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

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A R T I C L E   I N F O

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Dengue
TBEV

A B S T R A C T

The laboratory confirmation of Zika virus (ZIKV) infection, and the differential diagnosis from other flavivirus infections such as dengue virus (DENV), often requires the use of several diagnostic test types. Cross-reactions and secondary infections complicate the serological diagnosis and specific viral RNA detection assays are often needed for confirming the diagnosis.

The aim of this study was to validate serological and molecular methods for diagnosing ZIKV infection. This included the evaluation of a ZIKV RT-qPCR assay for diagnostics that was previously set up for research use and to compare the ZIKV, DENV and TBEV EIA methods. External and in-house controls and pre-characterized sample panels were tested, and also automated and manual nucleic acid extraction methods were compared.

A total of ten Finnish traveler patients were diagnosed with acute ZIKV infection during 2015–2017 including one suspected dual DENV and ZIKV infection. These samples along with panels of DENV and tick-borne encephalitis virus (TBEV) infections were used to test the cross-reactive properties of ZIKV, DENV and TBEV IgM assays. Additionally, the diagnosed acute ZIKV patient samples were tested using commercially available diagnostic DENV NS1 antigen assay and a ZIKV NS1 antigen assay intended for research use.

The ZIKV RT-qPCR assay was demonstrated to be both specific and sensitive (one genome per reaction) and suitable for routine diagnostic use utilizing automated nucleic acid extraction. Of the tested IgM tests the NS1 antigen-based ZIKV IgM (Euroimmun) assay performed with least cross-reactivity with a specificity of 97.4%. The DENV IgM assay (Focus Diagnostics) had specificity of only 86.1%. The results are in line with previous studies and additionally highlight that also acute TBEV patients may give a false positive test result in DENV and ZIKV IgM assays.

1. Introduction

Family \textit{Flaviviridae}, genus \textit{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...
description in Jääskeläinen et al. (2015) and final template amount of 10 step qRT-PCR System (Invitrogen, Carlsbad, CA, USA), using a protocol from Faye et al., 2013), 200 nm probe [VIC- CTYAGACCAGCT- causing severe fetal brain abnormalities (Driggers et al., 2016). In brief, a Finnish traveler subsequently leading to congenital ZIKV infection used previously in studying a case of ZIKV infection in a pregnant woman. 2. Materials and methods

Assays for their cross-reactivity properties. Manual nucleic acid extraction methods and testing the flavivirus IgM 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- CTYAGACCAGCT- GAA-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 polymeravirus and human herpesvirus 6 screening (Table 1). The patient samples were used 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 Zeptometrix 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 (Mögli et al., 2016) 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 7 min at room temperature. Both DENV and ZIKV IFAs were carried out using serially diluted patient sera. The diluted samples were incubated for 30 min at 37°C followed by washings with 1 x PBS and conjugation using FITC-conjugated Affini Pure Fab(’b)2 Fragment Goat Anti-Human IgG (H+L) (Jackson ImmunoResearch, UK) for 30 min 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 ELIA (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 LOD was one ZIKV RNA template copy per ZIKV-RT-qPCR reaction using quantified in vitro transcribed RNA from ZIKV NS5.
Table 2
The IgM cross-testing of serum samples from patients with acute DENV, ZIKV, TBEV and malaria infection.

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

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).

1Dengue Virus IgM Capture DxSelect™ (Focus Diagnostics, Diasorin Molecular LLC, California, USA).
2Pan-flavi-PCR positives (Korhonen et al. unpublished).
3Anti-Zika virus IgM ELISA (Euroimmun, Lübeck, Germany).
4μ-capture TBEV IgM EIA (in-house; HUSLAB, Helsinki University Hospital, Helsinki, Finland).
5Diagnosis based on blood-smear samples: 11 Plasmodium falciparum, one P. vivax, and one plasmodium spp.

Tested negative for ZIKV and DENV RNA.

