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Collaborative Study to Evaluate a Candidate World Health Organization International Standard for Zika Virus for Nucleic Acid Amplification Technique (NAT)-Based Assays

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NOTE:
This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by **16 September 2016** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: **Dr C M Nübling** (nueblingc@who.int)

Summary

The aim of the collaborative study was to assess the suitability of a candidate International Standard (IS) for Zika virus (ZIKV) for nucleic acid amplification technique (NAT)-based assays. Potency of the candidate IS and related reference preparations was evaluated using a range of NAT-based assays for ZIKV RNA with the aim of assigning an internationally agreed unitage to the candidate WHO IS.

The candidate IS consisted of an inactivated, lyophilized ZIKV preparation formulated in a stabilizing, neutral solution and intended for dilution using a range of different types of sample matrix. The virus strain used for the preparation of the candidate IS originated from a ZIKV-infected patient from French Polynesia, closely related to ZIKV strains currently circulating in the Asia-Pacific region and central and South America. Further strains from the Asian ZIKV lineage were included in the study as well as two preparations derived from African ZIKV isolates. The samples consisted of a mixture of inactivated ZIKV reference materials as well as clinical materials (urine or plasma) from ZIKV-infected patients. In addition, a panel of *in vitro* transcribed RNAs covering partial ZIKV genome sequences were included in the study.

The samples for evaluation were distributed to 24 laboratories from 11 different countries. The samples were assayed on three separate days and the data were collated and analysed at the Paul-Ehrlich-Institut (PEI). Data were returned by 21 of the participating laboratories, in total 37 sets of data were analysed; 19 from quantitative assays and 18 from qualitative assays. The assays used consisted of a mixture of in-house developed and commercial assays (currently available or in development). The results showed that all samples were detected consistently by the majority of participants. The candidate standard is very stable under recommended storage conditions, i.e. at or below -20ºC, and is therefore suitable for long term use. On-going real-time and accelerated stability studies of the candidate IS are in progress.

It is proposed that the heat-inactivated and lyophilized preparation with cell culture-derived French Polynesian ZIKV strain be established as the 1st IS for ZIKV RNA with an assigned unitage of 50,000,000 International Units per ml.

Introduction

Zika virus (ZIKV) was first identified in 1947 and circulated in Africa between the 1960s and 1980s, emerging in 2007 in Yap Island, Micronesia (Duffy *et al*., 2009). In 2013/2014 ZIKV appeared in Tahiti, French Polynesia (Cao-Lormeau *et al*., 2014), spreading to New Caledonia and Cook Islands in 2014 (Musso *et al*., 2014), and subsequently causing the on-going outbreak in Brazil (Zanluca *et al*., 2015; Campos *et al*., 2015) with wider dissemination of the virus in Central and South America and continued circulation in Pacific region. In other parts of the world, for example North America and Europe, there are reports of ZIKV infection in travellers returning from affected areas. ZIKV is commonly transmitted by *Aedes aegypti* and *Aedes albopictus*, the same vectors that transmit Chikungunya and Dengue viruses.
Infection with ZIKV is usually asymptomatic or results in usually mild disease typically presenting with fever, headache, malaise, conjunctivitis and skin rash (Musso and Gubler, 2016), however, complications with ZIKV infection include microcephaly and brain abnormalities in the fetus (Rasmussen *et al*. 2016; Driggers *et al*., 2016) as well as other neurological conditions in adults such as Guillain-Barré syndrome (Cao-Lormeau *et al*., 2016). ZIKV, like other arboviruses, has the potential to be transmitted by transfusion of blood and blood products

(Cunha *et al*., 2016; Barjas-Castro *et al*., in press). Sexual transmission of ZIKV is being increasingly reported (Musso *et al*., 2015; Deckard *et al*., 2016).
There are no specific therapies or vaccines for ZIKV. Suspected clinical cases of ZIKV infection can only be confirmed by detection of virus RNA in plasma, or other bodily fluids such as urine (Anonymous, 2016) and saliva using nucleic acid amplification techniques (NAT). False positive results may occur with serological testing due to cross-reactivity with other flaviviruses such as Dengue virus; testing algorithms for discrimination are restricted to specialist laboratories. Currently, there is no standardization of NAT-based assays for the detection of ZIKV RNA. In early 2016, the World Health Organization (WHO) asked the Paul-Ehrlich-Institut (PEI), a WHO collaborating centre for quality assurance of blood products and *in vitro* diagnostic devices, to develop an International Standard (IS) for ZIKV RNA for use in NAT assays.

