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Relative analytical sensitivity of donor nucleic acid amplification technology screening and diagnostic real-time polymerase chain reaction assays for detection of Zika virus RNA

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BACKGROUND: Zika virus (ZIKV) has spread rapidly in the Pacific and throughout the Americas and is associated with severe congenital and adult neurologic outcomes. Nucleic acid amplification technology (NAT) assays were developed for diagnostic applications and for blood donor screening on high-throughput NAT systems. We distributed blinded panels to compare the analytical performance of blood screening relative to diagnostic NAT assays.

STUDY DESIGN AND METHODS: A 25-member, coded panel (11 half-log dilutions of a 2013 French Polynesia ZIKV isolate and 2015 Brazilian donor plasma implicated in transfusion transmission, and 3 negative controls) was sent to 11 laboratories that performed 17 assays with 2 to 12 replicates per panel member. Results were analyzed for the percentage reactivity at each dilution and by probit analysis to estimate the 50% and 95% limits of detection (LOD_{50} and LOD_{95}, respectively).

RESULTS: Donor-screening NAT assays that process approximately 500 μL of plasma into amplification reactions were comparable in sensitivity (LOD_{50} and LOD_{95}, 2.5 and 15-18 copies/mL) and were approximately 10-fold to 100-fold more sensitive than research laboratory-developed and diagnostic reverse transcriptase-polymerase chain reaction tests that process from 10 to 30 μL of plasma per amplification. Increasing sample input volume assayed with the Centers for Disease Control and Prevention reverse transcriptase-polymerase chain reaction assays increased the LODs by 10-fold to 30-fold.

CONCLUSIONS: Blood donor-screening ZIKV NAT assays demonstrate similar excellent sensitivities to assays currently used for screening for transfusion-transmitted viruses and are substantially more sensitive than most other laboratory-developed and diagnostic ZIKV reverse transcriptase-polymerase chain reaction assays. Enhancing sensitivities of laboratory-developed and diagnostic assays may be achievable by increasing sample input.

ABBREVIATIONS: BSRI = Blood Systems Research Institute; ECDC = European Center for Disease Prevention and Control; EFS = Etablissement Français du Sang; EUA = emergency use authorization; HI = high input; IC = internal control; ILM = Institut Louis Malarde; IND = investigational new drug; LI = low input; LOD(s) = limit(s) of detection; PFU(s) = plaque-forming unit(s); RMS = Roche Molecular Systems; TT(s) = transfusion transmission(s); VL = viral load; ZIKV = Zika virus.

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Zika virus (ZIKV), a single-stranded, positive-polarity RNA virus, belongs to the *Flavivirus* genus within the *Flaviviridae* family along with other clinically relevant arboviruses, such as yellow fever, Japanese encephalitis, West Nile, and dengue viruses (DENVs).[1] Although ZIKV is mostly transmitted from human to human through the bite of *Aedes* mosquitoes,[2] intrauterine,[3,4] perinatal,[5] sexual,[6-9] and probable transfusion transmissions (TTs)[10,11] have been documented. In addition to high levels of viremia in plasma, ZIKV RNA and infectious ZIKV have been found in urine,[12-17] breast milk,[5] saliva,[18] and semen.[9,19] Considering the risk for severe clinical disease outcomes after maternal ZIKV infection,[20-24] monitoring and mitigation strategies are being deployed to alleviate the risk of transmission to pregnant women and conceiving couples. After the confirmation of increasing numbers of imported ZIKV infection cases in the United States,[25] including women delivering babies with ZIKV congenital syndrome after a history of travel to countries reporting active ZIKV transmission,[26] the US Centers for Disease Control and Prevention (CDC) released a travel advisory discouraging pregnant women from traveling to countries currently experiencing ZIKV outbreaks and new guidelines for the testing of pregnant women and their newborns living in or returning from epidemic areas.[27-30]

After reports of probable cases of transfusion and sexual transmission in late 2015 and early 2016,[10,11] and in light of high rates of viremic blood donations reported during the French Polynesia outbreak in 2013 and 2014,[31] the US Food and Drug Administration (FDA) issued a "Guidance to Industry" in February 2016 precluding blood donations for 28 days after returning from countries with active ZIKV transmission or sexual contact with men returning from active transmission areas.[32] In active transmission areas, the February 2016 FDA guidance required blood to be imported from areas with no reported local transmission unless donations either were screened for ZIKV RNA by nucleic acid amplification technology (NAT) assays or were pathogen reduced using FDA-approved technologies for plasma and platelets.[32]

Given the necessity of detecting donors in the preseroconversion viremic phase of infection, NAT-based, rather than serology-based, assays were recommended by the FDA,[32] the European Center for Disease Prevention and Control (ECDC),[33] and the World Health Organization (WHO)[34] for sensitive and specific detection of acute ZIKV infection, and particularly for donor screening.[35,36] In response to this rapidly emerging threat,[36] government and academic laboratories and industry partners have developed ZIKV NAT assays. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) diagnostic tests developed by the CDC for the detection of ZIKV RNA (the Trioplex assay, which amplifies ZIKV, DENV and chikungunya virus [CHIKV] RNA, or a modified assay that detects only ZIKV) received emergency use authorization (EUA) from the FDA in March 2016.[37] This assay protocol and key reagents were distributed by the CDC to public health laboratories throughout the United States. Alternative versions of diagnostic RT-PCR and other NAT assays were developed and implemented in the United States and other countries, some of which subsequently received FDA EUA[37] or European Union CE (Conformité Européen) marking. To facilitate blood donor screening, high-throughput and highly sensitive, ZIKV-specific NAT assays were developed for automated NAT testing systems manufactured by Roche Molecular Systems, Inc. (RMS) (the cobas Zika test for use on the cobas 6800/8800 Systems) and Hologic, Inc. (the Procleix ZIKV assay on the Panther system), which FDA has allowed the clinical studies to proceed under Investigational New Drug (IND) authorization for use to screen the blood supply in Puerto Rico and the 50 US states.

With limited access to clinical samples and virus isolates, and no international standards, the evaluation of relative sensitivities of ZIKV NAT assays was initially very challenging. After the FDA released its February 2016 guidance to safeguard the blood supply in areas with active transmission,[32] and with the impending mandate for ZIKV screening of blood donations in Puerto Rico and potentially US states, there was an urgent need to evaluate the performance (analytical sensitivity and specificity) of newly developed, investigational donor NAT screening assays to be implemented under IND authorization, relative to other diagnostic and blood screening assays developed by the CDC and commercial and national laboratories in the United States, France, French Polynesia, and Brazil.

We took advantage of our access to clinical samples from Brazil and ZIKV virus isolates from collaborators in French Polynesia and Brazil to design and execute a blinded panel study to characterize the analytical performance of these assays. We focused on establishing the relative sensitivities of donor-screening NAT assays, which process approximately 500 μL plasma into each amplification relative to laboratory-developed, diagnostic PCR assays, which generally process less than 50 μL of plasma-derived RNA into PCR reactions; and subsequently developed modifications of PCR assays to enhance sensitivity with increased sample input volume. Using well characterized virus stocks and clinical samples with estimated RNA copy numbers, serially diluted samples were created and compiled into blinded panels, which were made available to 11 testing laboratories in the United States (CDC, FDA, Blood Systems Research Institute [BSRI], RMS, and Hologic), France (Etablissement Français du Sang [EFS]), French Polynesia (Institut Louis Malarde [ILM]), and Brazil. Results were compared across testing laboratories to evaluate the relative sensitivities of assays currently used in various settings, including clinical diagnosis, research, and blood donor screening.

MATERIALS AND METHODS

Source and characterization of analytical standards

A French Polynesia ZIKV-positive plasma sample collected during the Zika outbreak in 2013 (PF13/251013-18)[38,39] was cultured to generate an isolate and was expanded in vitro in Vero cells. A French Polynesia isolate supernatant was collected and stored at −80°C and was shared with the BSRI for further expansion to high titer in Vero cells, followed by aliquoting, and storage at −80°C (Fig. 1).[10,11,32,33,40-45] Plasma was acquired from a Brazilian blood donation during the Zika outbreak in 2015 that was implicated in the first reported cases of ZIKV TT.[10] The donation sample was identified as ZIKV RNA-positive after the donor reported symptoms several days postdonation; a recipient of a cellular blood component derived from this donation was tested retrospectively and determined to have acquired ZIKV from the transfusion based on the development of ZIKV viremia posttransfusion. The ZIKV RNA-positive plasma component from the implicated donation was aliquoted and retained in a repository at −80°C in Brazil until shipment on dry ice to BSRI for panel construction (Fig. 1).

