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Validation of serological and molecular methods for diagnosis of zika virus infections

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Highlights:

- The developed ZIKV-RT-qPCR was a core tool for diagnosis of ZIKV infection.
- The diagnosis should be based on both molecular and serological methods.
- Cross-reactions in commercial flaviviral IgM tests were common.

Abbreviations:

NS1 (non-structural protein 1), NS5 (non-structural protein 5), LOD (limit of detection), EVAg (European Virus Archive)

1. Introduction

Family *Flaviviridae*, genus *Flavivirus*, includes human pathogens transmitted by mosquitoes or ticks. Due to the similarities in their antigenic structures, flaviviruses cross-react in serological tests. This is especially problematic in cases where the infecting virus cannot be identified from patient samples by detection of specific viral RNA or antigen. A particular challenge is the differential diagnostics of dengue virus (DENV 1-4) and Zika virus (ZIKV), which are circulating in overlapping geographical areas. As symptoms, epidemiological situation or patient travel history are not helpful for the diagnosis, specific laboratory tests, such as viral RNA detection and IgM assays, are needed to differentiate acute ZIKV infection from DENV. In acute phase samples, viral RNA detection from blood, serum or urine in ZIKV and DENV infections is possible and enable rapid confirmation of the diagnosis (Erra et al. 2013, Driggers et al. 2016, Barzon et al. 2018). However, viral RNA is not always detected in ZIKV patients due various reasons, such as suboptimal timing of sampling. The levels of viremia in ZIKV infections are reported to be lower than those of DENV both in naïve ZIKV infection and co-infections of DENV and ZIKV (Driggers et al., 2016, Azeredo et al. 2018). Although several manufacturers provide diagnostic DENV NS1 antigen tests, only one ZIKV NS1 antigen test for research use is currently commercially available.

The specific diagnosis of an acute flavivirus infection requires detection of viral RNA and/or antigen, or cultivation of the virus. In dengue, the first days of fever coincide with the DENV viremia and NS1 antigenemia, which can both last for a number of days (Erra et al. 2013). A

prolonged detection time window for ZIKV RNA has been observed in whole blood and urine, but also in semen (Barzon et al. 2018). Concerning serological assays, the IgM antibodies become detectable usually by the end of the first week after onset of a primary acute flaviviral infection, followed by the rise of IgG antibodies (Driggers et al. 2016, Erra et al. 2013, Pasquier et al. 2018). A convalescent sample is often required for seroconversion or diagnostic antibody titer rise. The possibility of DENV and ZIKV co-infection or, for example, prolonged persistence of DENV IgM for months after acute infection makes interpreting the serological results difficult without specific RNA detection (Azeredo et al. 2018, Chien et al. 2018). In secondary flaviviral infection, such as subsequent DENV or a ZIKV infection after primary DENV infection, the IgM response may be very low or even undetectable (Sa-Ngasang et al. 2006, Peeling et al. 2010, Barzon et al. 2018). Additionally, patients with potential exposure for multiple flaviviruses such as ZIKV, DENV, West Nile virus (WNV), Japanese encephalitis virus (JEV) and also tick-borne encephalitis viruses (TBEV) due to cocirculation and international travel, as well as flavivirus vaccinations, are nowadays a common challenge in diagnostic settings. In addition to classical neutralization assays, serological assays based on e.g. NS1- antigen have been developed to distinguish ZIKV and DENV serologically, yet the multiple flavivirus exposures leads to their limited specificity or sensitivity.

In order to improve the diagnosis of acute ZIKV infections, we validated the in-house ZIKV RT-qPCR and commercial or in-house IgM tests for ZIKV, DENV, TBEV. This included the evaluation of a ZIKV RT-qPCR assay for routine diagnostics, comparison of automated and manual nucleic acid extraction methods and testing the flavivirus IgM assays for their cross-reactivity properties.

2. Materials and methods

2.1 ZIKV-RT-qPCR

An in-house ZIKV RT-qPCR targeting the NS5 gene was set up and used previously in studying a case of ZIKV infection in a pregnant Finnish traveler subsequently leading to congenital ZIKV infection causing severe fetal brain abnormalities (Driggers et al. 2016). In brief, the ZIKV-RT-qPCR was carried out using 500 nm of primers (modified from Faye et al. 2013), 200 nm probe [VIC- CTYAGACCAGCTGAA-MGBNFQ; Driggers et al. 2016], Superscript® III Platinum® One-step qRT-PCR System (Invitrogen, Carlsbad, CA, USA), using a protocol described in Jääskeläinen et al. (2015) and final template amount of 10 µl. The determination of limit of detection (LOD), and intra- and inter assay repeatability/reproducibility were carried out using quantified in vitro transcribed ZIKV NS5 region RNA (described in Driggers et al. 2016) and quantified ZIKV RNA (strain MR766), respectively, using seven parallel reactions.