Study materials

Candidate International Standard – 11468/16

The ZIKV strain PF13/251013-18, selected for the preparation of the candidate IS, was isolated in 2013 from the serum of a French Polynesian patient (Aubry *et al*., 2016; Trösemeier *et al*., 2016). For preparation of the candidate IS, ZIKV was propagated in Vero E6 cells using Dulbecco's modified Eagle medium, without serum. Supernatants were harvested 8 to 10 days post-infection, clarified by low speed centrifugation and stored at -80°C prior to final formulation and lyophilization.

Heat inactivation of the stock virus was performed by initially filtering through a 0.45 µm filter and allowing 30 ml aliquots of the stock to equilibrate to 56°C in a water bath and then incubating for a period of 1 hour. The titre of the bulk virus stock before heat-inactivation was 7.07 $\log_{10}$ $TCID_{50}$/ml. The heat inactivated ZIKV stock was tested for infectious virus by large volume plating/bulk titration (28.8 ml). The $\log_{10}$ reduction factor for the heat treatment was $\geq 7.89 \pm 0.31$. The candidate IS was dispensed in 0.5 ml aliquots per vial for lyophilization (see below), and the calculated residual infectious virus load per vial, after heat-inactivation and dilution of the bulk preparation, is ≤ -1.98 $\log_{10}$ $TCID_{50}$.

For the lyophilization the inactivated virus stock was diluted 1:7.2 in a solution containing hydroxyectoin (Bitop AG, Witten, Germany) at a final concentration of 0.6 M. The filling and lyophilization was performed by an ISO 13485:2003 accredited Swiss company and processing took place between the 4th and 7th of April, 2016. For processing, 0.5 ml volumes were dispensed into 4 ml screw-cap glass vials. After completion of the freeze-drying procedure, the vacuum was broken by the introduction of nitrogen gas and the vials sealed. The vials were further secured with screw caps prior to storage at -20°C. A total of 4092 vials were prepared. The coefficient of variation (% CV) of the filled vials was 0.69% (n=27). Residual moisture was determined by Karl Fischer analysis and was 1.35% (n=14). Testing of samples of 11468/16 post-lyophilization revealed that there was an approximate drop in ZIKV RNA titre of 0.7-0.8 $\log_{10}$ copies/ml compared to the bulk.

Because the candidate IS was to be assigned a unitage with respect to the ZIKV RNA content, homogeneity of the filling/freeze-drying was assessed using real time-PCR. Extraction of RNA was performed using 200 µL of the sample using the QIAamp MinElute Virus Spin Kit (Qiagen GmbH, Hilden, Germany). Elution of the viral nucleic acid was performed using 70 µL of elution buffer, and 5 µL of the eluate was used for the RT-PCR. ZIKV RNA was detected using reverse-transcription real time PCR. Amplification reactions were performed using the

LightMix® Modular Zika Virus kit (TIB Molbiol GmbH, Berlin, Germany) and the LightMix® Modular Equine Arteritis Virus (EAV) RNA extraction control. Detection of ZIKV and EAV RNA was performed using the LightCycler 480 (Roche Applied Science GmbH, Mannheim, Germany) in accordance with the manufacturer's instructions. The assay is able to detect 10 copies of ZIKV RNA per reaction. A standard curve was prepared using an *in vitro* ZIKV RNA transcript (GenExpress Gesellschaft für Proteindesign mbH, Berlin, Germany). The crossing threshold or cycle threshold (C_T) values were determined for 30 vials of the candidate IS; the mean C_T value was 20.09, with a CV of 1.96%, indicating that the filling was of acceptable homogeneity.

