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ORIGINAL ARTICLE

# Challenges in diagnosing Zika—experiences from a reference laboratory in a non-endemic setting

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## Abstract

Diagnosing a patient with Zika infection is not always straightforward. Here, we aim to describe our data collected from December 2015 to December 2017 and discuss the implemented algorithm and diagnostic challenges we encountered. At the National Reference Center for Arboviruses at the Institute of Tropical Medicine, Antwerp, Belgium (ITM), a commercial Zika virus (ZIKV) enzyme-linked immunosorbent assay (ELISA) detecting immunoglobulin (Ig) M and IgG, a commercial ZIKV immunofluorescence assay (IFA) detecting IgM, and an in-house Zika virus neutralization test (VNT) were implemented. For molecular detection of ZIKV, an in-house and a commercial real-time RT-PCR were applied. An algorithm, adapted from the European Centre for Disease Control and Prevention (ECDC), was implemented. Between December 2015 and December 2017, we tested 6417 patients for ZIKV. Of those, according to ECDC criteria, 127 (2.0%) were classified as a *confirmed* Zika infection of which 39 by RT-PCR (0.6%), 15 (0.2%) as a *probable* Zika infection, 73 (1.1%) as *undefined*, and 65 (1.0%) as *false positive reactions*. Main challenges were the brief window for detection of IgM, cross-reactivity of antibodies with other flaviviruses and malaria, and low VNT titers in the acute phase. In RT-PCR negative samples, classification of ZIKV infection as recent or past proved difficult, when IgM was negative. The majority of patients could be classified according to ECDC criteria, though 1.1% of patients remained "undefined" and 1.0% were ELISA false positive reactions. Complementary IFA IgM was of added value to increase IgM detection rates. Improved serological assays and more longitudinal data on antibody kinetics are needed.

**Keywords** Zika · Diagnosis · Serology · Challenges

## Introduction

Zika virus (ZIKV) is a mosquito-borne flavivirus found for the first time in monkeys from the Zika forest in Uganda in 1947 [1]. Though outbreaks had already occurred in Micronesia in 2007 [2] and French Polynesia in 2013 [3], ZIKV came to the fore-front of global public health following the outbreak in 2015–2016 in the Americas and the Caribbean. The virus spread to other regions, and by December 2017, over 70 countries had been affected [4].

Based on symptoms alone, it is difficult to differentiate between ZIKV, dengue virus (DENV), and Chikungunya virus (CHIKV) infection. Accurate diagnosis of arbovirus infections requires laboratory testing. Establishing a diagnosis of ZIKV infection is clinically important because of possibly severe complications, such as poor neonatal outcome of ZIKV infection in pregnancy and Guillain-Barré syndrome. Indeed, ZIKV diagnostics are frequently requested for couples with child wish or in case of pregnancy. When the World Health Organization declared the Zika outbreak in the Americas as a Public Health Emergency of International Concern in February 2016, the number of lab requests rose extensively and laboratories struggled as they did not have the necessary diagnostic capacity [5]. As a result, new diagnostic tests, molecular as well as antibody detection assays, were developed and several (interim) guidelines for laboratory testing were published [6–8].

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In general, ZIKV RNA detection is performed in the acute phase of the disease up to around 5 days post symptom onset (dpso) for serum [9] and 14 to 21 dpso for urine [10], while the virus can be detected until 4 months post symptom onset (pso) in whole blood [11–14]. ZIKV RNA has been reported to be detectable in semen up to 370 dpso [15]. Antibodies develop within 1 week pso. Different formats of serologic assays for ZIKV are now available such as enzyme-linked immunosorbent assays (ELISA), immunofluorescence assays (IFA), immunoblots, and (chemiluminescent) microsphere immunoassays ((C)MIA). Confirmation of results by an assay detecting neutralizing antibodies is warranted to exclude cross-reactivity with antibodies against other flaviviruses, most commonly DENV.