2.1.1 Sample material and control strains

For assessing the specificity of the ZIKV-RT-qPCR, panels of pre-characterized human samples were used, including sera, urine and whole blood from Finnish patients sent for polyomavirus and human herpesvirus 6 screening (Table 1). The patient samples used were tested under code and according to research permits of Helsinki University Hospital. For further specificity testing to distinguish ZIKV from related infections, DENV 1-4 [Robert Koch Institute (RKI), Germany, and Zeptomatrix Corporation, Buffalo, New York, USA], yellow fever virus (YFV, strains 17D and Asibi, RKI), WNV (strain Egypt 101), TBEV [both European and Siberian subtypes, and in vitro transcribed TBEV NS5 ssRNA, in-house], JEV (strain Nakayama), Usutu virus (USUV), and chikungunya virus [CHIKV, strains Caribbean, European Virus Archive (EVAg) and Ross] RNA were tested using ZIKV-RT-qPCR. Furthermore, 12 quality control samples from EVD-Labnet (Mögling et al. 2017) were tested (Table 1).

2.1.2 Extraction methods

Nucleic acids were extracted from serum, EDTA-blood and urine by QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) and/or MagNa Pure LC system and kits (Total Nucleic acid Kit, Roche, Espoo, Finland) following manufacturers' instructions. Starting volumes included 200 µl of serum and 100 µl of EDTA-blood, both with final elution volumes of 50 µl (QIAamp Viral mini kit, Qiagen, and MagNa Pure, Roche). A total of 140 µl of urine was used in the extraction with elution volume of 60 µl of elution buffer (QIAamp Viral mini kit, Qiagen) or 50 µl urine with elution volume of 50 µl (MagNa Pure, Roche). To compare different extraction methods and inhibitory factors in urine or sera, dilution series of ZIKV (strain MR766; cell culture supernatant) were spiked to 10 urine and 10 serum samples and extracted in parallel using QIAamp Viral RNA mini kit (Qiagen) and MagNa Pure LC system (Roche).

2.2 Serological methods

IgG antibody titers against DENV were determined using an in-house immunofluorescence assay (IFA) as previously described (Vene et al, 1995, Jääskeläinen et al. 2014). ZIKV IFA was also set up for ZIKV IgG detection using ZIKV MR766 infected Vero E6 cells according to the same protocol as for DENV described in Vene et al. (1995). Briefly, the Vero E6 cells were infected with ZIKV and after cytopathic effect was observed the cells were detached and washed followed by adding non-infected Vero E6 cells, prior to diluting and pipetting to immunofluorescence slides. The slides were air-dried, and fixed with ice-cold acetone for 7min at room temperature. Both DENV and ZIKV IFAs were carried out using serially diluted patient sera. The diluted samples were incubated for 30min at 37°C followed by washings with 1×PBS and conjugation using FITC-conjugated Affini Pure F(ab')₂ Fragment Goat Anti-Human IgG (H+L) (Jackson ImmunoResearch, UK) for 30min at 37°C. After

final washings, the IFA slides were dried and read using microscope. To examine potential IgM-cross-reactions among different flaviviruses, samples from previously diagnosed 73 acute DENV, 6 ZIKV and 82 TBEV cases (HUSLAB, Helsinki University Hospital) were tested in parallel with Anti-Zika virus IgM ELISA (Euroimmun, Lübeck, Germany), Dengue Virus IgM Capture DxSelect™ (Focus Diagnostics, Diasorin Molecular LCC, California, USA), and μ -capture TBEV IgM EIA (in-house, HUSLAB, Helsinki, Finland).

Also, an additional panel of 67 serum samples from 52 individuals diagnosed for acute dengue fever by both viral RNA detection and serology during years 2011-2016 (HUSLAB) were tested using Anti-Zika virus IgM ELISA (Euroimmun). The DENV serotype information was available from 42 out of 52 patients including DENV-1 ($n=16$), DENV-2 ($n=11$), DENV-3 ($n=10$) and DENV-4 ($n=5$) (Korhonen *et al.*, unpublished; determined by using pan-flavi RT-PCR described by Moureau *et al.* 2007 and 2010 and subsequent sequencing). In addition, a panel of 13 serum samples from acute malaria patients (Table 2) was included in the testing.

2.3 NS1 Antigen assays and sequencing

The Dengue NS1 antigen test (SD BIOLINE, Alere/ Abbott GmbH & Co. KG, Wiesbaden, Germany) was used for acute DENV diagnosis and differential diagnosis of ZIKV. The ZIKV NS1 Ag ELISA (BioFront Technologies, FL, USA) was tested using samples from acute ZIKV patients.

The ZIKV-RT-qPCR positive patient test results were confirmed by direct sequencing of the amplification products (102 bp) at DNA Sequencing Service, DNA Sequencing and Genomics, Institute of Biotechnology, University of Helsinki, Finland (data not shown).

3. Results

3.1 Evaluation of ZIKV-RT-qPCR

Negative panel of pre-characterized human samples (sera, urine and whole blood), or control samples containing RNA of DENV1-4, YFV, WNV, TBEV, JEV, USUV, CHIKV were tested negative by ZIKV RT-qPCR assay (100%; Table 1). The results from quality control samples (EQA, EVD-Labnet; Table 1) were in 100% concordance with the expected results.

