



**UNDER EMBARGO UNTIL
JUNE 13, 2017, 12:01 AM ET**

**the Journal of
Molecular
Diagnostics**

jmd.amjpathol.org

Highly Multiplex Real-Time PCR—Based Screening for Blood-Borne Pathogens on an OpenArray Platform

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Accepted for publication
March 28, 2017.

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Molecular diagnostics are increasingly used in the blood bank industry. A device that can combine simultaneous detection of multiple targets with the flexibility of inclusion of emerging pathogens is desirable for testing blood products. A highly multiplexed blood-borne pathogen panel (BBPP) using dual-label probe chemistry (TaqMan assays) was developed for simultaneous detection and discrimination of 17 viral pathogens in human plasma samples and 13 bacterial and protozoan pathogens in human blood samples on the OpenArray platform. The custom BBPP OpenArray plate was tested for specificity and analytical sensitivity with purified nucleic acids from each pathogen and with pathogen-spiked human blood and plasma samples. The results of analytical validation of known samples yielded decision trees for identification of coded samples: pathogens spiked in human plasma or whole blood. Results from coded samples demonstrated no false positives among the plasma or whole blood specimens. Samples not detected were at the lower limit of the detectable range or qualified for retesting as indeterminate. Further demonstration of the performance of the BBPP OpenArray was achieved with clinical samples from a blood donor testing organization. Ninety-five percent of virus-positive samples were correctly identified. These results show that a high-throughput OpenArray PCR platform can be expanded and adapted for higher discrimination and newly emerging agents, enabling consideration for development as a next-generation device for testing blood products. (*J Mol Diagn* 2017, 19: 549–560; <http://dx.doi.org/10.1016/j.jmoldx.2017.03.004>)

The quality and safety of blood products used in transfusions are of significant public concern. The number of emerging pathogens that affect blood safety has steadily increased in the recent decades and has triggered a rapid development of molecular tests for detection of blood-borne pathogens such as West Nile virus,¹ dengue virus (DENV),² and *Babesia microti*³ to name a few. Some highly virulent pathogens may have a low prevalence rate and/or be restricted seasonally or geographically, suggesting they may not be of concern for blood safety. However, the impact of transfusion-transmitted infection of such agents can have fatal consequences in highly vulnerable populations such as

Supported by the Food and Drug Administration Medical Countermeasures Initiative (R.D.).

Disclosures: E.G. is an employee of Diatherix Laboratories; S.P. is an employee of Thermo Fisher Scientific, the manufacturer of the OpenArray; V.W. and P.W. are employees of Creative Testing Solutions, the source of blood donor specimens.

The comments in this publication are an informal communication and represent the authors' own best judgments. These comments do not bind or obligate the Food and Drug Administration (FDA). The devices described in this publication are for research use only and have not been formally cleared or approved by FDA for the uses discussed herein.

All samples and Thermo Fisher Scientific (Applied Biosystems) reagents and materials are for research use only, not for use in diagnostic procedures.

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neonates, the elderly, or immunocompromised persons. Therefore, approaches to blood donor testing that can include these pathogens are needed. Molecular diagnostic tests for assessment of the quality of blood products require a high sensitivity to ensure blood safety and high specificity because too many blood donations would be lost as a result of a test with too many false positives. Although many singleplex tests are approved or cleared by the Food and Drug Administration (FDA), there are a limited number of tests that accomplish simultaneous detection for multiple pathogens.⁴ Application of novel and emerging technologies offers the prospect of developing the next generation of superior performance tests that will detect an increasing number of transfusion-transmissible agents in multiplex format with high sensitivity and specificity, robustness, and adaptability to accomplish detection of new agents once issues of throughput, proper format for clinical trials, and requirement for FDA approval of new more complex devices are solved. The successful utilization of a high-throughput multiplex PCR platform (OpenArray; Thermo Fisher Scientific, South San Francisco, CA) for detection of a limited number of viral and bacterial pathogens was reported earlier,⁵ and its performance was comparable with existing singleplex assays currently used for donor screening.

In this study, we present the evaluation of a highly multiplex blood-borne pathogen panel (BBPP) built on an OpenArray platform with capacity for simultaneous detection and discrimination of 17 viral strains in human plasma samples and 13 bacterial and protozoan pathogens in human blood samples. The specificity and sensitivity of the custom BBPP was evaluated using pathogen-spiked blood and plasma. The ultimate test of the device performance was achieved by obtaining and blindly processing (E.G. and S.P.) 92 characterized blood donor specimens. The results of this study yielded a decision tree that can be used for identification of unknown samples on an OpenArray BBPP.

Materials and Methods

Viral Specimen Preparation

Specimens positive for HIV-1 clades, HIV-2, hepatitis C virus (HCV)-1b, and hepatitis B virus (HBV) were assembled from reference material prepared by the FDA (Supplemental Table S1).^{6–13} The viral concentrations of these frozen vials were established by the FDA, and we used these concentrations in the spiked specimens without further verification. The concentration of HCV-2a genomes in copies per milliliter of plasma was established at 120,000 by quantitative RT-PCR (7500 RT-PCR Guide; Applied Biosystems, Foster City, CA) in the source specimen, and the concentrations in the specimens reported in the results were obtained by diluting the source sample with human plasma. West Nile virus (WNV) strains NY99 and FDAHu2002 were quantified by both plaque-forming assay¹⁴ and TaqMan

quantitative RT-PCR assay (Applied Biosystems).^{15,16} DENVs were molecularly quantified in PCR-detectable units by TaqMan quantitative RT-PCR.¹² Influenza virus stocks were quantified by plaque-forming assay on Madin-Darby canine kidney epithelial cells.¹³

Bacterial and Protozoan Specimen Preparation

Three bacterial species, *Escherichia coli*, *Yersinia enterocolitica*, and *Staphylococcus aureus* (Supplemental Table S2)^{17–24} were quantified by measuring optical density at 600 nm and converting to cells per milliliter by the formula $5.0 \times 10^8 \text{ cell/mL} = 1 \text{ absorbance unit}$ in the SmartSpec 3000 spectrophotometer (Bio-Rad, Hercules, CA). *Babesia microti* were quantified by microscopic determination of the percentage of red blood cells infected and determination of the number of red blood cells per milliliter with a Coulter particle counter (Beckman Coulter, Brea, CA), then the *Babesia* cells per milliliter were calculated by the product of these two numbers. *Leishmania braziliensis*, *Leishmania donovani*, *Leishmania infantum*, *Leishmania major*, *Leishmania mexicana*, and *Leishmania tropica*; promastigotes (Supplemental Table S2); and *Trypanosoma cruzi* were quantified with a Coulter particle counter. *Plasmodium falciparum* and *Plasmodium vivax* were quantified microscopically, as with *B. microti*.