The two virus strains used in the panel had been extensively characterized by sequencing.[39,46] Both viral stock of the French Polynesia ZIKV isolate and ZIKV-contaminated Brazilian plasma were quantified at BSRI for ZIKV RNA viral load (VL) by real time RT-PCR and for infectious particle titer in plaque-forming units (PFUs) by plaque assay on Vero cells. The RNA copy number of the French Polynesia ZIKV isolate was calculated from serial dilution, real-time RT-PCR data using a computational method based on maximum-likelihood estimation for copy number and assay sensitivity estimation. The ZIKV RNA endpoint was first determined based on 10-fold dilutions of the viral stock in plasma. Subsequently, eight 3-fold dilutions around the endpoint were prepared by diluting in plasma. Twenty replicate RNA extractions were performed for each of the two highest and two lowest dilutions, whereas 40 replicate RNA extractions were performed for each of the four intermediate dilutions. RNA was reverse transcribed and amplified in quadruplicate wells using two-step, real-time RT-PCR, and the number of positive replicates was recorded for each dilution. Maximum-likelihood estimation was used to combine the Poisson distribution estimators for all dilutions and to generate a copy number estimate that optimally fit the overall serial dilution data. The RNA copy number of the Brazilian ZIKV RNA-positive plasma was calculated by one-step real-time RT-PCR compared with a standard curve of the quantified French Polynesia ZIKV isolate.

Construction of the analytical performance panel

A 25-member, blinded ZIKV analytical performance panel was constructed. It consisted of 11 serial half-log dilutions of the two viral standards (Polynesia isolate and Brazilian plasma) with estimated initial concentrations of ZIKV RNA in defibrinated human serum (Gemini Biosciences), resulting in panels with ZIKV VLs ranging from approximately 10^4 to 10^{-1} copies/mL; the panels also included three replicate negative control donor plasma samples. The dilutions were performed in a single procedure at BSRI to generate a sufficient number of 1-mL aliquots of each dilution, such that identical frozen panels could be used for testing at the originally planned testing laboratories/assays (Fig. 1). When testing was expanded beyond the anticipated number of participating sites, there was insufficient volume of the highest concentration panel members; consequently, 22-member or 20-member panels were created and tested by several laboratories. Panels were stored and shipped at −80°C until testing. Panels were sent to government, research, and commercial laboratories performing a total of 17 different assays for direct comparison of the relative sensitivities of ZIKV RNA detection assays (Fig. 1, Table 1). Each panel member was tested in replicate under code, with a range of 2 to 12 replicates. Results were reported to BSRI for decoding, analysis of the percentage reactivity at each dilution, and probit analysis to estimate the 50% and 95% limits of detection (LOD_{50} and LOD_{95}, respectively).

ZIKV NAT assays

Table 1 summarizes the key characteristics of the assays evaluated in this study. The Appendix provides more detailed summaries of methods for each assay.

Statistical methods

Probit regression was performed using copy numbers as the dependent variable and positive or negative readings as independent variables. The R (version 3.1.1; R Core Team 2014; http://www.R-project.org/) glm module with probit link function was used for probit regression. The fitted probit regression model and its standard errors were used to estimate LOD_{50}, LOD_{95}, and confidence intervals. In addition to analyses of results from individual assays, the results from assays were grouped, based on similar intended applications and methodologies and comparable results, into the following 10 categories:

1. Blood screening NAT assays combined (RMS and Hologic)
2. CDC Puerto Rico (CDC-PR) Trioplex low-input (LI) assays combined
3. CDC-PR high-input (HI) Trioplex assay
4. CDC Fort Collins (CDC-FC) 1087 LI assay
5. CDC-FC 1087 HI assay
6. BSRI/UC Davis
7. FDA/Center for Biologics Evaluation and Research (in house test using CDC-FC 1087 LI assay)

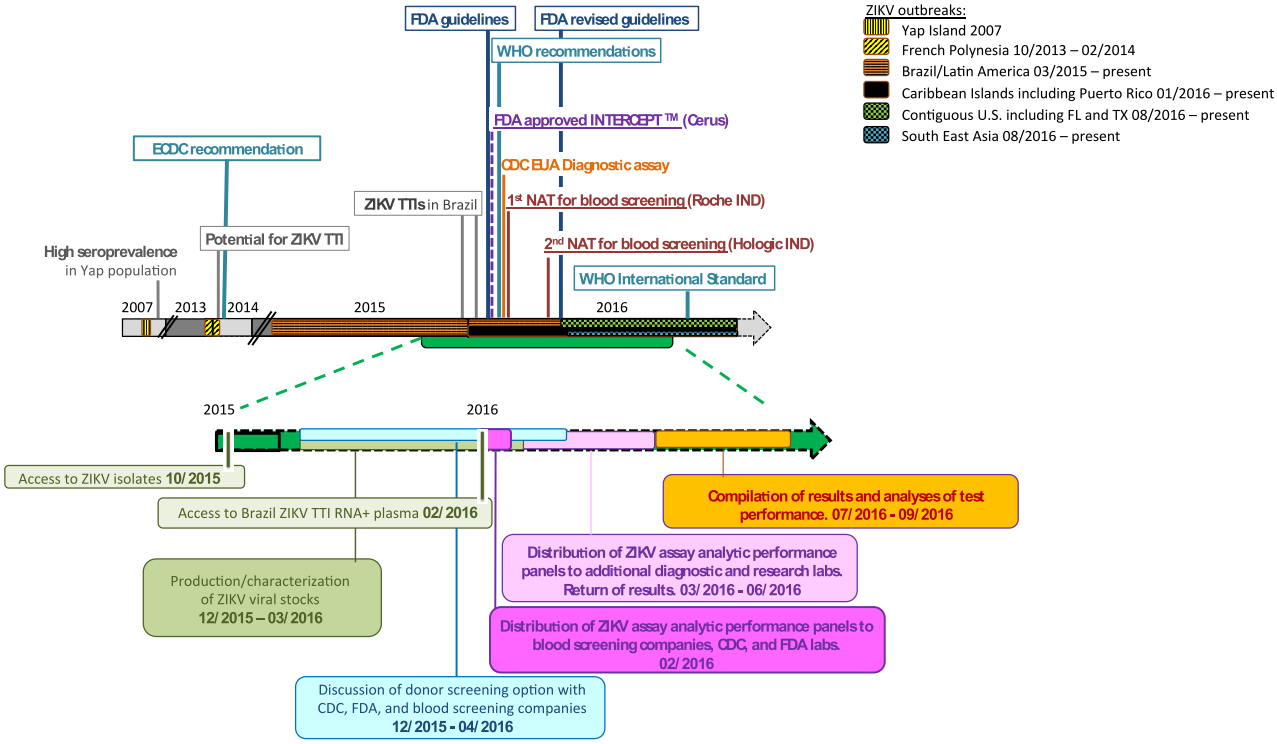

Fig. 1. Key study events are displayed on a timeline relative to: 1) successive ZIKV outbreaks, 2) evidence for ZIKV transfusion-transmission risk, and 3) public health and blood safety responses. The central part of the timeline shows the succession of ZIKV outbreaks starting with the Yap Island outbreak in 2007,[40] followed by the French Polynesia outbreak in 2013 and 2014,[41] the Brazil[42] and Caribbean outbreaks since 2015, and subsequent spread to the contiguous United States in the second half of 2016.[43] Evidence of the potential for and the first cases of demonstrated ZIKV TTI[10,11] are displayed along with the initial releases of guidelines by the ECDC,[33,44] FDA,[32,42] and WHO[45] to mitigate the risk of ZIKV transfusion transmission. The timeline also displays the response from the blood bank industry to the FDA guidelines with the implementation of FDA-approved pathogen-reduction technology and the use under IND approval of investigational NAT assays for blood donor screening. The lower line represents the activities related to the current study and includes, on a magnified timeline: the early access to ZIKV strains; viral expansion and viral stock characterization; access to ZIKV TT, RNA-positive plasma; discussion with government agencies, industry, and research laboratories; construction and distribution of panels; receipt and compilation of test data; and comparative analyses of results.