The LOD was one ZIKV RNA template copy per ZIKV-RT-qPCR reaction using quantified in vitro transcribed RNA from ZIKV NS5 construct and Probit Analysis (95CI; SPSS Statistics, IBM, Helsinki, Finland). Total PCR reaction volume was 25 μ l which includes 10 μ l of RNA dilution as a template. Calculated average value of intra-assay repeatability was 22.92 Ct (ZIKV strain MR766, control ID1), standard deviation (STDEV) of 0.48, and for inter-assay reproducibility (ZIKV strain MR766, control ID2) 21.0 Ct, 0.39 STDEV.

In comparison of two sample matrixes (urine and serum) and two different extraction methods [MagNa Pure LC system (Roche) and QIAamp Viral RNA mini kit (Qiagen)], both extraction methods were successful in extraction of viral RNA and no inhibitory effects were detected (Table 1). However, the QIAamp kit performed slightly better with urine samples than the MagNa Pure system (Ct difference average 3.3, STDEV 0.8), and the MagNa Pure LC system was better than QIAamp kit with serum samples (Ct difference average 0.9, STDEV 0.5).

3.2 Antibody and antigen tests

Serum samples from 241 patients diagnosed with acute DENV ($n=140$), ZIKV ($n=6$), TBEV ($n=82$) or malaria ($n=13$) (Table 2) infection were tested using DENV, ZIKV, and TBEV IgM ELISAs, and

the results were compared (Table 2). Eight out of the 82 of the tested acute TBEV patient samples and 4/6 of acute ZIKV patients gave false positive IgM test results in the Dengue Virus IgM Capture DxSelect™ (Focus Diagnostics) test. In addition, 2/13 confirmed malaria cases gave either false positive or borderline result in DENV IgM test with an overall specificity of 86.1% (87/101). In the cross-reactivity panel, Anti-Zika virus IgM ELISA (Euroimmun) had specificity of 97.4% (229/235) and in-house TBEV IgM EIA 97.8% (90/92) (Table 2).

There were no differences observed in the IgG titers of acute ZIKV patients using DENV or ZIKV IgG IFAs (data not shown), and therefore only a single IgG titer value for each sample is listed in Table 3 (DENV/ZIKV IgG IFA).

The testing of the acute ZIKV patient samples using ZIKV NS1 Ag ELISA (BioFront Technologies) was positive only in 4/10 acute ZIKV patients with range of 0.23 – 0.68 ng/ml.

3.3 Acute ZIKV cases

Ten acute ZIKV infections, including one possible dual infection of DENV and ZIKV were diagnosed in Finnish travellers (Table 3). The available data (5/10 patients) included symptoms of maculopapular rash and fever, headache, eye pain, arthralgia, joint pain, myalgia and sore throat.

In five cases, ZIKV-RT-qPCR was positive (3 sera and 1 whole blood), followed by confirmatory sequencing and serology. In the rest of cases, the ZIKV infections were diagnosed using serological methods only (Table 3).

In the possible dual infection of DENV and ZIKV (patient 3, Table 3), the acute phase serum sample, was positive in both ZIKV-RT-qPCR (this study) and DENV specific RT-qPCR (Huhtamo et al. 2010) in addition to DENV IgM and NS1 antigen tests. The IgG IFA titer was 320 in acute phase but the titer increased to 40,960 in the sample taken 11 days later, when DENV NS1 assay was tested

negative (Table 3). Additionally the ZIKV NS1 Ag ELISA (BioFront Technologies) was negative (0.10 ng/ml) in the first sample but borderline in the second (0.18 ng/ml). Dengue IgM test turned from low positive in acute phase (1.392 index) to moderately positive (3.593 index) whereas the ZIKV IgM EIA was negative in both samples. However, although attempted, the sequencing confirmation of the dual infection was not successful.

4. Discussion and conclusions

The viremia in acute ZIKV infections is typically lower than in acute DENV infection, and therefore requires a more sensitive RNA detection methodology (Waggoner et al. 2016, Azeredo et al. 2018). Here, the validation of the ZIKV RT-qPCR method included the assay specificity and LOD determination (1 RNA copy per PCR reaction), which indicated the method to be suitable for daily diagnostics with excellent specificity. This was demonstrated using in house and external controls, including the EVD-Labnet quality control samples with 100% concordance in the results. The testing of nucleic acid extraction methodologies provided promising results for setting up faster and easier workflows using automated systems with large sample numbers and different sample materials. The developed method was used in confirming ZIKV infection in 4/10 of the tested travellers suspected for ZIKV infection. In one patient tested parallel from serum and whole blood samples, only the whole blood sample was found positive in ZIKV RT-qPCR. This is in line with the previous studies which have indicated that serum may not be the best sample type and whole blood and urine samples should also be tested in order to detect ZIKV or other flavivirus RNA (Cabral-Castro et al. 2016, Lustig et al. 2016, Waggoner et al. 2016, Mansuy et al. 2017, Murray et al. 2017).