Spiking

HIV-1 group M subtype A, subtype B, and subtype G; HIV-2; HCV-1b; and HBV were not further diluted, but the Lot Release Panel members were used directly as test specimens. Chikungunya virus strain R91064-purified RNA was estimated for genome copy number by RNA concentration and diluted into RNA samples extracted from the appropriate volumes of human plasma. DENV 1 to 4, HCV-2a, influenza H1N1, and influenza H5N1 obtained as cultured virus stock were diluted into human plasma that was free of infectious agents. After quantification, an appropriate dilution of WNV stock was made in phosphate-buffered saline, then further diluted at least 10-fold into 1 mL of human plasma to prepare the test specimens. The six species of *Leishmania*, *P. falciparum*, *P. vivax*, *T. cruzi*, *E. coli*, *Y. enterocolitica*, and *S. aureus*, obtained as cultured cells, were similarly diluted and spiked into whole heparinized human blood. *B. microti* as intraerythrocytic parasites at 1000 infected cells/ μL were further diluted in whole human blood. The blood and plasma were obtained from deidentified donors to the research program at the Department of Transfusion Medicine, NIH, under an approved protocol that is reviewed by the FDA Research in Human Subjects Committee (principal investigator R. Duncan). All donations are tested by the collection center with FDA-approved donor tests for HIV, HCV, HBV, WNV, *Treponema palladium*, and *T. cruzi*.

Nucleic Acid Extraction, Volume, Quantification

Nucleic acid from plasma spiked with WNV was extracted using the QIAamp Viral Mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Nucleic acid from all other plasma specimens was extracted with the QIAamp MinElute Virus Spin kit (Qiagen) according to the manufacturer's protocol, eluted in 180 μ L. DNA from whole blood specimens was extracted with the QIAamp DNA Blood Mini kit (Qiagen) according to the manufacturer's protocol, eluted in 180 μ L.

Primer Design and Selection

The panel for detection of a broad range of blood-borne pathogens consisted of primers and TaqMan probes for viral, bacterial, and parasitic targets ([Supplemental Tables S3 and S4](#))^{2,5,22,25–31} designed for this study, published in the literature, or commercially available from Thermo Fisher Scientific (for research use only, not for use in diagnostic procedures). TaqMan assays were designed using a proprietary algorithm developed by Thermo Fisher Scientific. This algorithm evaluates a set of optimal assays, considering criteria of melting temperature and nucleotide composition of primer-pair combinations. The algorithm selects the assays with highest specificity, based on nucleic acid sequence comparison of assay primers and probes with genomic sequences from other closely related species. From the sequence comparison, the assay with the highest mismatch score with other organisms was chosen, which minimizes the possibility of generation of false-positive results during the testing. Primers and probes designed by the FDA laboratory as part of this study were selected from genomic sequences using MacVector software version 15.1.3 (MacVector, Inc., Cary, NC), avoiding hairpin loops, primer duplexes, and optimizing melting temperatures.

Reverse Transcription Reaction and Preamplification

Extracted viral RNAs (10 μ L of each sample) were reverse transcribed using the High Capacity RT kit (Applied Biosystems) with random hexamer primers or gene-specific primers (pool of all assay primers diluted 1:5 relative to the concentration in the PCR reaction) and incubated at 37°C for 2 hours per manufacturer's protocol in a 20- μ L reaction volume. To increase sensitivity for targets present at low concentration, the target-specific preamplification protocol was applied. A sample of cDNA (10 μ L) was mixed with 10 μ L of a mixed pool of PCR assay oligonucleotides at 180 nmol/L/50 nmol/L primer pair/probe concentration and 20 μ L of TaqMan preamplification master mix (Applied Biosystems). The preamplification reaction was performed on an ABI9700 (Applied Biosystems) at the following cycling conditions: single cycle at 95°C for 10 minutes; 18 cycles at 95°C for 15 seconds, 60°C for 4 minutes; single cycle at 99°C for 10 minutes in a total volume of 25 μ L.

Preamplification products were diluted 1:10 in 0.1 $\times$ Tris and EDTA buffer (Quality Biological, Gaithersburg, MD) and were used on the BBPP OpenArray platform.

Real-Time PCR on BBPP OpenArray Platform

The OpenArray technology (for research use only, not for use in diagnostic procedures) is based on a metal plate the size of a microscope slide that has been photolithographically patterned and etched to form a rectilinear array of 3072 through-holes, organized in 48 subarrays with 64 through-holes each. Each through-hole is loaded with an individual TaqMan assay ([Supplemental Tables S3 and S4](#)) and contains 33 nL of PCR reaction mixture. Previous work has shown that the PCR assay performance in the nanoplates is equivalent to the same assay in microplates,³² but with >150-fold lower reaction volume (33 nL versus 5 μ L PCR reaction volumes) and with the ability to profile multiple targets using the same sample. Blood collected from a donor can be separated into a plasma sample and a whole blood sample, providing the opportunity to target plasma-residing pathogens with one panel and whole blood-residing pathogens with a separate panel. The primer and probe sets targeting viruses were configured in triplicate on each subarray of one set of plates (plasma panel), and the primer and probe sets targeting bacteria and protozoans were similarly configured on a separate set of plates (whole blood panel). Thus, plasma samples were applied to the virus-type plates, whereas whole blood samples were applied to the bacteria/protozoan plates. Each sample was tested on multiple subarrays to achieve a higher level of replicates for each assay.