Little evidence is available on the kinetics of ZIKV antibodies and persistence of viral RNA in different body fluids. In the National Reference Center (NRC) for Arboviruses at the Institute of Tropical Medicine, Antwerp, Belgium (ITM), a diagnostic algorithm specific to our non-endemic reference laboratory setting was implemented. Based on our experience combined with evidence from literature, National Public Health guidelines (issued by the Belgian Superior Health Council [16]) were published in May 2016. Here, we aim to describe our data collected from Dec. 2015 to Dec. 2017, a time interval spanning the epidemic and approximately 1 year after the official end of the epidemic in Latin America. Concurrently, we discuss the implemented algorithm and diagnostic challenges we encountered.

## Methods

### Serology

A ZIKV ELISA detecting IgM and IgG (Euroimmun AG Lübeck, Germany) and an in-house Zika virus neutralization test (VNT) were implemented as of February 2016. Initially, out of caution and later as it became clear that the sensitivity of the ELISAs was not optimal, not only positive samples but also samples with undetermined results (ratio between 0.80 and 1.10) were subjected to VNT. Virus neutralization testing was performed applying a CPE (cytopathic effect) based assay in a 96-well format using Vero cells (ATCC® CCL-81™) and ZIKV strain MR766 with results interpreted at 90% neutralization. VNT titers between 1/10 and 1/100 were cautiously interpreted. From internal validation data, it was shown that these low VNT titers could be the result of cross-reacting dengue antibodies. Performing the VNT too early in the course of the disease is also a possible cause of low VNT titers, in which case we asked for a convalescent sample.

As in-house validation data revealed that IFA ZIKV IgM (Euroimmun AG) was more sensitive than ELISA ZIKV IgM, this complementary test was mainly performed when ELISA was IgM negative/IgG positive, generally up to 4 months after infection.

### RT-PCR

Throughout 2016, two specific ZIKV RT-PCRs were used: an in-house ZIKV RT-PCR targeting NS5, as described by De Smet et al. [17], and the commercial RealStar® Zika Virus RT-PCR Kit (Altona diagnostics GmbH, Hamburg, Germany) targeting NS1. As from January 2017, to reduce costs, only the RealStar® RT-PCR kit was used as it proved to be more sensitive than the in-house ZIKV RT-PCR (data not shown).

### Diagnostic flow

The implemented diagnostic algorithm, adapted from the European Centre for Disease Prevention and Control (ECDC), is illustrated in Fig. 1. This was later used in the national guideline published by the Belgian Superior Health Council (SHC).

### Case definitions

All samples with any test positive for ZIKV between December 2015 and December 2017 were included for retrospective case classification. The travel history and clinical information were obtained from the medical file and from a questionnaire sent to or by direct contact with colleagues from the referring laboratory for patients receiving medical care outside the ITM.

Criteria from the European Center for Disease Prevention and Control interim case definition published 21 July 2016 were used to classify ZIKV cases [18]. A case was considered a *confirmed* case when ZIKV RNA was detected, ZIKV IgM and VNT were positive, a seroconversion, or a fourfold rise in antibody titers from paired serum samples was found. Cases were labeled *probable* when clinical and epidemiological criteria according to ECDC were met or when IgM was positive without a VNT result. A reaction was considered false positive when both IgM and IgG ELISA ratios were negative in a follow-up sample, when VNT was negative or when cross-reaction with another flavivirus was confirmed through comparison of VNT titers. Patients who did not meet the criteria for a confirmed or probable case and whose results were not considered false positive were classified as "undefined."

### Statistical analysis

Positive detection rates (PDR = positive test results / all samples from confirmed cases) with 95% confidence intervals were calculated by Analyse-it Software for Microsoft Excel (Leeds, England).