Secondary flavivirus infections and co-infections of ZIKV and DENV have been described (Iovine et al. 2016, Chia et al 2017, Carrillo-Hernández et al. 2018), that complicate the interpretation of serological test results. One dual infection of DENV and ZIKV was also suspected in this study but could not be confirmed by sequencing. Although only a small number of ZIKV patients were available for testing in this study, the cross-reactivity testing of the flavivirus IgM tests including larger DENV and TBEV patient sample panels provided interesting results. The recombinant ZIKV NS1 antigen based IgM ELISA (Euroimmun) showed some cross-reactivity or non-specific reactions with IgM positive DENV and TBEV patients, but also with samples of malaria patients. The unspecific IgM reaction due to acute malaria in ZIKV IgM tests had been reported also elsewhere (Steinhagen et al. 2016, Van Esbroeck et al. 2016, Kadkhoda et al. 2017). The DENV IgM assay (Focus Diagnostics) gave positive results in two cases diagnosed as acute malaria and was also positive in 66.7% of cases diagnosed with acute ZIKV infection (4/6) and in 10.8% (8/82) of TBEV cases, leading to final specificity of 86.1% (Table 2). The cross-reactions of acute ZIKV cases in DENV IgM tests have also been reported by others (Cabral-Castro et al. 2016, Felix et al. 2017, Korhonen et al. 2016). We observed that samples from acute TBEV patients gave false positive results especially in the DENV IgM assay, and one sample also in the ZIKV IgM assay. Although the typical symptoms of TBE infection differ from acute DENV or ZIKV infection, the cross-reactivity should be taken into account in interpretation of the serological results in patients who may have been exposed to DENV/ZIKV/TBEV. Although there is a need for specific antibody or antigen test to discriminate DENV and ZIKV, these are currently not available. The ZIKV NS1 antigen assay intended for research use did not give promising results for diagnostic use based on the small number of acute ZIKV samples tested in this study. Further development of specific antibody and antigen detection assays would be needed for laboratory diagnosis and typing of flavivirus infections, especially for discriminating DENV from ZIKV. As this relies currently mostly in specific RNA detection methodologies, more information of ZIKV RNAemia and its

timing in acute ZIKV infection, and in different sample types, would be needed for optimized sampling.

Competing interest

None declared.

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Ethics approval

The patient samples used were tested anonymously and according to research permits (for projects TYH2016258, TYH2017257 and TYH2018322) of Helsinki University Hospital, Helsinki, Finland, and THL/104/5.05.00/2017 of National Institute for Health and Welfare, Finland.

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References

- Driggers, R.W., Ho, C.Y., Korhonen, E.M., Kuivanen, S., Jääskeläinen, A.J., Smura, T., Rosenberg, A., Hill, D.A., DeBiasi, R.L., Vezina, G., Timofeev, J., Rodriguez, F.J., Levanov, L., Razak, J., Iyengar, P., Hennenfent, A., Kennedy, R., Lanciotti, R., du Plessis, A., Vapalahti, O., 2016. Zika Virus Infection with Prolonged Maternal Viremia and Fetal Brain Abnormalities. *N. Engl. J. Med.* 374, 2142-2151.
- Azeredo, E.L., Dos Santos, F.B., Barbosa, L.S., Souza, T.M.A., Badolato-Corrêa, J., Sánchez-Arcila, J.C., Nunes, P.C.G., de-Oliveira-Pinto, L.M., de Filippis, A.M., Dal Fabbro, M., Hoscher Romanholi, I., Venancio da Cunha, R., 2018. Clinical and Laboratory Profile of Zika and Dengue Infected Patients: Lessons Learned From the Co-circulation of Dengue, Zika and Chikungunya in Brazil. *PLoS. Curr.* 15, 10.
- Erra, E.O., Korhonen, E.M., Voutilainen, L., Huhtamo, E., Vapalahti, O., Kantele, A., 2013. Dengue in travelers: kinetics of viremia and NS1 antigenemia and their associations with clinical parameters. *PLoS. One* 8, e65900.
- Barzon, L., Percivalle, E., Pacenti, M., Rovida, F., Zavattoni, M., Del Bravo, P., Cattelan, A.M., Palù, G., Baldanti, F., 2018. Virus and Antibody Dynamics in Travelers With Acute Zika Virus Infection. *Clin. Infect. Dis.* 66, 1173-1180.
- Pasquier, C., Joguet, G., Mengelle, C., Chapuy-Regaud, S., Pavili, L., Prisant, N., Izopet, J., Bujan, L., Mansuy, J.M., 2018. Kinetics of anti-ZIKV antibodies after Zika infection using two commercial enzyme-linked immunoassays. *Diagn. Microbiol. Infect. Dis.* 90, 26-30.

Chien, Y.W., Liu, Z.H., Tseng, F.C., Ho, T.C., Guo, H.R., Ko, N.Y., Ko, W.C., Perng, G.C., 2018. Prolonged persistence of IgM against dengue virus detected by commonly used commercial assays. *BMC Infect. Dis.* 18, 156.