A sample of genomic DNA or cDNA across a range of concentrations (1.2 μ L of each) was mixed with 1.3 μ L of PCR-grade water and 2.5 μ L of TaqMan OpenArray real-time PCR master mix (Applied Biosystems). Then, the reaction mixture was dispensed on a BBPP OpenArray plate using the automated sample loading system, AccuFill (Applied Biosystems). Real-time PCR occurred in an OpenArray custom computer-controlled imaging thermal cycler under software control (manufacturer's proprietary software), where imaging data for up to 12,168 individual reaction wells (four plates) was collected during 40 cycles of PCR. The step after acquisition includes calculation of C_q ³³ and the C_T confidence parameter, which were used for further data analysis and in the decision tree for pathogen identification. The C_T confidence parameter is calculated using Thermo Fisher Scientific's proprietary algorithm, and its value reflects the quality of the amplification curve generated during PCR. The C_T confidence values >300 indicate good quality of amplification, C_T confidence values between 200 and 300 are considered marginal, and each amplification curve with such C_T confidence values has to be manually checked before the result can be

Table 1 Analytical Sensitivity Study Test Results of the Plasma Panel

Pathogen	Copies/mL	Assay 1				Assay 2			
		Name	Mean Cq	95% CI	TH, pos/total	Name	Mean Cq	95% CI	TH, pos/total
Chikungunya virus	1000	CHIK	18.85	18.45–19.25	12/12	NA			
Chikungunya virus	100	CHIK	22.81	22.67–22.95	12/12	NA			
DENV-1	10 ⁴	DENV-1	22.16	21.81–22.51	9/9	PAN DENV	26.22	±0.43	9/9
DENV-1	10 ³	DENV-1	23.00	22.74–23.26	9/9	PAN DENV			
DENV-2	10 ⁴	DENV-2	18.35	17.90–18.80	12/12	PAN DENV	24.79	±0.49	12/12
DENV-2	10 ³	DENV-2	22.93	22.54–23.32	12/12	PAN DENV	0		0
DENV-3	10 ⁴	DENV-3	20.02	19.88–20.16	9/9	PAN DENV	25.00	±0.14	9/9
DENV-3	10 ³	DENV-3	22.78	22.69–22.87	9/9	PAN DENV	0		0
DENV-4	10 ⁴	DENV-4	20.14	20.01–20.27	9/9	NA			
DENV-4	10 ³	DENV-4	19.72	19.65–19.79	9/9				
HBV	500	HBV-A	22.15	21.96–22.34	12/12	NA			
HCV-1b	500	HCV-B	24.25	24.10–24.40	12/12	NA			
HCV-2a	2000	HCV-B	14.09	13.91–14.27	12/12	NA			
HIV-1 group M subtype B	500	HIV-1A	22.11	21.77–22.45	12/12	HIV-1	24.23	±0.32	12/12
HIV-1 group O	1000	HIV-1 O	19.93	19.67–20.19	12/12	NA			
HIV-1 group O	100	HIV-1 O	24.52	24.22–24.82	12/12	NA			
HIV-1 group M subtype A	1000	HIV-1 O/M	23.51	23.13–23.89	12/12	HIV-1	22.73	±0.20	12/12
HIV-1 group M subtype A	100	HIV-1 O/M	25.08	24.77–25.39	11/12	HIV-1	26.37	±0.29	12/12
HIV-1 group M subtype G	1000	HIV-1	21.59	21.52–21.66	12/12	NA			
HIV-1 group M subtype G	100	HIV-1	24.25	23.71–24.79	12/12	NA			
HIV-2	1000	HIV2_1	14.50	14.25–14.75	12/12	HIV2_2	14.50	±0.25	12/12
HIV-2	100	HIV2_1	17.62	17.29–17.95	12/12	HIV2_2	17.58	±0.30	12/12
Influenza H1N1	1000	H1N1-HA	13.80	13.71–13.89	12/12	INF-M	15.16	±0.08	12/12
Influenza H1N1	100	H1N1-HA	16.89	16.64–17.14	12/12	INF-M	18.13	±0.21	12/12
H5N1	1000	H5N1-HA	15.40	15.21–15.59	12/12	INF-M	14.33	±0.19	12/12
H5N1	100	H5N1-HA	19.32	18.98–19.66	12/12	INF-M	18.49	±0.38	12/12
WNV-FDAHu2002	1000	WNV-B	20.93	20.82–21.04	12/12	NA			
WNV-FDAHu2002	100	WNV-B	22.61	22.21–23.01	12/12	NA			
WNV-NY99	1000	WNV-B	24.28	23.77–24.79	12/12	DENV-2	23.23	±0.50	12/12
WNV-NY99	100	WNV-B	25.22	24.98–25.46	12/12	DENV-2	22.60	±0.14	12/12
Neg control plasma		None	NA	NA	0/9				
No-template control		HIV-1A	34.42	32.20–36.64	2/36	INF-M	34.96	2.18	3/36

DENV, dengue virus; HBV, hepatitis B virus; HCV, hepatitis C virus; NA, not applicable; Neg, negative; pos, positive; TH, through-hole, cumulative, from multiple OpenArray plates; WNV, West Nile virus.

considered valid. Reactions with C_T confidence values <200 are not considered valid.

The blind testing (E.G. and S.P.) of coded specimens was assured by nucleic acid extraction of the spiked specimens in the FDA laboratory, the eluted samples were placed into tubes that were all identically labeled with a code. Coded specimens were shipped to the Thermo Fisher Scientific laboratory where they were tested with the BBPP OpenArray, and the result were interpreted. The code was broken only after the final interpretation had been recorded.