Vials of the candidate WHO IS are held at the Paul-Ehrlich-Institut, Paul-Ehrlich-Straße 51-59, D-63225 Langen, Germany. The vials are kept at -20°C with continuous temperature monitoring.

All manufacturing records are held by PEI and are available on request by the WHO Expert Committee on Biological Standardization.

Inactivated reference materials

11469/16

This preparation was provided by ZBS 1 - Highly Pathogenic Viruses, Robert Koch Institute (RKI), Germany. Zika virus strain Uganda MR766 (Dick *et al*. 1952) was kindly provided by Dr. Maria João Alves (CEVDI/INSA National Institute of Health Dr. Ricardo Jorge, Portugal) via the European Network for Diagnostics of "Imported" Viral Diseases – (ENIVD). For thisreference material, the virus has been propagated in cell culture at the RKI, inactivated by heat-treatment and gamma irradiation, and diluted in a proprietary matrix and lyophilized. The standard is stored at +4°C. Details are given in the following link: https://www.rki.de/DE/Content/Institut/OrgEinheiten/ZBS/ZBS1/Zika_PCR_standard.pdf?__blob=publicationFile

11470/16 and 11471/16

These preparations were provided by the Center for Biologics Evaluation and Research/U.S. Food and Drug Administration (CBER/FDA). The ZIKV strains were isolated in Cambodia (11470/16) and Puerto Rico (11471/16) in 2010 and 2015, respectively. The isolates were propagated in cell culture and heat-inactivated, diluted in pooled human plasma and stored frozen at ≤ -60°C.

11474/16

This was prepared by the PEI using the same inactivated virus preparation and dilution as the candidiate IS, however, this preparation was diluted in pooled human plasma and lyophilized. Vials of 11474/16 were stored frozen at -20°C

114579/16

This was prepared by the National Institute for Biological Standards and Control (NIBSC), United Kingdom. This preparation (NIBSC code 16/110) has been produced using the MP 1751 ZIKV strain from Uganda (Haddow *et al*., 1964) as supplied by Public Health England Culture Collection. Details are given in the following link: https://www.phe-culturecollections.org.uk/products/viruses/detail.jsp?refId=1308258v&collection=ncpv. The virus was propagated in cell culture, heat-inactivated and diluted in pooled human plasma prior to lyophilization. Vials of 11579/16 were stored frozen at -20°C.

Clinical materials

11467/16
This material was a pool of two ZIKV-positive urine samples from Europe, one patient developed ZIKV infection after travelling to South America and the other was obtained from a patient who became infected after sexual intercourse with a partner who had visited South America. The pooled material was stored frozen at ≤ -60ºC.

11472/16
This viraemic plasma sample came from a Brazilian patient infected with ZIKV. Plasma has been stored as liquid/frozen material at ≤ -60ºC after ~1:50 dilution in pooled human plasma which tested negative for ZIKV RNA at the PEI.

11473/16
This viraemic plasma sample came from a Brazilian blood donor infected with ZIKV (Cunha *et al*., 2016; Barjas-Castro *et al*., 2016). Plasma has been stored as liquid/frozen material at ≤ -60ºC after ~1:10 dilution in pooled human plasma which tested negative for ZIKV RNA at the PEI.

In vitro transcribed RNAs

11475/16-11478/16

Two sets of *in vitro* transcribed RNAs (IVTs) were included in the study and these were prepared by the University of Bonn, Germany. The IVTs have been described elsewhere: http://www.who.int/bulletin/online_first/16-175950.pdf, futher information is provided in Appendix 2. For use in the study, the IVTs were diluted in 10 mM Tris pH 8.0 containing carrier nucleic acid and provided at 2 different concentrations i.e. 10,000 copies/µl and 1,000 copies/µl.

The phylogenetic relationship of the ZIKV strains included in the collaborative study are shown in Figure 1. Sequences were not available for all strains included in the study.