Sa-Ngasang, A., Anantapreecha, S., A-Nuegoonpipat, A., Chanama, S., Wibulwattanakij, S., Pattanakul, K., Sawanpanyalert, P., Kurane, I., 2006. Specific IgM and IgG responses in primary and secondary dengue virus infections determined by enzyme-linked immunosorbent assay. *Epidemiol. Infect.* 134, 820-825.

Peeling, R.W., Artsob, H., Pelegriano, J.L., Buchy, P., Cardoso, M.J., Devi, S., Enria, D.A., Farrar, J., Gubler, D.J., Guzman, M.G., Halstead, S.B., Hunsperger, E., Kliks, S., Margolis, H.S., Nathanson, C.M., Nguyen, V.C., Rizzo, N., Vázquez, S., Yoksan, S., 2010. Evaluation of diagnostic tests: dengue. *Nat. Rev. Microbiol.* 8, S30-8.

Faye, PPO., Faye, O., Diallo, D., Diallo, M., Weidmann, M., Sall, A.A., 2013. Quantitative real-time PCR detection of Zika virus and evaluation with field-caught mosquitoes. *Virol. J.* 10, 311.

Jääskeläinen, A.J., Moilanen, K., Aaltonen, K., Putkuri, N., Sironen, T., Kallio-Kokko, H., Vapalahti, O., 2015. Development and evaluation of a real-time EBOV-L-RT-qPCR for detection of Zaire ebolavirus. *J. Clin. Virol.* 67, 56-58.

Mögling, R., Zeller, H., Revez, J., Koopmans, M., ZIKV reference laboratory group and Reusken R. Status, quality and specific needs of Zika virus (ZIKV) diagnostic capacity and capability in National Reference Laboratories for arboviruses in 30 EU/EEA countries, May 2016. Source: *Eurosurveillance* 22, 36.

Vene, S., Mangiafico, J., Niklasson, B., 1995. Indirect immunofluorescence for serological diagnosis of dengue virus infections in Swedish patients. *Clin. Diagn. Virol.* 4, 43-50.

Jääskeläinen, A.J., Huhtamo, E., Kivioja, R., Domingo, C., Vene, S., Kallio-Kokko, H., Niedrig, M., Tienari, P.J., Vapalahti, O., 2014. Suspected YF-AND after yellow fever vaccination in Finland. *J. Clin. Virol.* 61, 444-447.

Moureau, G., Temmam, S., Gonzalez, J.P., Charrel, R.N., Grard, G., de Lamballerie, X., 2007. A real-time RT-PCR method for the universal detection and identification of flaviviruses. *Vector Borne Zoonotic Dis.* 7, 467-477.

Moureau, G., Ninove, L., Izri, A., Cook, S., De Lamballerie, X., Charrel, R.N., 2010. Flavivirus RNA in phlebotomine sandflies. *Vector Borne Zoonotic Dis.* 10, 195-197.

Huhtamo, E., Hasu, E., Uzcátegui, N.Y., Erra, E., Nikkari, S., Kantele, A., Vapalahti, O., Piiparinen, H., 2010. Early diagnosis of dengue in travelers: comparison of a novel real-time RT-PCR, NS1 antigen detection and serology. *J. Clin. Virol.* 47, 49-53.

Waggoner, J.J., Gresh, L., Vargas, M.J., Ballesteros, G., Tellez, Y., Soda, K.J., Sahoo, M.K., Nuñez, A., Balmaseda, A., Harris, E., Pinsky, B.A., 2016. Viremia and Clinical Presentation in Nicaraguan Patients Infected With Zika Virus, Chikungunya Virus, and Dengue Virus. *Clin. Infect. Dis.* 63, 1584-1590.

Cabral-Castro, M.J., Cavalcanti, M.G., Peralta, R.H.S., Peralta, J.M., 2016. Molecular and serological techniques to detect co-circulation of DENV, ZIKV and CHIKV in suspected dengue-like syndrome patients. *J. Clin. Virol.* 82, 108-111.

Lustig, Y., Mannasse, B., Koren, R., Katz-Likvornik, S., Hindiyeh, M., Mandelboim, M., Dovrat, S., Sofer, D., Mendelson, E., 2016. Superiority of West Nile Virus RNA Detection in Whole Blood for Diagnosis of Acute Infection. *J. Clin. Microbiol.* 54, 2294-2297.

Mansuy, J.M., Mengelle, C., Pasquier, C., Chapuy-Regaud, S., Delobel, P., Martin-Blondel, G., Izopet, J., 2017. Zika Virus Infection and Prolonged Viremia in Whole-Blood Specimens. *Emerg. Infect. Dis.* 23, 863-865.