8. EFS (Altona RealStar ZIKV RT-PCR assay)
9. ILM (1087 E partial primers)
10. Brazil (two assays performed in separate laboratories combined)

RESULTS

Figure 1 presents a timeline with key study activities, including early access to ZIKV strains,[39,46] viral expansion and viral stock characterization, access to ZIKV RNA-positive plasma from Brazil,[10] compilation and distribution of panels, gathering of test result data, and comparative analyses of results. These study-related events are displayed on a timeline relative to key events in the global ZIKV pandemic, including successive ZIKV outbreaks since 2007; first reports of ZIKV TT cases[10,11]; release of initial guidelines to mitigate the risk of ZIKV transmission by the ECDC,[33] the WHO,[47] and the FDA[32,48]; ongoing collaborations with blood screening companies; and implementations of IND NAT assays and FDA-approved pathogen-reduction technology.[49,50]

Results of replicate testing for all 17 individual assays on the decoded analytical performance panels are reported in Table 2. Table 3 presents the decoded results with assays grouped into 10 categories based on similar methods and/or findings (LODs). The assays had good specificity based on no reported reactive results on replicate analysis of the three negative control samples (except for one false-positive result with one of the assays performed at ILM), whereas the sensitivity of the assays varied by one to three logs based on comparisons of the percentage reactivity on serial dilutions and probit analyses to derive LOD_{50} and LOD_{95}.

TABLE 1. Summary of key characteristics, including extraction methods, input sample volumes, cutoffs, reagents, and instruments, of the 17 assays performed at the 11 laboratories that participated in this study

Testing site	Assay	Assay group	Plasma extraction volume	Extraction method	Elution volume	RNA input volume	Derived plasma input*	Primers	Ct cutoff	Reagents	Instrument
Hologic	Procleix TMA	Donor screening NAT	500 μL	Magnetic bead target capture	N/A	500 μL	500 μL	Proprietary	N/A	Proprietary	Procleix Panther
Roche	cobas Zika	Donor screening NAT	850 μL	Magnetic glass particles	Proprietary	Proprietary	Approx 500 μL	Proprietary	Proprietary	Proprietary	cobas 6800/8800 Systems
CDC Ft Collins	CDC Singleplex SI	CDC Ft Collins SI	100 μL	MagMAX	100 μL	5 μL	5 μL	1087	38.5	QuantiTect Probe RT-PCR Kit	iCycler
	CDC Singleplex SI	CDC Ft Collins SI	100 μL	MagMAX	100 μL	5 μL	5 μL	4481	38.5	QuantiTect Probe RT-PCR Kit	iCycler
	CDC Singleplex HI	CDC Ft Collins HI	300 μL	MagMAX	90 μL	20 μL	67 μL	1087	38.5	QuantiFast Pathogen RT-PCR +IC Kit	iCycler
CDC Puerto Rico	CDC Singleplex SI	CDC PR SI	200 μL	MagNApure 96	60 μL	10 μL	33 μL	1087	38	SuperScript III Platinum One-Step qRT-PCR	ABI7500Dx
	CDC Trioplex SI	CDC PR SI	200 μL	MagNApure 96	60 μL	10 μL	33 μL	1087	38	SuperScript III Platinum One-Step qRT-PCR	ABI7500Dx
	Trioplex HI	CDC PR HI	1000 μL	MagNA Pure 96	100 μL	10 μL	100 μL	1087	38	SuperScript III Platinum One-Step qRT-PCR	ABI7500Dx
BSRI	CDC PR SI	BSRI/UC Davis	140 μL	QIAamp Viral RNA Mini Kit	50 μL	5 μL	14 μL	1087	38	SuperScript III Platinum One-Step qRT-PCR	LightCycler 480 real-time PCR
	MTC two step	BSRI/UC Davis	140 μL	QIAamp Viral RNA Mini Kit	50 μL	4.2 μL	12 μL	1087	40	MuLV RT and FastStart Taq	LightCycler 480 real-time PCR
Davis	CDC PR SI	BSRI/UC Davis	140 μL	QIAamp Viral RNA Mini Kit	60 μL	9.6 μL	22 μL	1087	38	Taqman Fast Virus 1-Step Master Mix	7300 Real-time PCR System (Applied Biosystems)
FDA	LDT	FDA	140 μL	QIAamp Viral RNA kit with QIACube	60 μL	5 μL	12 μL	1087	38	One-step RNA-to-Ct kit (Applied Biosystems)	7300 Real-time PCR System (Applied Biosystems)
EFS	LDT (Altona)	EFS	140 μL	NucleoSpin 96	50 μL	10 μL	28 μL	1087	45	RealStar Zika Virus RT-PCR Kit 1.0	Microlab STARlet and CFX96 (Bio-Rad)
ILM	LDT (CDC modified)	ILM	200 μL	NucliSENS easyMAG Biomerrieux	50 μL	5 μL	20 μL	1087	45	2X RT-PCR Reaction Mix for Probes (BioRad)	Automate CFX96 (Bio-Rad)
	LDT (CDC modified)	ILM	200 μL	NucliSENS easyMAG Biomerrieux	50 μL	5 μL	20 μL	835	45	2X RT-PCR Reaction Mix for Probes (BioRad)	Automate CFX96 (Bio-Rad)
Fundação Pró-Sangue	LDT (CDC modified)	Brazil	250 μL	MagNA pure Compact	60 μL	10 μL	42 μL	1087	36	4X Taqman Fast Virus mix (Thermo Fisher)	Step-One Plus machine from Applied Biosystems
Laboratório Richet	LDT (CDC modified)	Brazil	140 μL	QIAamp Viral RNA Mini Kit	60 μL	5 μL	12 μL	1087	38	BIO GENe ZIKV PCR KiT	Thermo Fisher 7300 RT

* The derived plasma input is a calculated parameter that represents the equivalent volume of plasma subjected to amplification: the volume of plasma from which RNA is extracted, multiplied by the proportion of the eluate used for amplification (plasma extraction volume × RNA input volume /elution volume).
N/A = not applicable.

TABLE 2. Results of replicate testing of (top) Brazil plasma and (bottom) Polynesia isolate coded panels, presented as the individual percentage reactivity for each assay

		Donor screening NAT		CDC PR LI			CDC Ft Collins LI			BSRI/UC Davis					ILM		Brazil	
Plasma cp/mL	PFU/mL	Roche, % N = 3	Hologic, % N = 4	CDC PR Singleplex-LI, % N = 3	CDC PR Trioplex-LI, % N = 3	CDC PR Trioplex-HI, % N = 4	CDC FC 1087-11, % N = 3	CDC FC 4481-11, % N = 3	CDC Ft Collins 1087-HI, % N = 3	BSRI MTC, % N = 4	BSRI CDC, % N = 4	Davis CDC, % N = 12	FDA, % N = 12	EFS Altona RealStar, % N = 8	ILM E partiel, % N = 4	ILM jonction M-E, % N = 4	Fundação Pró-Sangue, % N = 2	Laboratório Richet, % N = 2
3.5E+04	3.81E+02	100	100	100	100	NA	100	100	100	100	100	NA	100	100	100	100	100	100
1.1E+04	1.21E+02	100	100	100	100	NA	100	100	100	100	100	100	100	100	100	100	100	100
3.5E+03	3.81E+01	100	100	100	100	100	100	100	100	100	100	100	75	100	100	100	100	100
1.1E+03	1.21E+01	100	100	100	100	100	100	67	100	100	75	100	83	100	100	100	100	100
3.5E+02	3.81E+00	100	100	33	0	100	100	33	100	25	50	25	50	100	100	0	100	100
1.1E+02	1.21E+00	100	100	0	0	100	67	0	100	0	75	0	0	63	100	0	50	0
3.5E+01	3.81E−01	100	100	0	0	100	67	0	67	0	25	8	8	50	50	0	0	0
1.1E+01	1.21E−01	100	100	0	0	0	0	0	0	0	0	0	0	13	50	0	0	0
3.5E+00	3.81E−02	33	75	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.1E+00	1.21E−02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3.5E−01	3.81E−03	0	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Supernatant cp/mL	PFU/mL	Roche, % N = 3	Hologic, % N = 4	CDC PR Singleplex-LI, % N = 3	CDC PR Trioplex-LI, % N = 3	CDC PR Trioplex-HI, % N = 4	CDC FC 1087-LI, % N = 3	CDC FC 4481-LI, % N = 3	CDC 1087 HI, % N = 3	BSRI MTC, % N = 4	BSRI CDC, % N = 4	Davis CDC, % N = 12	FDA, % N = 12	EFS Altona RealSta, %r N = 8	ILM E partiel, % N = 4	ILM jonction M-E, % N = 4	Fundação Pró-Sangue, % N = 2	Laboratório Richet, % N = 2
4.4E+04	5.76E+01	100	100	100	100	100	100	100	100	100	100	100	100	100	75	100	100	100
1.4E+04	1.82E+01	100	100	100	100	NA	100	100	100	100	100	100	100	100	100	100	100	100
4.4E+03	5.76E+00	100	100	100	100	NA	100	100	100	100	100	100	100	100	100	100	100	100
1.4E+03	1.82E+00	100	100	100	100	100	100	100	100	100	100	100	67	100	100	100	100	100
4.4E+02	5.76E−01	100	100	100	100	100	100	67	100	100	75	92	67	100	100	25	100	0
1.4E+02	1.82 E−01	100	100	33	0	100	33	0	100	50	75	58	25	63	100	0	100	0
4.4E+01	5.76E−02	100	100	33	0	100	0	33	100	25	0	17	8	63	50	0	0	0
1.4E+01	1.82E−02	67	100	0	0	0	0	33	33	0	25	8	8	0	50	0	0	0
4.4E+00	5.76E−03	33	100	33	0	0	0	0	0	25	50	0	0	13	0	0	0	0
1.4E+00	1.82E−03	67	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4.4E−01	5.76E−04	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Negative cp/mL	PFU/mL	Roche, % N = 3	Hologic % N = 4	CDC PR Singleplex-LI % N = 3	CDC PR Trioplex-LI % N = 3	CDC PR Trioplex-HI % N = 4	CDC FC 1087-LI % N = 3	CDC FC 4481-LI % N = 3	CDC 1087 HI % N = 3	BSRI MTC % N = 4	BSRI CDC, % N = 4	Davis CDC, % N = 12	FDA, % N = 12	EFS Altona RealStar, % N = 8	ILM E partiel, % N = 4	ILM jonction M-E, % N = 4	Fundação Pró-Sangue, % N = 2	Laboratório Richet, % N = 2
Neg	Neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Neg	Neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Neg	Neg	0	0	0	0	0	0	0	0	0	0	0	0	0	25	0	0	0