Statistical Analysis

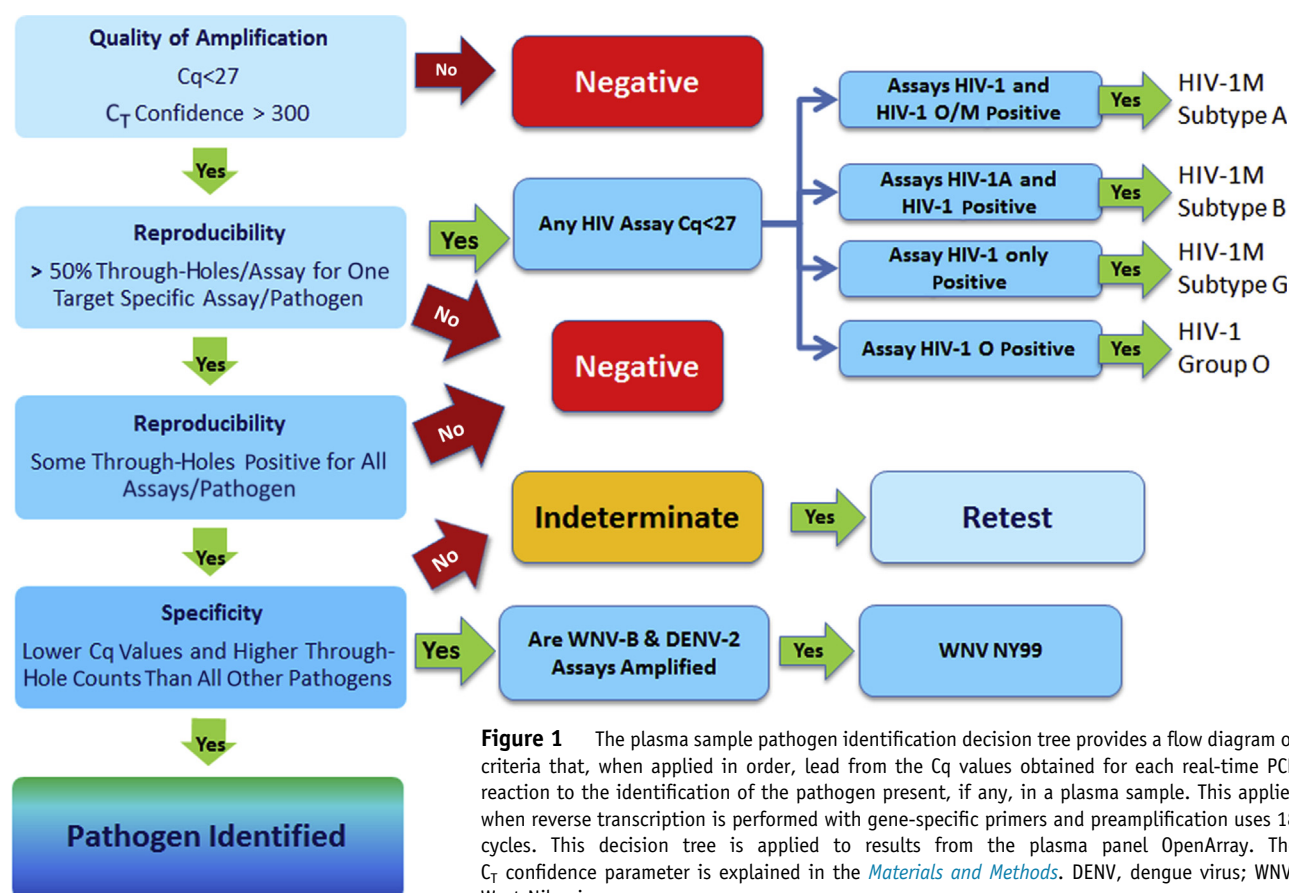
The means and confidence intervals of C_q values were calculated with the functions AVERAGE and CONFIDENCE.NORM(0.05, STDEV.S, N) in Microsoft Office Excel (Microsoft Inc., Redmond, WA). The overall correct and incorrect percentages of unknowns detected are best represented by the binomial distribution, so the 95%

confidence intervals were calculated by the Wilson score interval.³⁴

Results

Evaluation of Plasma Specimens

A blood sample can be collected and separated into the plasma fraction and the cellular fraction. Thus, without prior knowledge of the infected status, the plasma fraction, where viruses can be detected, was applied to the plasma panel. The analytical sensitivity for each assay was determined as a lowest detected concentration of viral sample spiked into human plasma, and results are shown in Table 1. The range of concentrations of viral samples was chosen based on clinical requirements for pathogen detection and performance variables of existing diagnostic tests.^{35–37} One or two target-specific assays were used for detection: the C_q values generated with assay 1 were used for determination



of analytical specificity, whereas C_q values obtained with assay 2 were used as a confirmatory. Two types of negative controls were included, process control (negative plasma) and PCR no-template controls. The mean C_q values for tested targets ranged from 13.8 to 25.2. The total through-holes reported in [Table 1](#) for each sample represent the number of replicates of a sample multiplied by 3 through-holes that contain the same TaqMan assay for each sample replicate. Nearly all samples were detected in 100% of the through-holes (9 of 9 or 12 of 12, depending on the run) with exception of the HIV-1 group M subtype A at 100 copies/mL detected in 11 of 12 through-holes tested (91.6%). The reliable detection of chikungunya; HIV-1 group O, group M subtype A and subtype G; HIV-2; influenza H1N1 and influenza H6N1; and WNV-FDAHu2002 and -NY99 viruses were achieved at 100 copies/mL. HCV-2a was not detected <2000 copies/mL because of specimen availability, and the lowest detectable level has not been determined. Negative control plasma samples produced no positive signal, whereas 2 and 3 of 36 replicates of no-template controls produced positive signal with HIV-1A and influenza matrix assays, respectively ([Table 1](#)). The C_q values generated with no-template controls were at least nine C_q units higher than the C_q resulting from positive viral samples. This information was used for

determination of the C_q threshold to separate positive and negative interpretations when blinded specimens (E.G. and S.P.) were tested. In addition, a set of parameters (average C_q values, the C_T confidence that reflects the quality of the amplification curve, reproducibility among the replicates of an assay, reproducibility among the assays targeting a particular pathogen, and assay specificity) were compiled and applied for identification of unknown (coded) specimens. These criteria were expressed in a decision tree specific to the plasma panel for evaluation of plasma specimens ([Figure 1](#)). The decision tree was applied to all of the replicates of each assay for each sample to reach a decision about the presence or absence of each pathogen in each sample. Guided by this decision tree, we proceeded to evaluate blinded specimens (E.G. and S.P.).