Murray, K.O., Gorchakov, R., Carlson, A.R., Berry, R., Lai, L., Natrajan, M., Garcia, M.N., Correa, A., Patel, S.M., Aagaard, K., Mulligan, M.J., 2017. Prolonged Detection of Zika Virus in Vaginal Secretions and Whole Blood. *Emerg. Infect. Dis.* 23, 99-101

Iovine, N.M., Lednicky, J., Cherabuddi, K., Crooke, H., White, S.K., Loeb, J.C., Cella, E., Ciccozzi, M., Salemi, M., Morris, J.G. Jr., 2017. Coinfection With Zika and Dengue-2 Viruses in a Traveler Returning From Haiti, 2016: Clinical Presentation and Genetic Analysis. *Clin. Infect. Dis.* 64, 72-75.

Chia, P.Y., Yew, H.S., Ho, H., Chow, A., Sadarangani, S.P., Chan, M., Kam, Y.W., Chong, C.Y., Thoon, K.C., Yung, C.F., Li, J.H., Lye, D.C., De, P.P., Ng, L.F.P., Yeo, T.W., Leo, Y.S., 2017. Clinical features of patients with Zika and dengue virus co-infection in Singapore. *J. Infect.* 74, 611-615.

Carrillo-Hernández, M.Y., Ruiz-Saenz, J., Villamizar, L.J., Gómez-Rangel, S.Y., Martínez-Gutierrez, M., 2018. Co-circulation and simultaneous co-infection of dengue, chikungunya, and zika viruses in patients with febrile syndrome at the Colombian-Venezuelan border. *BMC Infect. Dis.* 18, 61.

Steinhagen, K., Probst, C., Radzimski, C., Schmidt-Chanasit, J., Emmerich, P., van Esbroeck, M., Schinkel, J., Grobusch, M.P., Goorhuis, A., Warnecke, J.M., Lattwein, E., Komorowski, L., Deerberg, A., Saschenbrecker, S., Stöcker, W., Schlumberger, W., 2016. Serodiagnosis of Zika virus (ZIKV) infections by a novel NS1-based ELISA devoid of cross-reactivity with dengue

virus antibodies: a multicohort study of assay performance, 2015 to 2016. *Eurosurveillance* 21, 50.

Van Esbroeck, M., Meersman, K., Michiels, J., Ariën, K.K., Van den Bossche, D., 2016. Letter to the editor: specificity of Zika virus ELISA: interference with malaria. *Eurosurveillance*, 21.

Kadkhoda, K., Gretchen, A., Racano, A., 2017. Evaluation of a commercially available Zika virus IgM ELISA: specificity in focus. *Diagn. Microbiol. Infect. Dis.* 88, 233-235.

Felix, A.C., Santiago Souza, N.C., Figueiredo, W.M., Costa, A.A., Inenami, M., da Silva, R.M., Levi, J.E., Pannuti, C.S., Romano, C.M., 2017. Cross reactivity of commercial anti-dengue immunoassays in patients with acute Zika virus infection. *J. Med. Virol.* 89, 1477-1479.

Korhonen, E.M., Huhtamo, E., Smura, T., Kallio-Kokko, H., Raassina, M., Vapalahti, O., 2016. Zika virus infection in a traveller returning from the Maldives, June 2015. *Eurosurveillance* 21, 2.

Bosch, I., de Puig, H., Hiley, M., Carré-Camps, M., Perdomo-Celis, F., Narváez, C.F., Salgado, D.M., Senthoo, D., O'Grady, M., Phillips, E., Durbin, A., Fandos, D., Miyazaki, H., Yen, C.W., Gélvez-Ramírez, M., Warke, R.V., Ribeiro, L.S., Teixeira, M.M., Almeida, R.P., Muñoz-Medina, J.E., Ludert, J.E., Nogueira, M.L., Colombo, T.E., Terzian, A.C.B., Bozza, P.T., Calheiros, A.S., Vieira, Y.R., Barbosa-Lima, G., Vizzoni, A., Cerbino-Neto, J., Bozza, F.A., Souza, T.M.L., Trugilho, M.R.O., de Filippis, A.M.B., de Sequeira, P.C., Marques, E.T.A., Magalhaes, T., Díaz, F.J., Restrepo, B.N., Marín, K., Mattar, S., Olson, D., Asturias, E.J., Lucera, M., Singla, M., Medigeshi, G.R., de Bosch, N., Tam, J., Gómez-Márquez, J., Clavet, C., Villar, L., Hamad-Schifferli, K., Gehrke, L., 2017. Rapid antigen tests for dengue virus serotypes and Zika virus in patient serum. *Sci. Transl. Med.* 9, 409.

Table 1. Sample panels, material and viruses used for ZIKV-RT-qPCR validation and results of the validation.