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
Table 3

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

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Age</th>
<th>Sex</th>
<th>Sampling date</th>
<th>DOS</th>
<th>ZIKV RNA</th>
<th>ZIKV IgM-ELISA (index)</th>
<th>ZIKV NS1 Ag ELISA* (Neg/Pos (ng/ml))</th>
<th>DENV/ZIKV IgG IFA (titer)</th>
<th>DENV IgM-ELISA (Neg/Pos)</th>
<th>DENV NS1 Ag (Neg/Pos)</th>
<th>Travelling history</th>
<th>Symptoms</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37</td>
<td>M</td>
<td>24.6.2015</td>
<td>7</td>
<td>ZIKV RNA Pos (urine; serum Neg)</td>
<td>Pos (4.1)</td>
<td>Pos (0.26)</td>
<td>1,280</td>
<td>Pos (low; 1.9)</td>
<td>Neg</td>
<td>Maldivé</td>
<td>flu-like, mild fever, rash, eye pain, arthralgia</td>
<td>Korhonen et al. 2015, Eurosurv.</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
<td>F</td>
<td>29.12.2015</td>
<td>30</td>
<td>ZIKV RNA Pos (serum**</td>
<td>Pos (3.4)</td>
<td>Neg (0.12)</td>
<td>10,240</td>
<td>Neg (&lt; 1)</td>
<td>Neg</td>
<td>Guatemala, Mexico, Belize</td>
<td>Pregnant, microcephaly, abortion, rash, fever, eye pain</td>
<td>Driggers et al., 2016, NEJM</td>
</tr>
<tr>
<td>3</td>
<td>44</td>
<td>F</td>
<td>16.1.2016</td>
<td>NA</td>
<td>ZIKV RNA Pos and DENV RNA Pos (serum)</td>
<td>Neg (0.10)</td>
<td>Neg (0.10)</td>
<td>320</td>
<td>Pos (low; 1.4)</td>
<td>Pos</td>
<td>NA</td>
<td>NA</td>
<td>This study</td>
</tr>
<tr>
<td>4</td>
<td>39</td>
<td>F</td>
<td>16.2.2015</td>
<td>3</td>
<td>ZIKV RNA Pos (serum)</td>
<td>Neg (0.5)</td>
<td>Pos (0.56)</td>
<td>&lt; 10</td>
<td>Neg (&lt; 1)</td>
<td>Neg</td>
<td>Brazil, Argentina</td>
<td>Fever, maculopapular rash, myalgia, sore throat</td>
<td>This study</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
<td>F</td>
<td>31.3.2016</td>
<td>27</td>
<td>ZIKV RNA Pos (serum)</td>
<td>Pos (2.6)</td>
<td>Neg (0.12)</td>
<td>80</td>
<td>Neg (&lt; 1)</td>
<td>Neg</td>
<td>Brazil</td>
<td>Fever, maculopapular rash, eye pain, headache, joint pain</td>
<td>This study</td>
</tr>
<tr>
<td>6</td>
<td>33</td>
<td>F</td>
<td>9.8.2016</td>
<td>9</td>
<td>ZIKV RNA Pos (EDTA-blood Pos; serum Neg)</td>
<td>Pos (3.1)</td>
<td>Neg (0.09)</td>
<td>80</td>
<td>Pos (2.5)</td>
<td>Neg</td>
<td>Costa Rica</td>
<td>Headache, maculopapular rash, tired</td>
<td>This study</td>
</tr>
<tr>
<td>7</td>
<td>33</td>
<td>F</td>
<td>29.7.2016</td>
<td>7</td>
<td>ZIKV RNA Pos (serum)</td>
<td>Pos (low; 1.4)</td>
<td>ND</td>
<td>&lt; 10</td>
<td>Neg (&lt; 1)</td>
<td>Neg</td>
<td>NA</td>
<td>NA</td>
<td>This study</td>
</tr>
<tr>
<td>8</td>
<td>26</td>
<td>M</td>
<td>16.11.2016</td>
<td>25</td>
<td>ZIKV RNA Pos (serum)</td>
<td>Pos (3.5)</td>
<td>Neg (0.07)</td>
<td>&gt; 2,560</td>
<td>Pos (low; 1.6)</td>
<td>Pos</td>
<td>ND</td>
<td>NA</td>
<td>This study</td>
</tr>
<tr>
<td>9</td>
<td>26</td>
<td>M</td>
<td>17.11.2016</td>
<td>NA</td>
<td>ZIKV RNA Pos (serum)</td>
<td>Pos (3.4)</td>
<td>Neg (0.23)</td>
<td>160</td>
<td>Neg (&lt; 1)</td>
<td>Neg</td>
<td>NA</td>
<td>NA</td>
<td>This study</td>
</tr>
<tr>
<td>10</td>
<td>59</td>
<td>F</td>
<td>15.12.2016</td>
<td>NA</td>
<td>ZIKV RNA Pos (serum)</td>
<td>Pos (4.7)</td>
<td>Neg (0.13)</td>
<td>5,120</td>
<td>Pos (low; 1.8)</td>
<td>Pos</td>
<td>Palawan, Philippines</td>
<td>NA</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pos (2.0)</td>
<td>Neg (0.05)</td>
<td>1,280</td>
<td>Neg (&lt; 1)</td>
<td>Neg</td>
<td>NA</td>
<td>NA</td>
<td>This study</td>
</tr>
</tbody>
</table>

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

* Based on Korhonen et al. 2015.

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

b ZIKV 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.

c In-house IgG IFA (HUSLAB, Helsinki University Hospital, Helsinki, Finland).

d Dengue virus IgM Capture DiSelect™ (Focus Diagnostics, Diasorin Molecular LLC, California, USA); < 1.0 Neg, > 1.0 Pos.

e Dengue NS1 antigen test (SD BIOLINE, Alere/Abbott GmbH & Co KG, Wiesbaden, Germany).
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., 2017; 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; Mansuy et al., 2017; Murray et al., 2017).

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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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