To evaluate the ability of the BBPP OpenArray plates to identify unknown viral specimens, plasma was spiked with measured amounts of cultured pathogens or selected Center for Biologics Evaluation and Research lot release panel members ([Materials and Methods](#)) near the limits of detection determined above. Specimens were extracted in one laboratory, coded, and sent to the other laboratory for testing. The testing of 24 coded nucleic acid specimens, including pathogen-free plasma on BBPP OpenArray plates, resulted in 19 true positives, two true negatives, three false

Table 2 Detection of Unknown Specimens, Plasma Spiked with Virus, or Control Plasma

Coded specimen type (copies/mL)	Total specimens	Outcome			
		True Positive	True Negative	False Positive	False Negative
Negative plasma control	2		2		
Chikungunya virus (10^2)	1	1			
Chikungunya virus (10^3)	1	1			
DENV1 (10^3)	1	1			
DENV1 (10^5)	1	1			
DENV2 (10^4)	1	1			
DENV3 (10^4)	1	1			
DENV4 (10^3)	1	1			
HBV (500)	1	1			
HCV-1b (100)	1				1
HCV-1b (500)	1				1
HCV-2a (2000)	1	1			
HIV-1 group M (500)	1	1			
HIV-1 group M (1000)	1	1			
HIV-1 group O (1000)	1	1			
HIV-1 subtype A (1000)	1	1			
HIV-1 subtype G (1000)	1	1			
HIV-2 (100)	1	1			
Influenza H1N1 (100)	1	1			
Influenza H1N1 (1000)	1	1			
Influenza H5N1 (100)	1	1			
WNV-2002 (100)	1	1			
WNV-NY99 (100)	1				1
Overall total	24	19	2	0	3
Overall correct/incorrect (%)		21 (87.5)		3 (12.5)	
Confidence limits (95%)		69.0%–95.7%		4.3%–31.0%	

Detailed results are found in [Supplemental Table S5](#).

DENV, dengue virus; HBV, hepatitis B virus; HCV, hepatitis C virus; WNV, West Nile virus.

negatives, and no false positives (Table 2), which is a success rate of 87.5% (21 of 24; 95% CI, 69%–96%). Most of the false negatives occurred in the specimens with the lowest virus concentrations (500–100 RNA copies/mL). Details of the OpenArray results for each coded specimen are shown in [Supplemental Table S5](#).

Evaluation of Whole Blood Specimens

Blood-borne pathogens of concern other than viruses are primarily found in whole blood such as bacteria and protozoans, which are organisms with DNA genomes. Thus, processing of whole blood specimens involved nucleic acid extraction optimized for DNA, and the samples were tested with a panel of TaqMan style PCR assays targeting bacteria and protozoans ([Supplemental Tables S3 and S4](#)). Volunteer donor whole blood was spiked with cultured pathogens at known concentrations, and results of detection and discrimination on the BBPP OpenArray platform were collected (Table 3). Whole blood samples were spiked with cultured pathogens to concentrations near the likely detectable level according to previous work.⁵ To enhance detection and discrimination, multiple primer/probe sets targeted each pathogen. Some were chosen which target sequences

that were unique to a species, whereas others targeted conserved regions that may amplify multiple species but would facilitate detection sensitivity. *B. microti* was easily detected at 100 cells/mL, suggesting the lower detection limit had not been reached. All of the *Leishmania* species could be detected at 100 cells/mL, and the unique pattern of amplification among the eight *Leishmania* assays allowed unambiguous discrimination of the species. The two *Plasmodium* species were detected at 100 cells/mL and were discriminated by species-specific assays. The two assays for *T. cruzi* are very sensitive because of the large number of repeats in the genome of the targeted sequence. The Gram-negative bacteria were detected at 100 cells/mL for *E. coli* and at 1000 cells/mL for *Y. enterocolitica*, although the result for *Y. enterocolitica* fell just below the cutoff at 100 cells/mL. The presence of a Gram-negative bacteria was identified, but the species were not discriminated. The Gram-positive bacterium, *S. aureus*, was not detected <1000 cells/mL. Twenty-seven different whole blood samples with no spiked pathogen were prepared and tested to evaluate the specificity of the BBPP OpenArray Whole Blood plates (Table 3). The results show that specificity is a property of each primer/probe set not the platform as a whole. The *Leishmania* (LEI)-1 assay targets a high-copy