Collaborative Study

The collaborative participants are listed in Appendix 1; 24 laboratories from 11 different countries agreed to participate in the study. For the purposes of data analysis, each laboratory has been referred to by a code number allocated at random and not representing the order of listing in Appendix 1.

All collaborative study materials were shipped to participating laboratories on dry ice and participants requested to store the materials at or below -60°C until use. Participants received samples representing both African and Asian ZIKV lineages and samples represented a mixture of inactivated reference materials as well as a small number of clinical samples and *in vitro* RNA transcripts.

The samples included in the panel are described above and listed in Table 1. The approximate titres of the study materials were provided to the participants for guidance (Table 2). Sufficient materials were provided for three separate assay runs. Additional vials were provided for laboratories using larger extraction volumes. In the case of sample 11467/16 (pooled ZIKV-positive human urine), because of limited sample volume, this sample was only distributed to

those laboratories that confirmed that they tested this type of sample routinely. Laboratory 13 only received inactivated samples and the IVTs.

Participants were asked to test the panel using their routine assay for ZIKV RNA, testing the panel of samples in three separate assay runs, using fresh vials of each sample for each run. Where laboratories performed quantitative tests, they were requested to report results in copies/ml, suggested dilutions to test for each sample were proposed in the study protocol (Table 2). In the case of qualitative assays, participants were requested to assay each sample by a series of one $\log_{10}$ dilution steps, to obtain an initial estimate of an end-point. For the two subsequent assays, they were requested to assay half-$\log_{10}$ dilutions around the end-point determined in their first assay. If, in the second assay, all dilutions were positive, or all negative, then the dilution series were to be adjusted accordingly for the final assay run. Participants were asked to note if replicate extractions and replicate amplification/detection steps were performed. Results were reported as either positive i.e. ZIKV RNA detected or negative. Electronic data sheets and a method reporting form were provided so that all relevant information (e.g. C_T values for the respective dilutions where real time PCR methods were used or signal to cut-off (S/Co) values - e.g. for transcription-mediated assays) could be reported.

For the preparation of dilutions, participants were requested to use their usual diluent representing the matrix of the normal test specimens e.g. ZIKV negative plasma. It was recommended to dilute sample 11468/16 in plasma, urine, or other types of sample matrix that might be used for ZIKV diagnostic testing. It was suggested to dilute sample 11469/16 using cell culture medium.

Several lyophilized preparations were evaluated in the study and these were reconstituted before use by participants using molecular grade, nuclease-free water. Samples 11468/16 and 11474/16 were reconstituted in 0.5 ml of water; samples 11469/16 and 11579/16 was reconstituted in 0.1 and 1.0 ml of water, respectively. After addition of water, it was recommended that the samples be left for a minimum of 20 minutes with occasional agitation before use. All other samples were provided as liquid/frozen materials.

Concerning the IVTs, participants were requested to test the IVTs by addition of 2 µl of RNA directly to the amplification/detection reaction. Because these IVTs do not cover the entire ZIKV genome, participants were asked to review the sequences (Appendix 2) to determine if they included the target region of their assay(s). In the case of quantitative assays participants were requested to report results as copies/ml. For the qualitative assays, results for testing of the IVTs were to be reported as C_T values, or as positive (RNA detected) or negative (RNA not detected). Because the IVTs were not intended to undergo extraction, it was obvious that fully automated systems might not be suitable for evaluation of these samples.

Statistical Methods

Quantitative Assays

Quantitative assays were performed over a range of specific dilutions expected to fall in the linear range of the majority of assays (Table 2).
Evaluation of quantitative results includes the complete dilution range used by the participants, as with correction of the dilution factor the data show comparable results between dilutions. Estimates (expressed in $\log_{10}$ NAT-detectable units/ml) for each laboratory, assay and matrix were derived by the arithmetic mean from up to 3 runs and 3 replicates.