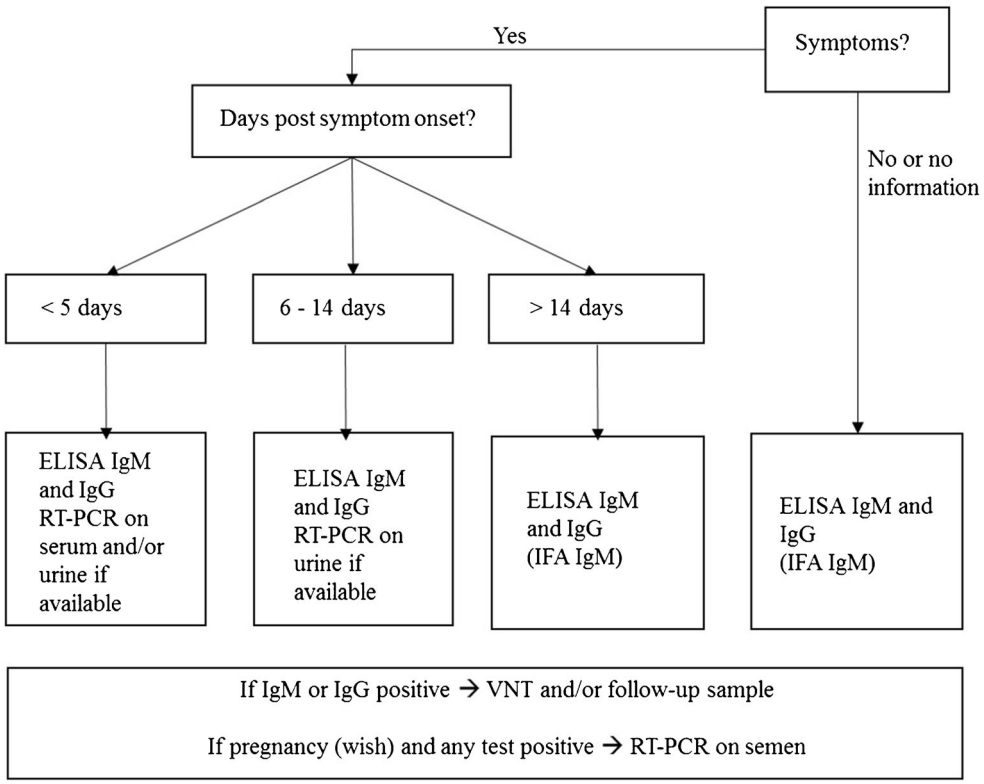


**Fig. 1** Diagnostic algorithm implemented at the NRC for arboviruses at the ITM

## Results

Between December 2015 and December 2017, the NRC received 6954 serum and/or urine samples from 6417 patients (0.79 male-to-female ratio) for analysis. Few samples ($n = 44$) from before February 2016 were analyzed retrospectively when ZIKV specific tests became available. The number of requests for diagnostic testing increased by 45% from 2834 in 2016 to 4120 in 2017. Of note, in 2017, only 31.5% (148/470) of the samples with a request for RT-PCR were known to be collected in the acute phase were analyzed, as opposed to 71.5% (331/463) in 2016.

Out of 6417 patients tested for ZIKV, 280 (4.4%) showed a positive reaction in any test, of which 127 (2.0%) were reported as having a *confirmed* Zika infection, 15 (0.2%) as a *probable* Zika infection, 73 (1.1%) as *undefined*, and 65 (1.0%) as *false positive reactions*.

Figure 2 illustrates all confirmed and probable cases per month with an obvious decrease of cases in 2017. Out of 127 patients with a confirmed infection, 39 were confirmed by RT-PCR and 83 by IgM and VNT positivity (8 with VNT lower than 1/100). Five patients were IgM negative, of which two showed an IgG seroconversion compared to a sample from before travel to an endemic region and three had a significant rise in neutralization titers from paired serum samples. Eleven out of 15 probable cases met clinical and epidemiological criteria according to ECDC. The remaining four were IgM positive without confirmation by VNT due to too little sample volume ($n = 1$, asymptomatic) or because VNT results were not reliable early in the course of disease and a follow-up sample was asked but not received ($n = 3$, no symptom information). The results of 65 patients were false positive, of which 25 were only IgM positive (ratios from 0.81 to 4.26, median 0.99) and 40 only IgG positive (ratios from 0.80 to 2.81, median 1.14). Of these, 9 became negative in a follow-up sample and 48 patients were ZIKV VNT negative. Cross-reactivity with DENV was shown for four samples by DENV VNT (all IgM negative/IgG positive (ratios from 0.80 to 2.20)). Cross-reactivity with malaria was demonstrated in four patients with a recent stay in Africa, which tested positive with ZIKV ELISA IgM (ratios from 0.82 to 6.24) but negative with VNT ($n = 3$) or ELISA ($n = 1$) in a follow-up sample. For all four patients with malaria, ZIKV IFA IgM remained negative. A group of 73 IgM negative/IgG positive patients with a positive VNT result could not be classified (undefined). Reasons were a VNT value below 1/100 ($n = 21$) without a follow-up sample ($n = 17$) or without an increase of the IgG ratio or the VNT titer in the follow-up sample ($n = 4$), the absence of symptoms ($n = 27$), or the lack of data on the presence of symptoms ($n = 25$). Although 21 had symptoms compatible with ZIKV infection, they did not meet ECDC criteria (e.g., a patient with rash and fever, but no mention of myalgia, arthralgia, or conjunctivitis) for classification as a probable case.