Neg = negative.

TABLE 3. Results from replicate testing of (top) Brazil plasma and (bottom) Polynesia isolate coded panels, presented as combinations of the percentage reactivity and LODs for related assays

Plasma cp/mL	PFU/mL	Donor screening NAT, %* N = 7	CDC PR Trioplex-LI, % N = 6	CDC PR Trioplex-HI, % N = 4	CDC FC 1087-LI, % N = 3	CDC FC 1087-HI, % N = 3	BSRI/UC Davis, % N = 20	FDA, % N = 12	EFS, % N = 8	ILM, % N = 4	Brazil, % N = 4
3.5E+04	3.81E+02	100	100	NA	100	100	100	100	100	100	100
1.1E+04	1.21E+02	100	100	NA	100	100	100	100	100	100	100
3.5E+03	3.81E+01	100	100	100	100	100	100	75	100	100	100
1.1E+03	1.21E+01	100	100	100	100	100	95	83	100	100	100
3.5E+02	3.81E+00	100	17	100	100	100	30	50	100	100	100
1.1E+02	1.21E+00	100	0	100	67	100	15	0	63	100	25
3.5E+01	3.81E−01	100	0	100	67	67	10	8	50	50	0
1.1E+01	1.21E−01	100	0	0	0	0	0	0	13	50	0
3.5E+00	3.81E−02	57	0	0	0	0	0	0	0	0	0
1.1E+00	1.21E−02	0	0	0	0	0	0	0	0	0	0
3.5E−01	3.81E−03	1%	0	0	0	0	0	0	0	0	0
	95% LOD	13.7 [4.7, 26.8]	540 [234, 846]	22.8 [13.6, 30.1]	220 [54.5, 490]	43.9 [16.7, 71.7]	2189 [1055, 3644]	6343 [2168, 13,181]	312 [105, 623]	107 [29.1, 233]	165 [66.9, 267]
	50% LOD	2.5 [1.4, 4.6]	411 [226, 766]	19.6 [13.4, 28.7]	43.9 [18.3, 105]	32.3 [16.1, 63.9]	326 [224, 477]	523 [299, 918]	46.3 [25.4, 84.5]	19.6 [8.9, 43.3]	124 [64.6, 241]

Supernatant cp/mL	PFU/mL	Donor screening NAT, %* N = 7	CDC PR Trioplex-LI, % N = 6	CDC PR Trioplex-HI, % N = 4	CDC FC 1087-LI, % N = 3	CDC FC 108-HI, % N = 3	BSRI/UC Davis, % N = 20	FDA, % N = 12	EFS, % N = 8	ILM, % N = 4	Brazil, % N = 4
4.4E+04	5.76E+01	100	100	100	100	100	100	100	100	75	100
1.4E+04	1.82E+01	100	100	NA	100	100	100	100	100	100	100
4.4E+03	5.76E+00	100	100	NA	100	100	100	100	100	100	100
1.4E+03	1.82E+00	100	100	100	100	100	100	67	100	100	100
4.4E+02	5.76E−01	100	100	100	100	100	90	67	100	100	50
1.4E+02	1.82E−01	100	17	100	33	100	60	25	63	100	50
4.4E+01	5.76E−02	100	17	100	0	100	15	8	63	50	0
1.4E+01	1.82E−02	86	0	0	0	33	10	8	0	50	0
4.4E+00	5.76E−03	71	17	0	0	0	15	0	13	0	0
1.4E+00	1.82E−03	43	0	0	0	0	0	0	0	0	0
4.4E−01	5.76E−04	0	0	0	0	0	0	0	0	0	0
	95% LOD	17.9 [5.6, 38.1]	1529 [362, 3829]	28.8 [17.3, 37.9]	205 [80, 337]	20.3 [8, 33.6]	1102 [466, 2053]	4918 [1596, 10,660]	466 [141, 1012]	135 [36.6, 293]	1351 [366, 2926]
	50% LOD	2.5 [1.3, 4.9]	123 [55.4, 273]	24.8 [17, 36.1]	152 [77.2, 301]	15.1 [7.7, 30.2]	81.7 [52.5, 127]	321 [179, 578]	49.6 [25.8, 95.1]	24.8[11.2, 54.8]	248 [112, 548]

* Donor screening NAT includes the RMS and Hologic NAT assays; CDC Puerto Rico (PR) low-input (LI) assay results were combined for Singleplex and Trioplex versions of the Trioplex assay (Trioplex-LI).
NA = not available.

Comparison of donor-screening NAT assays with standard-input RT-PCR assays

The analytical performance was compared between the RMS cobas Zika and Hologic, Inc. Procleix ZIKV blood donor-screening NAT assays and with various versions of RT-PCR diagnostic assays developed by the CDC, FDA, and other laboratories. The RMS and Hologic blood donor-screening NAT assays, which extract nucleic acid from 850 μL and 500 μL of plasma, respectively, were comparable in sensitivity, with 100% detection of dilutions of both standards at levels from 10 to 40 estimated copies/mL. Given the comparable performance of these donor-screening NAT assays based on the percentage reactivity on serial dilutions, their results were combined to allow for a more accurate assessment of LODs based on seven replicate determinations on each panel member. This analysis yielded LOD_{95} and LOD_{50} values of 13.7 (95% confidence interval [CI], 4.7-26.8) and 2.5 (95% CI, 1.4-4.6) copies/mL for the serially diluted Brazilian plasma and 17.9 (95% CI, 5.6-38.1) and 2.5 (95% CI, 1.3-4.9) copies/mL for the serially diluted viral stock from the French Polynesia ZIKV isolate.

These high-input donor-screening NAT assays were approximately 10-fold to 100-fold more sensitive than standard input diagnostic RT-PCR assays. These assays extract from 100 to 200 μL of plasma and process 12 to 42 μL of plasma-derived RNA per amplification ("Derived plasma input" in Table 1). We combined results from the CDC-PR LI assays; from the BSRI and UC Davis laboratories, which were performing similar versions of the CDC LI assays; and from the two Brazil PCR assays to compare the sensitivities relative to commercial NAT, FDA, EFS, and ILM assays (Table 3, Fig. 2). Combining results of similar assays provided more robust estimates for LODs: LOD_{95} ranged from 107 to 6343 copies/mL, and LOD_{50} ranged from 20 to 523 copies/mL for the diluted Brazil plasma panel; serially diluted French Polynesia isolate supernatant was detected at similar sensitivities, with LOD_{95} ranging from 135 to 4918 copies/mL and LOD_{50} ranging from 25 to 321 copies/mL. These findings confirmed the significantly better sensitivity of the commercial donor-screening NAT assays than other laboratory-developed tests and diagnostic PCR assays as well as PCR assays used to screen blood donors in French Polynesia and Brazil.