Qualitative Assays

For qualitative data analysis, results from all assays were pooled to give the number of positives out of the total number tested at each dilution. If it is assumed that a single 'detectable unit' will give a positive result, and that the probability of a positive result follows a Poisson distribution, the EC63 (the dilution at which 63% of the samples are expected to be positive) was chosen as the end-point. For each laboratory and sample, these end-points were estimated by means of probit analysis. For assays where the change from complete negative to complete positive results occurred in two or fewer dilution steps for all samples, the Spearman-Kaerber method was applied for EC63 estimation. The calculated end-point was used to give estimates expressed in $\log_{10}$ NAT-detectable units/ml after correcting for the equivalent volume of the test sample.

Combination of quantitative and qualitative data

Quantitative and qualitative data were combined by means of mixed linear models to calculate estimates per sample and laboratory, per sample and assay type (qualitative, quantitative), per sample and matrix (plasma, medium, saliva, serum, urine or direct testing for the IVTs), and overall estimates for each sample. Distribution of participants, matrix, and assay types were graphically presented in histogram form.

For assignment of the potency to the candidate IS, data from 70 assays were combined using a mixed linear model with random factors *laboratory*, *assay type* (qualitative/quantitative), and *matrix*. Data from all 21 laboratories was included with 17 participants providing data for more than one assay. Most assays were quantitative (41 assays, 29 qualitative assays) and tested in plasma (35 assays; urine: 23, saliva: 9, serum: 2, and medium: 1 assay). No assays had to be excluded.

Relative potencies

Potencies of all samples, for the quantitative assays, were estimated relative to the candidate IS 11468/16 using parallel line analysis of log transformed data. In the case of the qualitative assays, the relative potencies were determined using parallel line analysis of probit transformed data.

C_T values

For assays reporting C_T values, these were evaluated for both qualitative and quantitative methods (relative to the candidate IS 11468/16) using a parallel line model for each laboratory combined for all evaluable (i.e. valid) assay runs. Relative potencies from C_T values were shown as forest plots to allow for a comparison of participants, assay types, and, where applicable, matrix.

Parallel line and probit analysis were performed according to methods as described in chapter 5.3, "Statistical analysis of results of biological assays and tests", of the European Pharmacopoeia (8th Edition, Strasbourg, France Council of Europe; 2015). The statistical analysis was performed with SAS®/STAT software, version 9.4, SAS System for Windows, and CombiStats, version 5.0, EDQM, Council of Europe (www.combistats.eu).

Results

Data received

Data were received from a total of 21 of the 24 participating laboratories. In total, 37 sets of data were returned; 19 from quantitative assays and 18 from qualitative assays. Some laboratories reported results for more than one type of assay. Where a laboratory performed more than one assay method, the results from the different methods were analysed independently, as if from separate laboratories, and coded, for example, laboratory 16a and laboratory 16b.

The types of methods used by the participants are listed in Table 3. Assays included in the study targeted several different regions of the ZIKV genome – these included the propeptide, envelope, NS1, NS2A, NS2B, NS5 as well as the 3' UTR. The assays included in-house developed assays, ones based on scientific publications as well as commercial assays (including ones still in development). The vast majority of assays were based on real-time PCR. Different types of extraction method were used (Table 3). The types of matrix evaluated for the dilution of sample 11468/16 are indicated in Table 3 (these do not necessarily reflect the types of matrix validated for the respective tests).

The urine sample (11467/16) was distributed to 12 laboratories, of these 7 returned results that could be analysed.

Laboratory 2a used the ZIKV reference material provided by the RKI as a calibrator in their quantitative assay. Laboratory 5 used the European Virus Archive reference material as calibrator for three quantitative assays (5a-5c). Other laboratories performing quantitative assays either used IVTs or ZIKV with quantified genomic titres for quantitation.

Laboratory 10 did not test sample 11469/16.

Laboratory 15 performed three different types of assay (quantitative), in the case of 15c, results were reported only for the panel of IVTs.

Laboratory 16 used 2 different extraction kits, one for runs 1 and 2 (same extract) and a different kit for the third run.

The different ZIKV strains from both the African and Asian lineages were well detected by the assays used in the study. In some cases, differences in the efficiency of detection were observed