Panel/sample material	Microbial agent	No of samples, reference result		ZIKV-RT-qPCR	
		ZIKV RNA Neg	ZIKV RNA Pos	Neg	Pos
Serum	Negatives*	20		20	0
	Total	20		20	0
Urine	Negatives*	15		15	0
	Total	15		15	0
EQA¹	ZIKV (strain MRS_OPY_Martinique_PaRi_2015)		4	0	4
	ZIKV (strain MR766)		2	0	2
	YFV (strain BOL88/1999)	1		1	0
	DENV1 (strain CNR16079/2012)	1		1	0
	CHIKV (strain H20235/StMartin/2013)	1		1	0
	Negatives (2 plasma samples, 1 urine)	3		3	0
	Total	6	6	6	6
Plasmodium**	<i>P. falciparum</i>	11		11	0
	<i>P. vivax</i>	1		1	0
	Plasm. Spp.	1		1	0
	Total	13		13	0
Viruses²	YFV (strains 17D and Asibi)	4		4	0
	WNV (strain Eg101)	2		2	0
	DENV1 (RKI and Zeptomatrix)	6		6	0
	DENV2 (RKI and Zeptomatrix)	6		6	0
	DENV3 (RKI and Zeptomatrix)	6		6	0
	DENV4 (RKI and Zeptomatrix)	6		6	0
	JEV (in-house)	2		2	0
	TBEV (European and Siberian subtype, NS5 construct, in-house)	6		6	0
	USUV (in-house)	2		2	0
	CHIKV (strains Caribbean, EVAg, and Ross, in-house)	4		4	0
	Total	44		44	0
Spiked serum samples³	ZIKV (strain MR766)		10	0	10
	Total		10	0	10
Spiked urine samples³	ZIKV (strain MR766)		10	0	10
	Total		10	0	10
Altogether	ZIKV positives		26	0	26
	ZIKV negatives	98		98	0
	Concordance (%)			100%	100%
	Specificity (%)			100%	-

ZIKV, zika virus; DENV, denguevirus; YFV, yellow fever virus; WNV, West-Nile virus; JEV, Japanese encephalitis virus; TBEV, tick-borne encephalitis virus; USUV, usutuivirus; CHIKV, chikungunya virus; No, number; neg, negative; pos, positive.

* All 20 serum and 15 urine samples from 25 individuals were tested negative for flaviviruses using nested pan-flavi RT-PCR (Moureau,G. 2007; Moureau,G. 2010).

** Diagnosis based on blood-smear samples.

¹ EVD-Labnet (Mögling et al. Eurosurv 2017) samples: CHIKV (2.0E+5 copies/0.2ml), YFV (8.53E+6 copies/0.2ml), DENV1 (5.84E+4 copies/0.2ml), ZIKV [Asian lineage 5.54E+6, 3.62E+4, 3.62E+2, and 2.5E+8 (urine base) copies/0.2ml], ZIKV (African lineage, 1.2E+6 and 8.3E+3 copies/0.2ml). All samples were extracted using MagNA Pure LC system (Roche).

² DENV1-4 kindly provided by Prof. Niedrig [Robert Koch Institute (RKI), Germany]. Zeptomatrix Corporation, Buffalo, New York, USA; European Virus Archive, EVAg. Viral control strains were extracted using QIAamp Viral RNA Mini kit (Qiagen).

³ Spiked human serum and urine samples with ZIKV (MR766) using dilutions of 1.00E-02 - 1.00E-06. Five serum and five urine samples (one of each dilution of 1.00E-02 - 1.00E-06) were extracted manually with QIAamp Viral RNA mini kit (Qiagen) and analogous samples with automated MagNA Pure LC (Total NA Kit, Roche) system.

Table 2. The IgM cross-testing of serum samples from patients with acute DENV, ZIKV, TBEV and malaria infection.

Reference methods below (Total samples tested, <i>N</i>)	DENV IgM ¹ (<i>N</i>)			ZIKV IgM ³ (<i>N</i>)			TBEV IgM ⁴ in-house (<i>N</i>)		Total tested (<i>N</i>)
	Pos	Bord.	Neg	Pos	Bord.	Neg	Pos	Neg	
DENV IgM ¹ (73*)	73*	0	0	1	1	71	2	71	73
DENV PCR ² positives (67*)	67	0	0	0	0	67	ND	ND	67
ZIKV IgM ³ (6*)	4	0	2	6*	0	0	0	6	6
TBEV IgM ⁴ in-house (82*)	8	0	74	1	1	80	82*	0	82
Malaria ⁵ (13)	0	2	11	2	0	11	0	13	13
Specificity (neg, %)	87/101 (86.1%)			229/235 (97.4%)			90/92 (97.8%)		
Altogether tested (pos and neg, <i>N</i>)	241			241			174		

DENV, dengue virus; ZIKV, zika virus; TBEV, tick-borne encephalitis virus; NC, not calculated; pos, positive; neg, negative.

*Total number of acute DENV/ZIKV/TBEV samples studied. Criteria for acute DENV infection was positivity of Dengue NS1 antigen test, DENV IgG test (or seroconversion of it) and DENV IgM test (73 patients); acute ZIKV infection, positivity of ZIKV-RT-qPCR in acute phase or ZIKV IgM test, and IgG seroconversion (6 samples from 4 individuals); acute TBEV infection, positivity of TBEV total antibody test (hemagglutination inhibition test; HUSLAB) and μ -capture TBEV IgM EIA (82 patients; HUSLAB).