Enhanced-input assays increased sensitivity

The Trioplex assay was performed with both low inputs (LI) of plasma, as specified in the original EUA, and subsequently with increased or high inputs (HI) of plasma by the CDC laboratory in Puerto Rico. Compared with the original EUA input results from the US CDC laboratory in Puerto Rico, as well as results generated with the CDC protocol at the BSRI and UC Davis laboratories, increasing the sample extraction volume to 1.0 mL and amplification to 100 μL equivalent of plasma resulted in approximately 30-fold increased sensitivity for ZIKV RNA detection by the Trioplex HI assay. The CDC laboratory in Fort Collins also performed an enhanced sample extraction (300 μL vs. 100 μL) and increased RNA input (amplified 67 μL vs. 5 μL equivalent of plasma) and demonstrated a greater than 10-fold enhanced sensitivity of an HI assay relative to an LI assay. These results demonstrate that sensitivity is a function of sample input and can be improved with such assay modifications.

DISCUSSION

The rapidly expanding evidence for the severity of neurological complications associated with ZIKV infection, high rates of infection in travelers returning to the United States, and risk of autochthonous outbreaks in Puerto Rico and US states led to the urgency with which measures had to be taken to protect the US blood supply in early 2016.[32,48] Implementation of a ban on the collection of blood in areas with active transmission without appropriate screening methods, which briefly occurred in Puerto Rico in March 2016 with funding for purchase and transport of blood from the continental United States by the federal government, established an urgent need for the development and implementation of sensitive, specific, and high-throughput donor-screening NAT assays to prevent ongoing disruptions of the blood system in the face of an evolving epidemic.

In March 2016, BSRI and Creative Testing Solutions, which tests approximately 90% of the blood collected in Puerto Rico and Florida, actively considered implementing the CDC ZIKV PCR assay developed by the Puerto Rico CDC branch. However, concerns were raised over the sensitivity of the assay, such that implementation of the test as a donor-screening assay would likely not be sufficient to release locally collected, ZIKV PCR-negative blood for transfusion without additional recipient informed consent that included the option of providing recipients (and particularly pregnant women) with blood components imported from ZIKV low-risk regions of the United States. These concerns, and the expected availability of ZIKV assays from the major blood-screening NAT assay manufacturers, led to the development and execution of this study.

The results of this study suggest that the investigational donor-screening ZIKV NAT assays from RMS and Hologic had high and similar sensitivities for the detection of ZIKV RNA, with LOD_{50} values calculated from the combined results of less than 5 copies/mL and LOD_{95} of less than 20 copies/mL. The results of this study demonstrate that the ZIKV NAT assays from RMS and Hologic had analytical sensitivities similar to those of the NAT assays that target other TT viral infections, which are currently FDA-

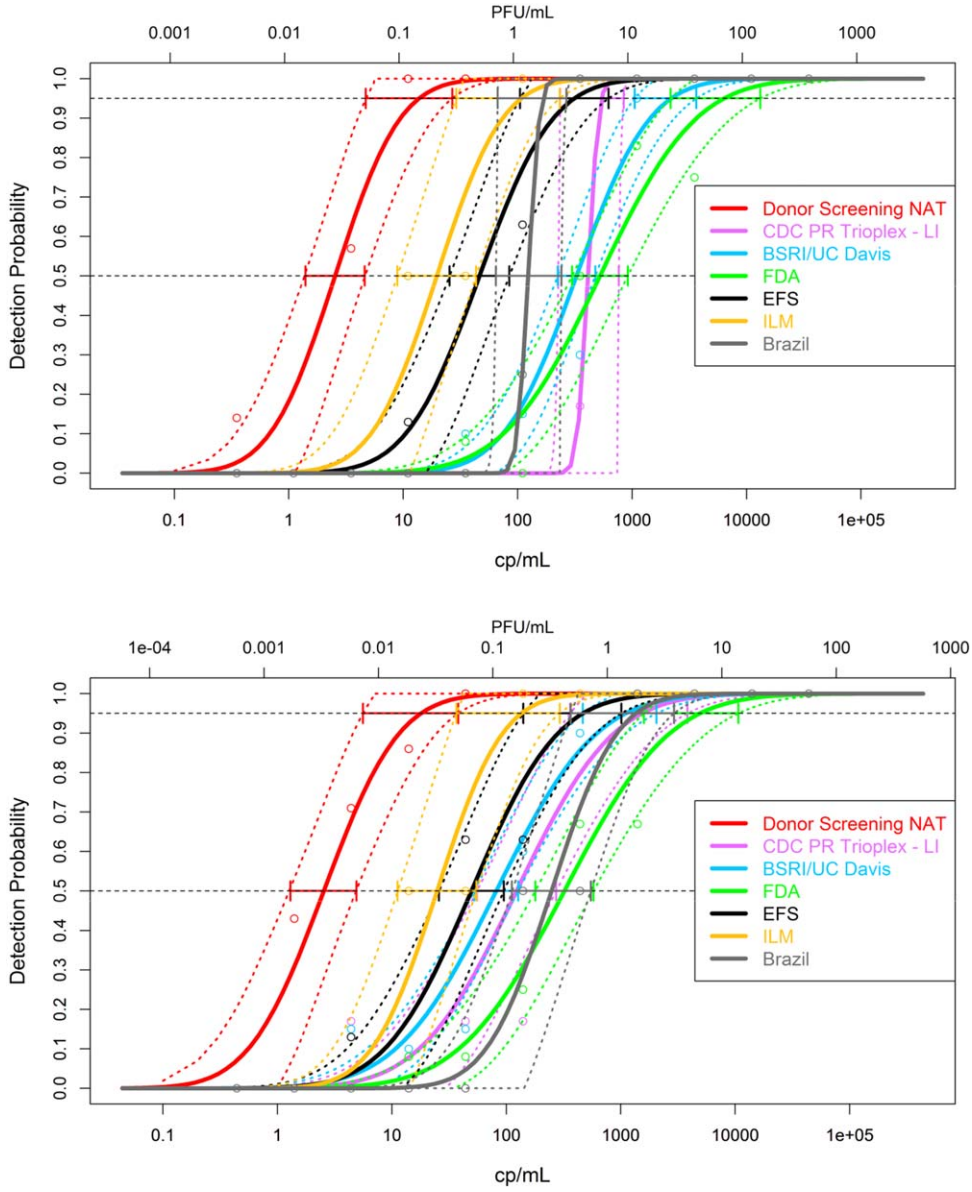

Fig. 2. Probit curves comparing the analytical sensitivity of assays evaluated in this study by replicate testing of (top) the Brazil plasma and (bottom) the French Polynesia isolate supernatant coded panels, grouped by assay category or laboratories (see Materials and Methods, Results, and Table 2). The figure does not include results from the high plasma input PCR assays subsequently performed by the CDC Fort Collins and Puerto Rico laboratories. The fitted probit regression model curve (solid lines) and 95% confidence intervals (dashed lines) were used to estimate LOD_{50}, LOD_{95}, and the confidence intervals presented in Table 3.

licensed and routinely used to screen the US and global blood supplies for human immunodeficiency virus, hepatitis B virus, hepatitis C virus, and West Nile virus. In addition, the analytical sensitivities of the investigational donor-screening NAT assays from RMS and Hologic are substantially higher than those of original CDC EUA Trioplex and the other laboratory-developed and diagnostic ZIKV PCR assays evaluated in this study, as well as assays that have been used in France, French Polynesia, and Brazil for donor-screening during ZIKV outbreaks. The differential sensitivities between assays correlated with and likely are primarily attributable to the volumes of plasma extracted and of eluted RNA that is amplified by different assays, so increasing sample input per assay may enhance sensitivities of diagnostic assays, as demonstrated by the relative sensitivities of LI and HI assays performed at the two participating CDC laboratories. The importance of differential sensitivities of assays on clinical diagnosis and

monitoring of pregnant women and travelers for ZIKV infection requires further study.