The male-to-female ratio of the 127 confirmed cases was 0.61. One third was between 21 and 30 years old. The age distribution is illustrated in Fig. 3. Over half of the patients

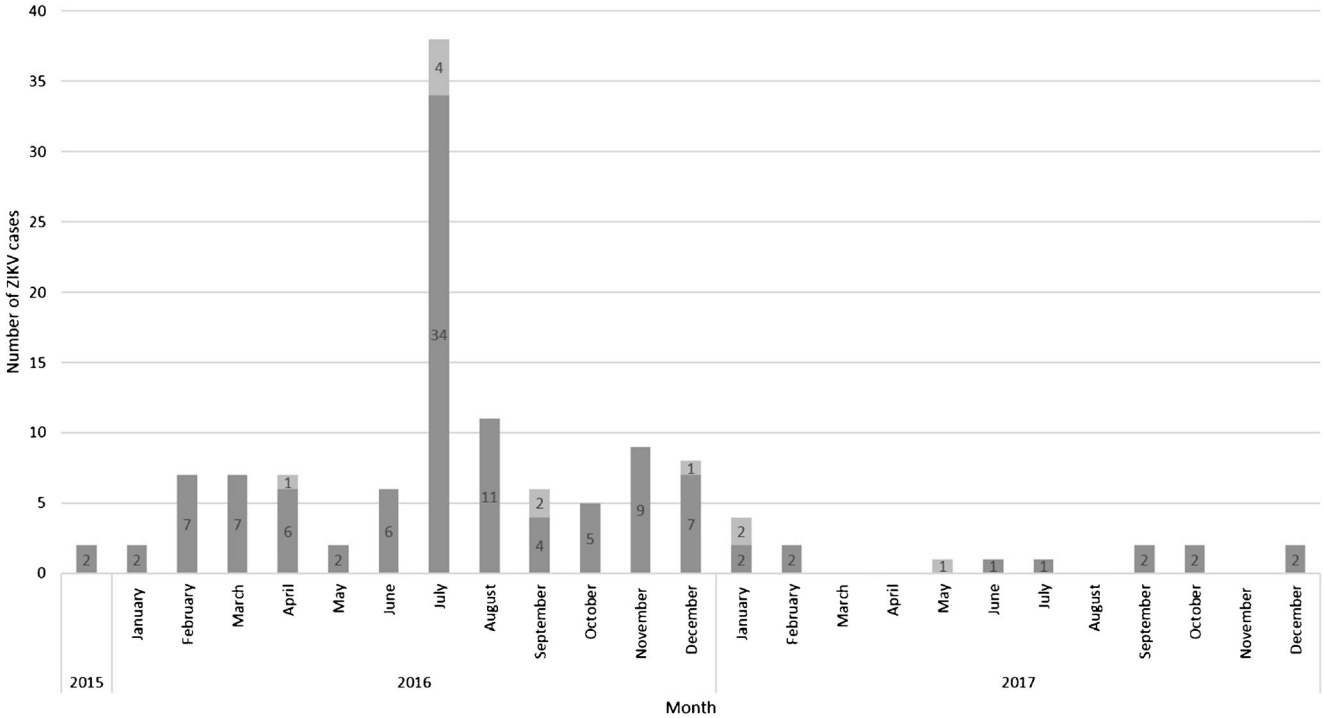


**Fig. 2** Confirmed (dark gray) and probable (light gray) ZIKV cases per month according to ECDC criteria

reported having traveled to or stayed in the Caribbean region (Fig. 4). Six patients with a confirmed ZIKV infection were mentioned to be pregnant or have a pregnancy wish; however, information was often lacking and this number is probably underestimated. The vast majority (114/127 or 89.8%) of patients were symptomatic (no information available for nine cases (7.1%)). For 102 patients (80.3%), the exact number of dpso was known. Serology (IgM and IgG combined) was positive in all 105 samples from 84 patients as from 7 dpso. PDRs of the different tests and their combinations are illustrated in Table 1. PDR of ELISA IgM was highest between 5 and 30 dpso (88.6% (95% CI 79.0–94.1%)) and decreased substantially between 31 and 120 dpso (30.4% (95% CI 15.6–50.9%)) and thereafter. Complementary detection of IgM antibodies by IFA between 5 and 30 dpso increased the detection from 88.6 to 95.7% (95%CI 88.1–98.5%). The added value was more striking between 30 and 120 dpso when IgM detection increased from 30.4 to 82.6% (95% CI 62.9–93.0%).

## Discussion

When we started to implement ZIKV diagnostic tests we encountered some challenges such as developing a diagnostic algorithm, difficulties in interpreting test results but also diagnostic capacity and cost.