Table 3 Analytical Sensitivity Study Results of Whole Blood Panel

Pathogen	Cells/mL	Assay 1				Assay 2			
		Name	Mean Cq	95% CI	TH Pos/Total	Name	Mean Cq	95% CI	TH Pos/Total
<i>Babesia microti</i>	1000	BAB-1	22.90	22.67–23.13	18/18				
<i>B. microti</i>	100	BAB-1	26.31	25.8–26.82	18/18				
<i>Leishmania braziliensis</i>	1000	LCHAG	26.31	25.66–26.96	18/18	LEI-1	23.58	±0.29	18/18
<i>L. braziliensis</i>	100	LCHAG	24.08	23.81–24.35	18/18	LEI-1	21.84	±0.24	18/18
<i>L. donovani</i>	1000	LINF	23.21	22.77–23.65	18/18	LTRO	22.18	±0.40	18/18
<i>L. donovani</i>	100	LINF	26.70	26.12–27.28	18/18	LTRO	25.82	±0.49	18/18
<i>L. infantum</i>	1000	LCHAG	21.39	21.15–21.63	18/18	LINF	24.70	±0.36	18/18
<i>L. infantum</i>	100	LCHAG	25.06	24.59–25.53	18/18	LINF	28.68	±0.46	18/18
<i>L. major</i>	1000	LMAJ	26.29	25.71–26.87	18/18	LTRO	28.58	±0.57	18/18
<i>L. major</i>	100	LMAJ	25.78	25.4–26.16	18/18	LTRO	28.62	±0.45	18/18
<i>L. mexicana</i>	1000	LMEX	24.18	24.01–24.35	18/18	LMEX-1	27.49	±0.48	17/18
<i>L. mexicana</i>	100	LMEX	28.25	27.96–28.54	12/18	LEI-1	26.03	±0.41	11/18
<i>L. tropica</i>	1000	LTRO	23.97	23.67–24.27	18/18	LEI-2	24.56	±0.31	18/18
<i>L. tropica</i>	100	LTRO	26.90	26.37–27.43	18/18	LEI-2	28.48	±0.50	17/18
<i>Plasmodium falciparum</i>	1000	PLA-3	21.58	21.19–21.97	17/18	PLA-2	19.95	±0.31	18/18
<i>P. falciparum</i>	100	PLA-3	25.12	24.5–25.74	18/18	PLA-2	23.06	±0.35	18/18
<i>Plasmodium vivax</i>	1000	PLA-4	23.49	23.11–23.87	18/18	PLA-2	18.88	±0.36	18/18
<i>P. vivax</i>	100	PLA-4	25.58	24.99–26.17	18/18	PLA-2	21.37	±0.32	18/18
<i>Trypanosoma cruzi</i>	1000	TCF-1	13.47	12.95–13.99	18/18	TCF-2	13.40	±0.53	18/18
<i>T. cruzi</i>	100	TCF-1	17.35	16.86–17.84	18/18	TCF-2	17.20	±0.46	18/18
<i>Escherichia coli</i>	1000	GNEG-1	24.39	24.1–24.68	17/18	GNEG-2	24.89	±0.36	16/18
<i>E. coli</i>	100	GNEG-1	27.31	26.81–27.81	18/18	GNEG-2	27.40	±0.36	18/18
<i>Yersinia enterocolitica</i>	1000	GNEG-1	25.21	24.87–25.55	18/18	GNEG-2	25.71	±0.27	18/18
<i>Y. enterocolitica</i>	100	GNEG-1	28.98	28.43–29.53	15/18	GNEG-2	29.24	±0.56	16/18
<i>Staphylococcus aureus</i>	10,000	STAU	25.11	24.73–25.49	18/18				
<i>S. aureus</i>	1000	STAU	27.67	26.91–28.43	5/18				
Neg control blood		LEI-1	28.66	28.26–29.06	15/27	GNEG-1	29.53	±0.37	4/27
No-template control		GNEG-1	29.38	29.03–29.73	10/36	GNEG-2	29.64	±0.45	9/36

BAB, *Babesia microti*; GNEG, Gram-negative bacteria; LCHAG, *Leishmania chagasi*; LEI, *Leishmania*; LINF, *Leishmania infantum*; LMAJ, *Leishmania major*; LMEX, *Leishmania mexicana*; LTRO, *Leishmania tropica*; Neg, negative; PLA, *Plasmodium* species; Pos, positive; STAU, *Staphylococcus aureus*; TH, through-hole, cumulative, from multiple OpenArray plates; TCF, *Trypanosoma cruzi*.

number DNA structure in the mitochondrion, unique to the class of protozoans that includes *Leishmania* and *Trypanosoma*, that makes it quite sensitive, but in a situation in which cross-reactivity is an issue, it may have to be disregarded. The Gram-negative assay is also subject to amplifying trace amounts of bacterial DNA known to be found in reagents.³⁸ However, establishing a Cq cutoff of 27 in the decision tree removed all of the amplification observed in negative control blood samples or no-template controls from consideration as pathogen positive (Table 3).

On the basis of comparison of amplification results for each assay, a set of criteria (mean Cq values and the reproducibility among the replicates of an assay, reproducibility among the assays targeting a particular pathogen, combinations of assays among related species, and assay specificity) were compiled that completely distinguished a true-positive result from a true-negative result and correctly identified the pathogen among the spiked blood specimens with known pathogen concentrations or blood alone. These criteria were expressed in a decision tree (Figure 2) that was applied to all of the replicates of each assay for each sample to reach a decision about the presence or absence of each

pathogen in each sample. Guided by this decision tree, we proceeded to evaluate blinded specimens (E.G. and S.P.). The decision tree for whole blood pathogens required some additional criteria to ensure specific detection and discrimination of all of the species in the panel. For example, *L. donovani* and *L. infantum*, two species that cause visceral leishmaniasis, are nearly identical at the genomic level; however, the Cq values that result from four of the *Leishmania* assays in the panel can be ordered by the magnitude of their Cq value that indicates which species is present in the specimen (Figure 2).

To evaluate the ability of the BBPP OpenArray plates to identify unknown bacteria and protozoan specimens, whole blood was spiked with measured amounts of cultured pathogens (Materials and Methods) near the limits of detection determined above. Specimens were extracted in one laboratory, coded, and sent to the other laboratory for testing. The testing of 24 coded nucleic acid specimens, including pathogen-free blood specimens on BBPP OpenArray plates, resulted in 19 true positives, one true negative, and four with indeterminate results. There were no false negatives and no false positives (Table 4), which is a