FDA allowed the clinical trials to proceed under IND in March 2016 for the RMS IND, and in June 2016 for the Hologic IND. The very rapid progress in assay development and manufacturing demonstrated by the NAT assay manufactures and the rapid response of the FDA and the blood-banking community enabled the implementation of ID-NAT donor screening (using the RMS assay) in Puerto Rico in April 2016[32] and in other locations in the continental United States with potential autochthonous ZIKV outbreaks (Texas and Florida) beginning in May 2016. Based on the high yield of infected donations in Puerto Rico, the increasing numbers of travel-related and sexual transmission cases in US states, and the detection of ZIKV-positive donations in Florida, the FDA issued an updated guidance on August 26 that mandated the immediate implementation of ID-NAT in areas affected by mosquito-borne transmission of ZIKV (i.e., Florida and Puerto Rico); the implementation of ID-NAT in 11 southern states with proximity to locally acquired, mosquito-borne cases and/or high numbers of travel-acquired cases (Alabama, Arizona, California, Georgia, Hawaii, Louisiana, Mississippi, New Mexico, New York, South Carolina, and Texas) within 4 weeks after the guidance issue date (by September 23, 2016); and implementation in the entire United States within 12 weeks after the guidance issue date (by November 18, 2016).[48] The results of early screening indicate excellent specificity of both assays and detection of low but significant numbers of ZIKV-infected donors, both due to travel-acquired and autochthonous infections.[51,52]

This study has several limitations. First, due to limited volumes of panel members (1-mL aliquots were provided to most laboratories, except for the donor-screening NAT assay manufacturers, each of which was provided with three 1-mL aliquots), the number of replicate tests of diluted panel members performed using each assay was relatively small (range, 2-12), precluding precise estimation of LODs (which usually requires more than 25 replicates testing of each dilution). In particular, this study was not designed to rigorously compare the analytical sensitivities of the RMS and Hologic NAT assays due to the limited availability of dilutions of the study panels. Second, the quantitation of VLs in copies and PFUs/mL in the viral stocks and of serial dilutions in the panels is not precise and should be considered only estimates, although we believe conclusions on the relative sensitivities of different assays are reliable. Third, there was an amendment to the CDC ZIKV PCR EUA protocol in August 2016 that included recommendations to extract and test larger volumes of serum and to add procedures for Trioplex testing of whole blood in addition to urine and serum, which were the only sample types in the original EUA protocol. These changes should significantly improve the diagnostic sensitivity of that assay. Moreover, FDA has also authorized a number of other ZIKV NAT assays under EUA, including diagnostic tests from RMS and Hologic; it is likely that many of these assays have enhanced analytical sensitivities relative to the assays evaluated in this study.

In conclusion, the findings of this study suggest that the investigational ZIKV NAT assays for blood donor screening have excellent sensitivities similar to the sensitivities of the assays currently used to screen for established TT viruses. The donor-screening NAT assays from RMS and Hologic appear to be substantially more sensitive than most other laboratory-developed and diagnostic ZIKV RT-PCR assays evaluated in this study. Enhancing sensitivities of laboratory-developed and diagnostic assays may be achievable by increasing sample input or testing alternative sample types. Rapid implementation of blood donor NAT screening for ZIKV in Puerto Rico and US states and in several other countries represents a major accomplishment of many involved parties to assure blood safety during the expanding ZIKV pandemic.

APPENDIX

DETAILED ASSAY METHODS

Roche Molecular Systems, Inc

The cobas Zika test for use on the cobas 6800/8800 Systems is a qualitative PCR test for the detection of ZIKV RNA in plasma specimens from individual human donors. The test includes assay-specific reagents and an external positive control, and it also shares cobas omni reagents and the negative control with other tests performed on the cobas 6800/8800 Systems. The cobas 6800/8800 Systems provide fully automated sample preparation (nucleic acid extraction and purification) followed by PCR amplification and detection.

During extraction of 850 μL of plasma, nucleic acids from the sample are processed simultaneously with added, armored RNA internal control (IC), which serves as the sample preparation and amplification/detection process control. Viral nucleic acids are released by the addition of proteinase and lysis reagent and bind with IC RNA to magnetic glass particles. Unbound substances and impurities, such as denatured proteins, cellular debris, and potential PCR inhibitors, are removed by subsequent wash steps. Purified nucleic acids are eluted from the glass particles, and eluate is added to PCR master mix.

The cobas Zika master mix contains primers and detection probes specific for ZIKV selected from highly conserved regions of the viral genome, and it also contains oligonucleotides to amplify and separately detect the IC. A thermostable DNA polymerase is used for reverse-transcription and amplification. The master mix includes AmpErase enzyme (uracil-N-glycosylase) and deoxyuridine triphosphate (dUTP), instead of

deoxythimidine triphosphate (dTTP), which is incorporated into the newly synthesized DNA (amplicon). Any potentially contaminating amplicons from previous PCR runs are destroyed by AmpErase and rendered nondetectable. The specific ZIKV and IC detection probes are labeled with unique fluorescent dyes, allowing simultaneous detection and discrimination of the amplified Zika target and the IC for each sample. Automated data analysis is performed by the cobas 6800/8800 software, which assigns a test result for each test as nonreactive, reactive, or invalid.

Hologic, Inc

The Procleix ZIKV assay, developed in partnership with Grifols Diagnostic Solutions, is a qualitative in vitro assay to detect Zika RNA on the fully automated Panther system. The same assay, marketed as the Aptima ZIKV assay by Hologic, was subjected to additional verification and validation studies and was granted EUA status from the FDA for use as a diagnostic NAT. The Procleix and Aptima assays are based on the same technology as other Procleix assays, which involve three main steps: sample preparation using magnetic-based target capture, RNA amplification by transcription-mediated amplification (TMA), and detection of the amplification products by the hybridization protection assay (HPA) using chemiluminescent probes. To mitigate the risk of false negative results, the assay targets 2 separate viral target regions of the ZIKV genome and includes an internal control to validate each reaction. The assay uses 0.5mL input volume and all reaction steps occur in one tube with an IC incorporated. The IC is added into samples via the target capture reagent, to monitor target capture, amplification, and detection, as well as operator or instrument error. The analyte and IC probes produce chemiluminescent signals with different light emission kinetics that are distinguishable from one another. Results are expressed in relative light units (RLU) values and signal-to-cutoff (S/CO) values. A floating cutoff value is obtained with calibrators included in each run. A sample is considered "Reactive" if the analyte S/CO is greater than or equal to 1.0. A sample is considered "Nonreactive" if the analyte S/CO is less than 1.0 and the internal control signal is greater than the internal control cutoff. The sample result is considered "Invalid" if the internal control signal is greater than the set maximum, or both the analyte S/CO is less than 1.0 and the internal control signal is below the internal control cutoff. The sample with invalid result must be retested.

CDC Puerto Rico

CDC Trioplex RT-PCR Assay was run in three modalities: Multiplex Low Input (LI) (200 μL), Multiplex High Input (HI—1000 μL), and the Singleplex Zika modality (LI—μL). In the multiplex modalities, the CDC Trioplex assay is used for the simultaneous and qualitative detection and differentiation of DNGVs, CHIVs, and ZIKVs from a single sample. Extraction of 200 μL or 1 mL was performed on MagNA Pure 96 Total Nucleic Acid Isolation Kit (Roche). PCR reactions were amplified with 10 μL extracted RNA using the SuperScript III Platinum One-Step qRT-PCR Kit (Thermo Fisher) on 10 replcates using an Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument (Thermo Fisher Scientific).

CDC Fort Collins

Two assays using two separate real-time primer/probe sets specific for the ZIKV 2007 strain (1087; 4481) were designed by using ZIKV 2007 nucleotide sequence data in the PrimerExpress software package (Applied Biosystems) and tested independently.[40] Primers were synthesized by Operon Biotechnologies with 5-FAM as the reporter dye for the probe. Standard input real-time assays were performed using the QuantiTect Probe RT-PCR Kit (Qiagen) with 5 μl of RNA extracted from 100 μl of plasma and eluted in 100 μl. High input RT-PCR assays were performed using the QuantiFast Pathogen RT-PCR + IC kit with 20 μl of RNA extracted from 300 μl of plasma eluted in 90 μl. Amplification was performed for both assays in the iCycler instrument (Bio-Rad) following the manufacturer's protocol.

BSRI

Viral RNA extraction: RNA was extracted from 140 μL of sample using the QIAamp Viral RNA Mini Kit (Qiagen) and eluted in 50 μL of water. Duplicate extractions were performed for each panel member.

One-step real-time RT-PCR (CDC protocol): RNA was amplified in a 25-μL reaction mixture using the SuperScript III Platinum One-Step Quantitative RT-PCR System (Life Technologies Corporation) under the following conditions: 0.5 μL of the enzyme mixture, 12.5 μL of the 2x buffer, 0.25 μL of 100 μM each ZIKV 1086 (5′ CCGCTGCCCAACACAAG 3′) and ZIKV 1162c (5′ CCACTAACGTTCTTTTGCAGACAT 3′) primer, 0.15 μL of 25 μM ZIKV 1107 (5′ AGCCTACCTTGACAAGCAGTCAGACACTCAA 3′) 6-FAM/ZEN/IBFQ probe, 6.35 μL of nuclease-free water, and 5 μL of extracted RNA. Duplicate amplifications were performed for each extracted sample. Amplification was performed in a LightCyler 480 real-time PCR instrument (Roche Diagnostics) in 96-well plates overlaid with 10 μL mineral oil per well using the following thermal profile: 30 minutes at 50°C (combinational DNA [cDNA] synthesis), hold for 2 minutes at 95°C, followed by 45 amplification cycles of 15 seconds at 95°C and 1 minute at 60°C. Data analysis was performed using the LightCycler 480 software.