Clinical and epidemiological data helps to select the optimal tests to diagnose ZIKV and is essential for the interpretation of the test results. For RT-PCR on serum samples, which is only useful in the acute phase of disease, knowledge of the date of symptom onset is important to be strict in applying the diagnostic algorithm. RT-PCR on whole blood seems to offer an advantage over serum as ZIKV RNA can be detected for a longer period in whole blood [11–14], but our experience with ZIKV RNA detection in whole blood is limited. The number of samples suitable for molecular diagnostics (collection in the acute phase) in 2017 probably decreased because after an outbreak, patients tend to consult their physicians later in the course of the disease. Because of limited reimbursement of PCR, serology was performed in the absence of symptoms or if no information was provided. ELISA was chosen over other antibody detection assays because it allowed the higher throughput needed to process the high volume of samples during the outbreak. In Europe, the Euroimmun ELISA was the first commercially available ZIKV ELISA and internal validation and an initial report in April 2016 from Huzly et al. [19] demonstrated high specificity. The combination of the Euroimmun ZIKV ELISA and confirmation by VNT in the implemented diagnostic algorithm was maintained throughout the outbreak and thereafter. As it became rapidly clear that the sensitivity of the Euroimmun IgM ELISA was suboptimal, especially from a month after symptom onset, we added IgM testing with IFA. The limited sensitivity of the ZIKV Euroimmun IgM ELISA was also described by others [20–27]. Therefore, the window for detecting a recent infection by this NS1-antigen based IgM ELISA is very short. Here, PDR was 88.6% between 5 and