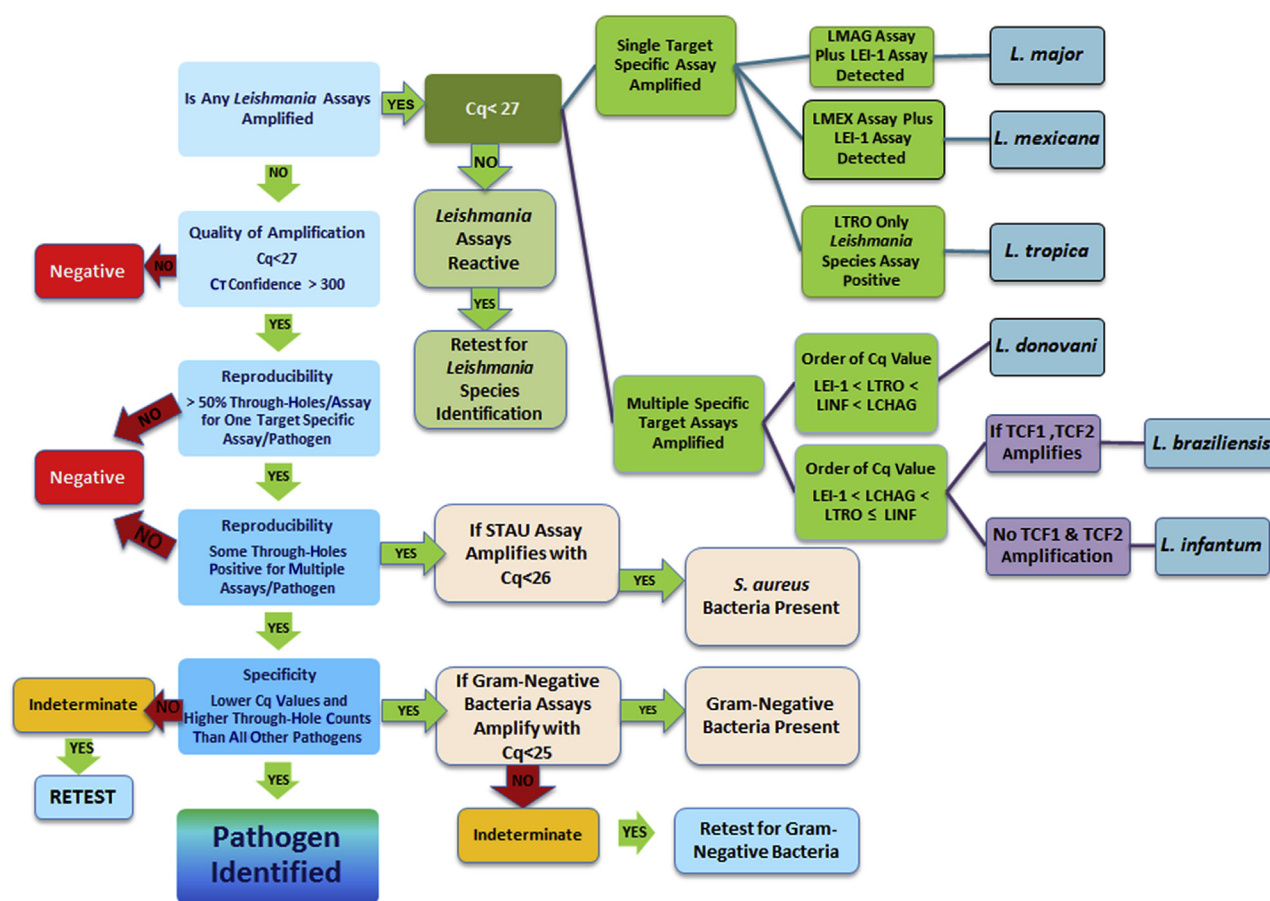


Figure 2 The whole blood sample pathogen identification decision tree provides a flow diagram of criteria that, when applied in order, lead from the Cq values obtained for each real-time PCR reaction to the identification of the pathogen present, if any, in a blood sample. This applies when gene-specific primers are used in pre-amplification for 14 cycles. This decision tree is applied to results from the whole blood panel OpenArray. LCHAG, *Leishmania chagasi*; LEI, *Leishmania*; LINF, *Leishmania infantum*; LMAG, *Leishmania major*; LTRO, *Leishmania tropica*; STAU, *Staphylococcus aureus*; *S. aureus*, *Staphylococcus aureus*; TCF, *Trypanosoma cruzi*.

success rate of 83% (20 of 24; 95% CI, 79.5%–99.3%). The indeterminates occurred in negative whole blood or specimens with the lowest pathogen concentrations (100 cells/mL), and all of them had a pattern of results that qualifies for retesting according to the decision tree. Details of the OpenArray results for each coded specimen are shown in Supplemental Table S6.

Evaluation of Blood Donor Plasma Specimens

To further the validation of the BBPP OpenArray, unlinked residual donor specimens after approval by the FDA Research in Human Subjects Committee (protocol number 13-022B) were obtained from Creative Testing Solutions, a centralized facility running FDA-licensed tests for blood donor screening. Creative Testing Solutions sent plasma samples with the infecting pathogen identified, nucleic acid was extracted in the FDA laboratory, and coded samples were sent to Thermo Fisher Scientific for processing on the plasma panel BBPP OpenArray. After final determinations were made on each specimen, the code was broken, and a

summary of the results is presented in Table 5. Detailed results for all 92 residual donor specimens showed the assay Cq values that allowed identification of the infecting agent according to the decision tree (Supplemental Table S7). Among the blood donor specimens, 8 of the 24 HIV positives were identified as subtype G (33.3%). In the literature, non-B subtypes are reported to represent about 2.5% of HIV infections in blood donors.^{39,40} Thus, these results are likely an overestimate of the proportion of HIV infections that were subtype G.

Discussion

A highly multiplex BBPP was built and extensively validated in this study. This is a first report in which an expanded spectrum of established and emerging pathogens (viruses, bacteria, and parasites) has been tested in a single PCR reaction. Our previous study reported the correct identification of a limited number of pathogens on an OpenArray platform.⁵ Experiments conducted in this report showed an increased capacity of an OpenArray platform for

Table 4 Detection of Unknown Specimens, Whole Blood Spiked with Bacterial or Protozoan Pathogens, and Negative Control Blood

