consideration should be given to testing samples collected beyond 3 MPEO from this specific demographic of patients by PRNT regardless of the M/G-ELISA screening results in order to ensure that ZIKV+ samples are not missed by the serological screening protocol. In addition, larger epidemiological studies are needed to determine the significance of the false-negative rate of the new algorithms. Future studies could also focus on the sensitivities of the M/G-ELISA combinations for ZIKV+ samples at time points beyond 3 MPEO in order to establish a cutoff time point at which samples require direct testing by PRNT.

5. Conclusions

The proposed algorithm in this study, which uses an M/G-ELISA combination as a preliminary serological screening tool prior to confirmation via PRNT, has potential to greatly reduce the logistical limitations and turnaround time for ZIKV diagnostic testing. However, to ensure that all convalescent ZIKV infections are detected by the algorithm, future studies could identify the sample collection time postexposure/onset at which the screening procedure is less effective. Until then, PRNT testing regardless of the result from the serological screening assays should be considered for samples collected beyond 3 MPEO from pregnant women who travelled to areas with active ZIKV transmission during their first trimester to assess the risk of CZS occuring in the newborn.

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Disclosures

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