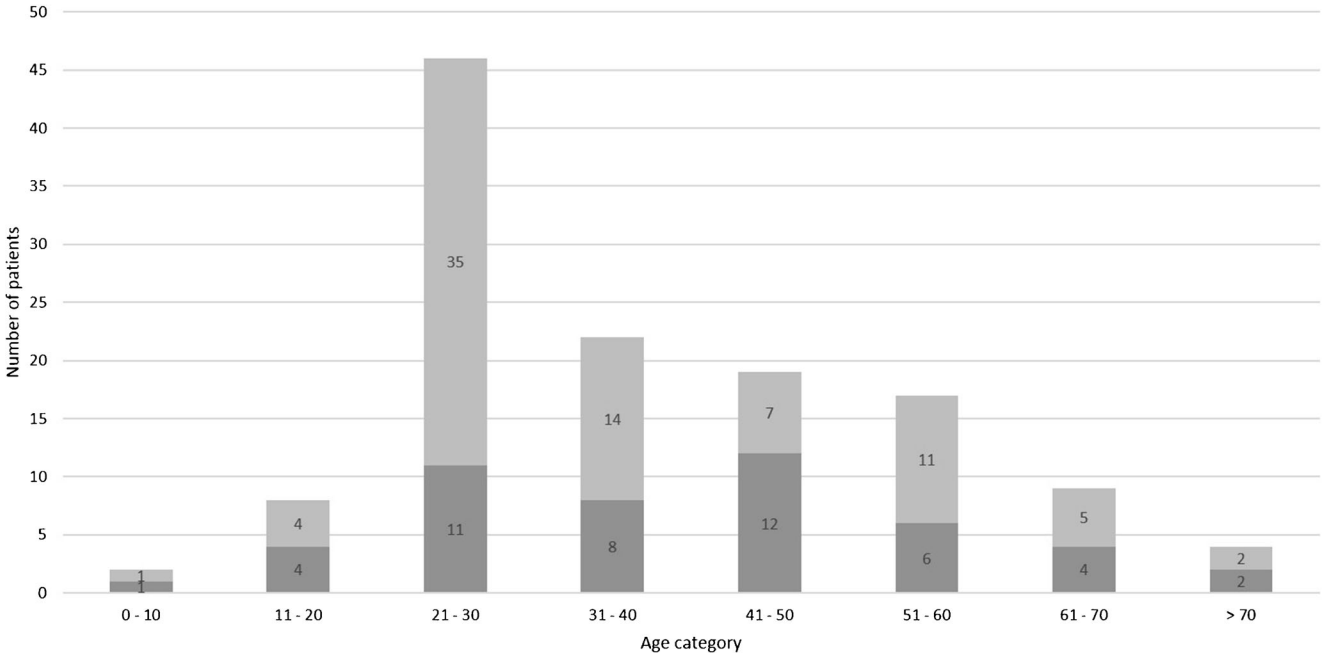


**Fig. 3** Age and gender (male: dark gray; female: light gray) distribution of all confirmed ZIKV cases

30 dpso but decreased substantially thereafter. Detecting IgM antibodies by performing the IFA as a complementary test, which utilizes Zika virus-infected cells as the antigenic substrate, allowed to increase the PDR from 30.4 to 82.6% between 31 and 120 dpso. When both IgM and IgG were negative, samples were not subjected to VNT; hence, we cannot calculate sensitivity, which is a limitation of our work. Tests detecting antibodies against the E antigen are more sensitive, but this is at the cost of a lower specificity. This can be explained by more cross-reactivity between ZIKV, DENV, and WNV antibodies against the E glycoprotein which is, e.g., used in the CDC-MAC-ELISA [28, 29]. We did not systematically investigate the cause of false reactivity in all 65/280 (23.2%) ELISA results unless this was required by the physician. We did find four cases to be caused by dengue cross-reactivity and four by malaria. Dissimilar numbers on the specificity of the Euroimmun ELISA have been reported, depending on the reference test used. In general, a high specificity (> 99%) is described, but there is some cross-reactivity with dengue antibodies [19, 21, 22, 30]. Others have demonstrated that specificity against other flaviviruses is higher for ZIKV IgM than for IgG [26, 31]. However, little data is available on how ZIKV IgM and IgG antibody assays react on acute versus convalescent anti-DENV positive samples [32]. Of note, in a primary ZIKV infection, anti-ZIKV IgM tends to react stronger compared to IgG, whereas when ZIKV is the secondary flavivirus infection, anti-ZIKV IgM can remain low or negative or increase with a time delay [21, 26]. Unfortunately, in our population, we did not have enough secondary infections to make any comments on this. Also,