Two-step real-time RT-PCR (MTC protocol): RNA was reverse transcribed in a 48-μL reaction mixture containing: 4.8 μL of 10x solution A + B (1 M KCl; 0.1 M Tris, pH 8.3; 25 mM $MgCl_2$; 10% Tween-20; 10% NP-40), 0.5 μL of 100 mM deoxynucleotide triphosphates,

0.2 μL of 100 μM ZIKV 1162c primer, 1.2 μL of 40 U/μL RNase inhibitor, 1.2 μL of 50 U/μL MuLV reverse transcriptase, and 40 μL of extracted RNA. cDNA synthesis was performed for 30 minutes at 42°C followed by reverse transcriptase inactivation for 10 minutes at 95°C. cDNA was amplified in a 15-μL reaction mixture containing: 9.54 μL of PCR buffer, 0.1 μL of 100 mM deoxynucleotide triphosphates, 0.1 μL of 100 μM each ZIKV 1086 and ZIKV 1162c primers, 0.02 μL of 100 μM ZIKV 1107 probe, 0.14 μL of 5 U/μL FastStart Taq DNA polymerase, and 5 μL of cDNA. Duplicate amplifications were performed for each extracted sample. Amplification was performed in a LightCycler 480 real-time PCR instrument in 96-well plates overlaid with 10-μL mineral oil per well using the following thermal profile: 1 minute at 95°C (DNA polymerase activation), followed by 45 amplification cycles of 30 seconds at 95°C and 1 minute at 60°C. Data analysis was performed using the LightCycler 480 software.

UC Davis

Each of 24 panel samples was divided into 4 aliquots of 140 μL and mixed with 560 μL of AVL buffer (Qiagen) for individual RNA extractions in parallel. This yielded a total of 96 samples, some of which were stored at 4°C for up to 1 week after the addition of AVL buffer before RNA was extracted. RNA was extracted from all samples using the QIAamp Viral RNA mini kit (Qiagen 52906) in accordance with the protocol provided and was then eluted in 60 μL of non-diethyl pyrocarbonate-treated water. After extraction, all RNA samples were stored at −80°C. Quantitative RT-PCR was performed with the Taqman Fast Virus One-Step Master Mix (Thermo 4444436), using the primers and probes published by Lanciotti et al.[40] All 96 samples were tested in triplicate using an Applied Biosystems ViiA 7 quantitative RT-PCR machine. A volume of 9.6 μL RNA was used in each sample. For results in which the cycle threshold (Ct) values did not match what was expected (e.g., loose triplicates), the multicomponent graph was analyzed visually using the Applied Biosystems software. False-positive values were identified by straight horizontal lines on the multicomponent graph, as opposed to a horizontal line with an upward curving tail at the very end (indicating amplification). Viral RNA levels were calculated in log copies by comparing the average of each samples' triplicate to the standard curve generated on each PCR plate.

FDA

RNA extraction from 140 μL of plasma was performed using the QIAamp Viral RNA mini kit with QIACube (Qiagen) and eluted in 60 μL. An extracted RNA volume of 5 μL was tested using Taqman Fast Virus One-Step Master Mix (Thermo Fisher) with primers and probe and was amplified on the ViiA 7 instrument (Applied Biosystems). The primer and probe sets used were as previously described.[40]

EFS

Individual NAT screening was performed on a semiautomated platform using a Microlab STARlet (Hamilton) for nucleic acid extraction and CFX96 (Bio-Rad Laboratories) automation for RT-PCR. Briefly, according to the manufacturer's instructions, nucleic acids were extracted in a final volume of 50 μL from 140 μL of sample using the NucleoSpin 96 Virus Extraction Kit (Macherey-Nagel GmbH & Company KG), and 10 μL of eluate was tested by RT-PCR using the RealStar Zika Virus RT-PCR Kit 1.1 (Altona Diagnostics GmbH). Each run was validated by positive and negative controls, and nucleic acid extraction for each sample was validated by an internal control.

ILM

RNA was extracted from 200 μL of plasma and eluted in 50 μL using the NucliSENS EasyMAG extraction system (bioMérieux), as previously reported.[12] ZIKV real-time RT-PCR using 5 μL input RNA was performed on a CFX BioRad real-time PCR analyzer using two real-time primers/probe amplification sets in two separate assays specific for ZIKV[40] with a Ct cutoff less than 38.5. Serial dilutions of an RNA synthetic transcript that covers the region targeted by the two primers/probe sets were amplified to monitor sensitivity. Results were reported as positive and blood products were discarded if any of the amplification was positive.

Brazil

Fundação Pró-Sangue. Extraction is performed on the MagNA pure Compact (RMS) with the large-volume kit of 250 μL of samples eluted in 60 μL. Real-time PCR is performed in the Step-One Plus machine from Applied Biosciences (now Thermo Fisher Scientific) or on the Light Cycler 480 from RMS. RNA input is 10 μL in a final volume of 20 μL, including 5 μL 4x Taqman fast virus mix, 5 μL water, 0.6 μL/0.4 μL 40x mix primers, and probe. Before extraction, samples were spiked with a diluted preparation of the Salk polio vaccine (used as the internal control), and run duplex Zika-polio virus PCR, with both sets using 500-nM primers with a 250-nM probe. Zika probe was labeled with FAM, and polio was labeled with VIC (or HEX). Primer/probe sets were used as previously described.[40]

Laboratório Richet. RNA was extracted from 140 μL of samples using the QIAamp Viral RNA Mini Kit (Qiagen), and RNA was eluted with 60 μL of RNase-free water. For control of the extraction process, 4 μL of an internal control was added in each sample in lysis buffer and, the Ct was considered valid between 25 and 31. For the ZIKV RT PCR, the Bio Gene ZIKV PCR kit (Bioclin) was used

with the RT primers and probes and master mix provided in the kit. The probes were designed with 5-FAM as the reporter dye for the target and with Vic for internal control. Five microliters of extracted RNA sample was used for a total reaction volume of 20 μL. All real-time assays were performed using amplification in the 7300 RT instrument (Thermo Fisher Scientific) following the manufacturer's protocol.

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CONFLICT OF INTEREST

The authors have reported the following conflicts of interest: MCL is now an employee of Cerus Corporation; SAG is an employee and shareholder of Roche Molecular Systems, Inc.; JML is an employee and shareholder of Hologic, Inc.; MPB is the Principal Investigator on contracts to Blood Systems Research Institute from Roche Molecular Systems, Inc. and Hologic, Inc. to support ZIKV confirmation testing for investigational blood donor screening, and from Grifols to support research related to ZIKV.