¹ Dengue Virus IgM Capture DxSelect™ (Focus Diagnostics, Diasorin Molecular LCC, California, USA)

² Pan-flavi-PCR positives (Korhonen et al. unpublished)

³ Anti-Zika virus IgM ELISA (Euroimmun, Lübeck, Germany)

⁴ μ -capture TBEV IgM EIA (in-house; HUSLAB, Helsinki University Hospital, Helsinki, Finland)

⁵ Diagnosis based on blood-smear samples: 11 *Plasmodium falciparum*, one *P. vivax*, and one plasmodium spp. Tested negative for ZIKV and DENV RNA.

Table 3. Acute zika virus cases from year 2015 to 2017, and results of the different tests carried out.

Patient No	Age	Sex	Sampling date	DOS	ZIKV-RT-qPCR (sample type)	ZIKV IgM-EIA ^a (index)	ZIKV NS1 Ag ELISA ^b [Neg/Pos (ng/ml)]	DENV/ZIKV IgG IFA ^c (titer)	DENV IgM EIA ^d (Neg/Pos)	DENV NS1 Ag ^e (Neg/Pos)	Travelling history	Symptoms	Ref.
1	37	M	24.6.2015	7	ZIKV RNA Pos (urine*; serum Neg)	Pos (4.1)	Pos (0.26)	1,280	Pos (low; 1.9)	Neg	Maldive	Flulike, mild fever, rash, eye pain, arthralgia	*Korhonen et al. 2015, Eurosurv.
2	32	F	29.12.2015	30	ZIKV RNA Pos (serum**)	Pos (3.4)	Neg (0.12)	10,240	Neg (<1)	Neg	Guatemala, Mexico, Belize	Pregnant, microcephaly, abortion, rash, fever, eye pain	Driggers et al. 2016, NEJM
			13.2.2016	66	Neg (serum; 13 days after abortion)	Neg (0.57)	Pos (0.68)	ND	ND	ND			
			17.3.2016	100	ND	Neg (0.42)	BL (0.13)	ND	ND	ND			
3	44	F	16.1.2016	NA	ZIKV RNA Pos and DENV RNA Pos (serum)	Neg (0.10)	Neg (0.10)	320	Pos (low; 1.4)	Pos	NA	NA	This study
			27.1.2016	NA	ND	Neg (0.14)	BL (0.18)	40,960	Pos (3.6)	Neg			
4	39	F	16.2.2016	3	ZIKV RNA Pos (serum)	Neg (0.5)	Pos (0.56)	<10	Neg (<1)	Neg	Brazil, Argentina	Fever, maculopapular rash, myalgia, sore throat	This study
			2.5.2016	75	ND	Neg (0.3)	Neg (0.06)	80	Neg (<1)	ND			
5	35	F	31.3.2016	27	Neg (serum)	Pos (2.6)	Neg (0.12)	10,240	Neg (<1)	Neg	Brazil	Fever, maculopapular rash, eye pain, headache, joint pain	This study
6	33	F	9.8.2016	9	ZIKV RNA Pos (EDTA-blood Pos; serum Neg)	Pos (3.1)	Neg (0.09)	80	Pos (2.5)	Neg	Costa Rica	Headache, maculopapular rash, tired	This study

7	33	F	29.7.2016	7	Neg (serum)	Pos (low; 1.4)	ND	<10	Neg (<1)	Neg	NA	NA	This study
			16.8.2016	25	Neg (serum)	Pos (3.5)	Neg (0.07)	>2,560	Pos (low; 1.6)	ND			
8	26	M	16.11.2016	NA	Neg (serum)	Pos (3.4)	Pos (0.23)	160	Neg (<1)	Neg	NA	NA	This study
9	26	M	17.11.2016	NA	Neg (serum)	Pos (4.7)	BL (0.13)	5,120	Pos (low; 1.8)	Neg	Palawan, Philippines	NA	This study
10	59	F	15.12.2017	NA	Neg (serum)	Pos (2.0)	Neg (0.05)	1,280	Neg (<1)	Neg	NA	NA	This study

DOS, days after onset of symptoms; Pos, posit; Neg, negative; M, male; F, female; NA, not available; ND, not done; ZIKV, zika virus; DENV, dengue virus;

*Based on Korhonen et al. 2015

^aAnti-Zika virus IgM ELISA (Euroimmun, Lübeck, Germany); 0.8 Neg, 0.8-1 borderline, >1.1 Pos.

^bZIKV NS1 Ag ELISA (BioFront Technologies, FL, USA), assay performance according to manufacturer: range of quantification: 0.13-8 ng/ml; results below 0.13 ng/ml were considered negative (based on negative human serum panel tested), borderline (BL) results 0.13-0.20 ng/ml and positive (Pos) >0.20 ng/ml.

^cIn-house IgG IFA (HUSLAB, Helsinki University Hospital, Helsinki, Finland)

^dDengue Virus IgM Capture DxSelect™ (Focus Diagnostics, Diasorin Molecular LCC, California, USA); <1.0 Neg, >1.0 Pos.

^eDengue NS1 antigen test (SD BIOLINE, Alere/ Abbott GmbH & Co. KG, Wiesbaden, Germany)