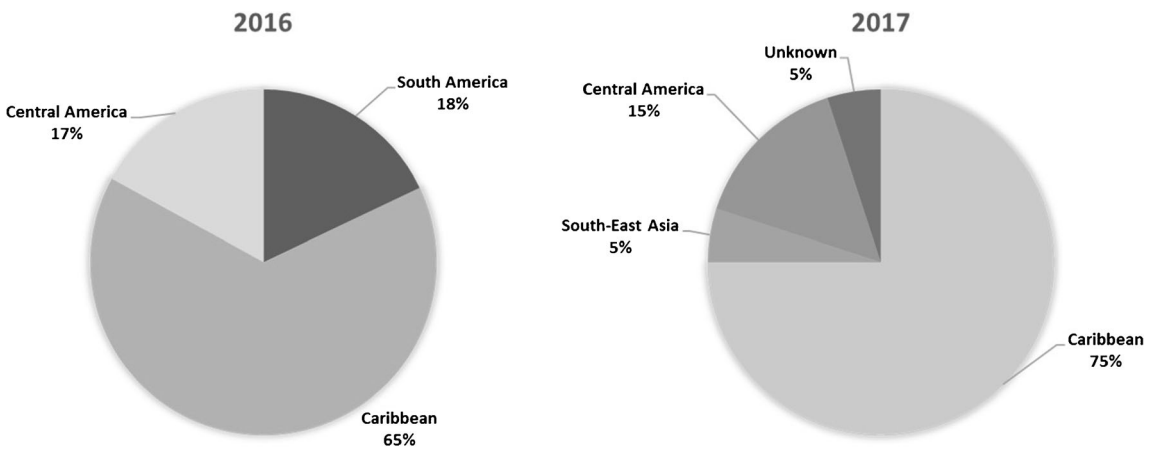


**Fig. 4** Geographic distribution of all confirmed ZIKV cases in 2016 ($n = 107$) and 2017 ($n = 20$)

**Table 1** Positive detection rates (% positive test results/ all samples from confirmed cases) with 95% confidence intervals for different test (combinations) compared to confirmation by RT-PCR or serological confirmed cases according to ECDC criteria. Dpso: days post symptom onset

| | PCR confirmed cases | | | | |
|---|---|---|---|---|---|
| | 0–5 dpso ($n = 21$) | 6–14 dpso ($n = 12$) | 15–30 dpso ($n = 13$) | 31–120 dpso ($n = 3$) | > 120 dpso ($n = 5$) |
| IgM ELISA | 14.3 (5.0–34.6) | 83.3 (55.2–95.3) | 100.0 (77.2–100.0) | 33.3 (6.1–79.2) | 0 (0–43.4) |
| IgM/IgG ELISA | 19.0 (7.7–40.0) | 91.7 (64.6–98.5) | 100.0 (77.2–100.0) | 100.0 (43.9–100.0) | 100.0 (56.6–100.0) |
| IgM ELISA and IFA | 19.0 (7.7–40.0) | 91.7 (64.6–98.5) | 100.0 (77.2–100.0) | 33.3 (6.1–79.2) | 0 (0–43.4) |
| IgM/IgG ELISA and IgM IFA | 23.8 (10.6–45.1) | 91.7 (64.6–98.5) | 100.0 (77.2–100.0) | 100.0 (43.9–100.0) | 100.0 (56.6–100.0) |
| | Serologically confirmed cases | | | | |
| | 0–5 pso ($n = 31$) | 6–14 dpso ($n = 17$) | 15–30 dpso ($n = 28$) | 31–120 dpso ($n = 20$) | > 120 dpso ($n = 11$) |
| IgM ELISA | 90.0 (59.6–98.2) | 94.1 (73.0–99.0) | 82.1 (64.4–92.1) | 30.0 (14.5–51.9) | 9.1 (1.6–37.7) |
| IgM/IgG ELISA | 100.0 (72.2–100.0) | 100.0 (81.6–100.0) | 100.0 (87.9–100.0) | 100.0 (83.9–100.0) | 100.0 (74.1–100.0) |
| IgM ELISA and IFA | 90.0 (59.6–98.2) | 100.0 (81.6–100.0) | 92.9 (77.4–98.0) | 90.0 (69.9–97.2) | 18.2 (5.1–47.7) |
| IgM/IgG ELISA and IgM IFA | 100.0 (72.2–100.0) | 100.0 (81.6–100.0) | 100.0 (87.9–100.0) | 100.0 (83.9–100.0) | 100.0 (74.1–100.0) |
| | All confirmed cases | | | | |
| | 0–5 dpso ($n = 10$) | 6–14 dpso ($n = 29$) | 15–30 dpso ($n = 41$) | 31–120 dpso ($n = 23$) | > 120 dpso ($n = 16$) |
| IgM ELISA | 38.7 (23.7–56.2) | 89.7 (73.6–96.4) | 87.8 (74.5–94.7) | 30.4 (15.6–50.9) | 6.3 (1.1–28.3) |
| IgM/IgG ELISA | 45.2 (29.2–62.2) | 96.6 (82.8–99.4) | 100.0 (91.4 - 100.0) | 100.0 (85.7–100.0) | 100.0 (80.6–100.0) |
| IgM ELISA and IFA | 41.9 (26.4–59.2) | 96.6 (82.8–99.4) | 95.1 (83.9–98.7) | 82.6 (62.9–93.0) | 12.5 (3.5–36.0) |
| IgM/IgG ELISA and IgM IFA | 48.4 (32.0–65.2) | 96.6 (82.8–99.4) | 100.0 (91.4–100.0) | 100.0 (85.7–100.0) | 100.0 (80.6–100.0) |