REFERENCES

1. Musso D, Gubler DJ. Zika virus. Clin Microbiol Rev 2016;29: 487-524.
2. Richard V, Paoaafaite T, Cao-Lormeau VM. Vector competence of French Polynesian Aedes aegypti and Aedes polynesiensis for Zika Virus. PLoS Negl Trop Dis 2016;10:e0005024
3. Oliveira Melo AS, Malinger G, Ximenes R, et al. Zika virus intrauterine infection causes fetal brain abnormality and microcephaly: tip of the iceberg? Ultrasound Obstet Gynecol 2016;47:6-7.
4. Sarno M, Sacramento GA, Khouri R, et al. Zika virus infection and stillbirths: a case of hydrops fetalis, hydranencephaly and fetal demise. PLoS Negl Trop Dis 2016;10:e0004517
5. Besnard M, Lastere S, Teissier A, Cao-Lormeau V, Musso D. Evidence of perinatal transmission of Zika virus, French Polynesia, December 2013 and February 2014. Euro Surveill 2014;19. pii: 20551
6. Davidson A, Slavinski S, Komoto K, et al. Suspected female-to-male sexual transmission of Zika Virus - New York City, 2016. MMWR Morb Mortal Wkly Rep 2016;65:716-7.
7. Deckard DT, Chung WM, Brooks JT, et al. Male-to-male sexual transmission of Zika Virus—Texas, January 2016. MMWR Morb Mortal Wkly Rep 2016;65:372-4.
8. Foy BD, Kobylinski KC, Chilson Foy JL, et al. Probable non-vector-borne transmission of Zika virus, Colorado, USA. Emerg Infect Dis 2011;17:880-2.
9. Musso D, Roche C, Robin E, et al. Potential sexual transmission of Zika virus. Emerg Infect Dis 2015;21:359-61.
10. Barjas-Castro ML, Angerami RN, Cunha MS, et al. Probable transfusion-transmitted Zika virus in Brazil. Transfusion 2016;56:1684-8.
11. Motta IJ, Spencer BR, Cordeiro da Silva SG, et al. Evidence for transmission of Zika virus by platelet transfusion. N Engl J Med 2016;375:1101-3.
12. Bingham AM, Cone M, Mock V, et al. Comparison of test results for Zika virus RNA in urine, serum, and saliva specimens from persons with travel-associated Zika virus disease—Florida, 2016. MMWR Morb Mortal Wkly Rep 2016;65:475-8.
13. Bonaldo MC, Ribeiro IP, Lima NS, et al. Isolation of infective Zika virus from urine and saliva of patients in Brazil. PLoS Negl Trop Dis 2016;10:e0004816.
14. Campos Rde M, Cirne-Santos C, Meira GL, et al. Prolonged detection of Zika virus RNA in urine samples during the ongoing Zika virus epidemic in Brazil. J Clin Virol 2016;77: 69-70.
15. Gourinat AC, O'Connor O, Calvez E, et al. Detection of Zika virus in urine. Emerg Infect Dis 2015;21:84-6.
16. Jayakumar P, Brar K, Lippmann S. Best Zika virus detection: saliva, serum, or urine? South Med J 2016;109:697.
17. Zhang FC, Li XF, Deng YQ, et al. Excretion of infectious Zika virus in urine. Lancet Infect Dis 2016;16:641-2.
18. Musso D, Roche C, Nhan TX, et al. Detection of Zika virus in saliva. J Clin Virol 2015;68:53-5.
19. Atkinson B, Hearn P, Afrough B, et al. Detection of Zika virus in semen [letter]. Emerg Infect Dis 2016;22:490.
20. Brasil P, Nielsen-Saines K. More pieces to the microcephaly-Zika virus puzzle in Brazil. Lancet Infect Dis 2016;16:1307-9.
21. Brasil P, Pereira JP Jr, Raja Gabaglia C, et al. Zika virus infection in pregnant women in Rio de Janeiro—preliminary report. N Engl J Med 2016;375:2321-4.
22. Mlakar J, Korva M, Tul N, et al. Zika virus associated with microcephaly. N Engl J Med 2016;374:951-8.
23. Nunes ML, Carlini CR, Marinowic D, et al. Microcephaly and Zika virus: a clinical and epidemiological analysis of the current outbreak in Brazil. J Pediatr (Rio J) 2016;92: 230-40.
24. Wang JN, Ling F. Zika virus infection and microcephaly: evidence for a causal link. Int J Environ Res Public Health 2016; 13. pii: E1031.
25. Centers for Disease Control and Prevention (CDC). Case counts in the US [monograph on the internet] [cited December 31, 2016]. Atlanta (GA): CDC; 2016. Available from: https://www.cdc.gov/zika/geo/united-states.html
26. Centers for Disease Control and Prevention (CDC). Pregnancy outcomes in the United States and the District of Columbia [monograph on the internet] [cited December 31, 2016]. Atlanta (GA): CDC; 2016. Available from: https://www.cdc.gov/zika/geo/pregnancy-outcomes.html

27. Fleming-Dutra KE, Nelson JM, Fischer M, et al. Update: interim guidelines for health care providers caring for infants and children with possible Zika virus infection—United States, February 2016. MMWR Morb Mortal Wkly Rep 2016; 65:182.
28. Oduyebo T, Petersen EE, Rasmussen SA, et al. Update: interim guidelines for health care providers caring for pregnant women and women of reproductive age with possible Zika virus exposure—United States, 2016. MMWR Morb Mortal Wkly Rep 2016;65:122-7.
29. Staples JE, Dziuban EJ, Fischer M, et al. Interim guidelines for the evaluation and testing of infants with possible congenital Zika Virus infection—United States, 2016. MMWR Morb Mortal Wkly Rep 2016;65:63-7.
30. Petersen EE, Staples JE, Meaney-Delman D, et al. Interim guidelines for pregnant women during a Zika virus outbreak—United States, 2016. MMWR Morb Mortal Wkly Rep 2016;65:30-3.
31. Musso D, Nhan T, Robin E, et al. Potential for Zika virus transmission through blood transfusion demonstrated during an outbreak in French Polynesia, November 2013 to February 2014. Euro Surveill 2014;19. pii: 20761.
32. US Food and Drug Administration (FDA). Recommendations for donor screening, deferral, and product management to reduce the risk of transfusion-transmission of Zika virus—guidance for industry. Silver Spring (MD): US Department of Health and Human Services, FDA, Center for Biologics Evaluaton and Research; 2016.
33. European Center for Disease Prevention and Control (ECDC). Zika virus and safety of substances of human origin. Stockholm (Sweden): ECDC; 2016.
34. World Health Organization (WHO). Maintaining a safe and adequate blood supply during Zika virus outbreaks. Geneva (Switzerland): WHO Press; 2016.
35. Musso D, Stramer SL; AABB Transfusion-Transmitted Diseases Committee, et al. Zika virus: a new challenge for blood transfusion. Lancet 2016;387:1993-4.
36. Lanteri MC, Kleinman SH, Glynn SA, et al. Zika virus: a new threat to the safety of the blood supply with worldwide impact and implications. Transfusion 2016;56:1907-14.
37. US Food and Drug Administraton (FDA). Emergency use authorizations [cited December 31, 2016]. Silver Spring (MD): US Department of Health and Human Services, FDA, Center for Biologics Evaluation and Research; 2016. Available from: http://www.fda.gov/MedicalDevices/Safety/EmergencySituations/ucm161496.htm
38. Baronti C, Piorkowski G, Charrel RN, Boubis L, Leparc-Goffart I, de Lamballerie X. Complete coding sequence of Zika virus from a French Polynesia outbreak in 2013. Genome Announc 2014;2. pii: e00500-14
39. Trösemeier JH, Musso D, Blümel J, Thézée J, Pybus OG, Baylis SA. Genome sequence of a candidate World Health Organization reference strain of Zika virus for nucleic acid testing. Genome Announc 2016;4. pii: e000917-16.
40. Lanciotti RS, Kosoy OL, Laven JJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerg Infect Dis 2008;14:1232-9.
41. Musso D, Nilles EJ, Cao-Lormeau VM. Rapid spread of emerging Zika virus in the Pacific area. Clin Microbiol Infect 2014;20:O595-6.
42. Musso D. Zika Virus transmission from French Polynesia to Brazil. Emerg Infect Dis 2015;21:1887.
43. Centers for Disease Control and Preventon (CDC). Areas with Zika [monograph on the internet] [cited December 31, 2016]. Atlanta (GA): CDC; 2016. Available from: https://www.cdc.gov/zika/geo/
44. European Center for Disease Prevention and Control (ECDC). Rapid risk assessment: Zika virus infection outbreak, French Polynesia. 14 February 2014. Stockholm (Sweden): ECDC; 2014.
45. Gulland A. Zika virus is a global public health emergency, declares WHO. BMJ 2016;352:i657.
46. Cunha MS, Esposito DL, Rocco IM, et al. First complete genome sequence of Zika virus (Flaviviridae, Flavivirus) from an autochthonous transmission in Brazil. Genome Announc 2016;4. pii: e000032-16.
47. World Health Organizationo (WHO). Maintaining a safe and adequate blood supply during Zika virus outbreaks—Interim guidance. Geneva (Switzerland): WHO Press; 2016.
48. US Food and Drug Administration (FDA). Revised recommendations for reducing the risk of Zika virus transmission by blood and blood components. Guidance for industry. Silver Spring (MD): US Department of Health and Human Services, FDA, Center for Biologics Evaluation and Research; 2016.
49. Aubry M, Richard V, Green J, Brolt J, Musso D. Inactivation of Zika virus in plasma with amotosalen and ultraviolet A illumination. Transfusion 2016;56:33-40.
50. Weiner E, Velez E, Alsina J, Chrebtow V, Bunch C. Implementation of the INTERCEPT Blood System for platelets in Puerto Rico to help safeguard against Zika. Presented at: 2016 AABB Annual Meeting; October 22-25, 2016; Orlando, FL.
51. Galel S, Williamson P, Busch M, et al. First Zika positive donations in the continental United States. Transfusion 2016;57:0.
52. Williamson P, Linnen J, Kessler D, et al. First cases of Zika virus infected US blood donors outside states with areas of active transmission. Transfusion 2016;57:0.