malaria can cause false IgM positivity, a phenomenon that we reported previously [30]. During this 2-year time span, all positive ZIKV IgM ELISA results from patients that traveled to Africa ($n = 4$) were confirmed to be cross-reactions with malaria. In these samples, ZIKV IFA IgM proved to be more specific.

Owing to the specificity problem of the ELISA, a virus neutralization test can help to confirm ZIKV infection. Virus neutralization tests are still considered the gold standard for serostatus determination; however, the specificity of these tests is limited at early time points after infection and upon sequential flavivirus infections [33-35]. With exception of previous flavivirus infection or vaccination against yellow fever, tick-borne encephalitis or Japanese encephalitis our population of European travelers is expected to have a low seroprevalence of antibodies against flaviviruses. Combining different flavivirus VNTs can be helpful but is too laborious and expensive for diagnostic laboratory routine. From internal validation data, it was noted that 90% neutralization titers below 1/100 were possibly due to cross-reactivity with other flaviviruses, but even this cutoff is uncertain because it is not entirely clear how cross-neutralization evolves following previous flavivirus exposure. Even in the ideal situation in which VNT of different flaviviruses could be compared, interpretation may be problematic. Low VNT values were also noted in the acute phase of the disease, as was the case for eight confirmed IgM-positive cases. In the absence of IgM positivity and without follow-up sample, a low VNT result does not allow to differentiate between cross-reactivity and early Zika infection.

Interpretation of results was often hampered by the limited IgM sensitivity and inconclusive VNT values, but also because of missing clinical and epidemiological information. A lot of effort was made to instruct and inform medical doctors by adding comments on the lab reports, by contacting physicians directly and by distributing the SHR guidelines. The ECDC Zika case definition of a confirmed case [18] states that presence of IgM antibodies should be confirmed by a neutralization assay, regardless of clinical and epidemiological information. This criterion caused difficulties in case of false negative IgM due to low sensitivity of the ELISA. When using ECDC case definitions, 73/280 (26.1%) patients with a negative IgM/positive IgG ELISA and positive VNT result remained undefined. For 17 out of 280 (6.1%) patients, the low neutralization titers left us inconclusive in the absence of a follow-up sample. If the 90% neutralization titer was > 1/100, we concluded that this was a true ZIKV antibody reaction, only we could not determine whether it was due to a recent or old infection though compatible ZIKV infection symptoms of 21 patients were indicative of a recent ZIKV infection. Determining whether a patient was infected recently or in the past is of the utmost importance for pregnant women and patients wishing to conceive. To resolve this issue, analyzing a previous sample (from before departure to endemic areas or from before pregnancy) or avidity testing may be useful.

In conclusion, ZIKV diagnosis is challenging and will become even more difficult with more flaviviruses spreading around the world and increasing vaccination coverage in endemic and non-endemic areas (e.g., Dengvaxia) [36]. The main problems we encountered were the low detection rate of the

Euroimmun IgM ELISA, mainly as from 1 month after symptom onset, cross-reactivity of antibodies with other flaviviruses and malaria, low neutralization titers in the acute phase, and interpretation as recent or past infection in case of a negative IgM result. In our approach, performing a complementary IFA IgM was of added value. Until more longitudinal data on the antibody kinetics and improved serological assays become available, concurrent testing—with more than one serologic assay and/or combined with molecular assays—remains necessary.

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## Compliance with ethical standards

The Institutional Review Board (IRB) of the ITM was notified on the publication of these data. As approved by the IRB, data from patients presenting at the ITM can be used for research purposes as long as the patient does not object and his/her identity is not disclosed to third parties. Some patient results were already published elsewhere (Steinhagen K ($n = 4$), Lustig ($n = 7$)).

**Conflict of interest** The authors declare that they have no conflict of interest.

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