Coded specimen type	Cells/mL	Total specimens	Outcome				Indeterminate
			True Positive	True Negative	False Positive	False Negative	
Negative blood	Control	2		1			1*
<i>Babesia microti</i>	1000	1	1				
<i>B. microti</i>	100	1	1				
<i>Leishmania braziliensis</i>	100	1	1				
<i>L. donovani</i>	1000	1	1				
<i>L. donovani</i>	100	1	1				
<i>L. infantum</i>	100	1	1				
<i>L. major</i>	100	1	1				
<i>L. mexicana</i>	1000	1	1				
<i>L. mexicana</i>	100	1					1*
<i>L. tropica</i>	100	1	1				
<i>Plasmodium falciparum</i>	1000	1	1				
<i>P. falciparum</i>	100	1	1				
<i>P. vivax</i>	1000	1	1				
<i>P. vivax</i>	100	1	1				
<i>Trypanosoma cruzi</i>	1000	1	1				
<i>T. cruzi</i>	100	1	1				
<i>E. coli</i>	1000	1	1				
<i>E. coli</i>	100	1					1*
<i>Yersinia enterocolitica</i>	1000	1	1				
<i>Y. enterocolitica</i>	100	1					1*
<i>Staphylococcus aureus</i>	10,000	1	1				
<i>S. aureus</i>	1000	1	1				
Total		24	19	1	0	0	4
Overall correct/incorrect/Ind, <i>n</i> (%)			20 (83)		0 (0)		4 (17)
[95% confidence limits]			[79.5%–99.3%]		[0.7%–20.5%]		[6.9%–36.2%]

Detailed results of each coded specimen are found in [Supplemental Table S6](#).

*Qualified for retest according to decision tree.

Ind, indeterminate.

detection and discrimination of 17 viral strains in human plasma samples and 13 bacterial and protozoan pathogens in human blood samples. The clear advantage of the Open-Array platform for this type of application is the ease of assembling singleplex TaqMan assays into the panel, having technical assay replicates and/or an additional assay for each detected target, limited or absence of assay cross-reactivity, and Cq for target detection within a linear range of PCR amplification. These facts played an important role in the construction of decision trees for identification of pathogens in plasma and whole blood samples ([Figures 1 and 2](#)).

Quality of amplification, based on established Cq threshold and the C_T confidence parameter, was a starting point in the decision tree for detection of viral pathogens in plasma samples. Because a single assay was used for viral subtyping within the HIV-1 group, we used it as a bifurcation point to separate the detected HIV-1 group subtypes from other viral pathogens in the panel. If more than one assay per target was positive and Cq values were below the established threshold, the identity of the pathogen can be correctly determined. Only one assay on this panel, WNV-B designed for detection of the WNV-NY99 strain, had a

unidirectional cross-reactivity with the DENV-2 assay, and another bifurcation in the decision trees was recommended for identification of WNV-NY99: if both assays are positives and Cq values are <27. This decision tree was tested on coded specimens ([Table 2](#)), and it correctly identified all samples with the exception of HCV-1b at 100 and 500 copies/mL and WNV-NY99 at 100 copies/mL which was incorrectly identified as DENV-2 because of the cross-reactivity mentioned above. When used in the training set ([Table 1](#)), both assays produced Cq values just within the threshold used in the decision tree. In addition, the concentration of 100 copies/mL for HCV-1b was not used in the training set and it is most likely outside of the limit of the detection for this target, so no positive signal detection would be expected for coded specimen P24 ([Supplemental Table S5](#)).

The decision tree constructed for identification of pathogens in a whole blood sample is more complicated because of several factors: assay cross-reactivity for identification of different *Leishmania* species and suboptimal performance of the *S. aureus* assay. The decision tree also had to set cutoffs that exclude detected amplification in negative

Table 5 Summary Results of Unlinked Residual Blood Donor Specimen Testing with the OpenArray Platform

Contaminating pathogen*	Result status	Number of calls	Percent
HIV	Correct	Subtype B 16 Subtype G 8	96
HIV	Incorrect	1 (FN)	4
HCV	Correct	28	93.3
HCV	Incorrect	2 (FP)	6.7
HBV	Correct	25	100
HBV	Incorrect	0	0
NEG	Correct	14	93.3
NEG	Incorrect	1 (FP)	6.7
Overall	Correct	91	94.8
Overall	Incorrect	4	5.2

Detailed results for each of the 95 calls made on 92 blood donor specimens are found in [Supplemental Table S7](#).

*Detected at Creative Testing Solutions using FDA-licensed donor screening tests.

FDA, Food and Drug Administration; FN, false negative; FP, false positive; HBV, hepatitis B virus; HCV, hepatitis C virus; NEG, negative.

control blood with the LEI-1 assay and in no-template control samples with the Gram-negative bacteria assay. The LEI-1 primers and probe constitute a pan-*Leishmania* genus assay that we have retained because it is sensitive for detection of this parasite. A positive result must always be confirmed because of the frequency of false positives with this primer pair. The LEI-2 assay detects both *Leishmania* and *Trypanosoma* genera, is slightly less sensitive than LEI-1, but much less prone to false-positive results. The starting point in this decision tree is separation of the analysis of positive *Leishmania* targets from other organisms. Previous studies on sequencing the genomes of kinetoplastid parasitic protozoa, such as *L. major* and *T. cruzi*, revealed significant genome conservation with differences in genes attributed to pathogenesis.^{41,42} The LEI-1 assay is targeting the detection of the multicopy minicircle kDNA structure (10,000 copies per parasite) which is conserved across multiple *Leishmania* species.²⁶ With the exception of *L. tropica*, DNA from all tested *Leishmania* species had a positive signal with the LEI-1 assay. Thus, *L. major* and *L. mexicana* can be identified if their target-specific assay had a Cq <27 in a combination with positive results for LEI-1. The identification of *L. donovani*, *L. braziliensis*, and *L. infantum* follows more complex patterns because of target cross-reactivity with multiple *Leishmania*- and *T. cruzi*-specific assays. However, such complexity of the decision tree allowed correct identification of all coded *Leishmania*-containing samples with the exception of one of *L. mexicana* at 100 cells/mL, a concentration that had Cq values just outside the threshold among the training sample set (Table 3).

The lowest detected levels for most bacteria with the BBPP OpenArray platform are too high to be expected in an

asymptomatic blood donor. However, most contaminations occur during venipuncture and rapidly grow in the blood product to as high as 10⁸ organisms/mL, and a rate of nearly 10 bacterial infections per million transfusions has been reported,⁴³ indicating the relevance of bacterial detection on this platform. Two separate paths for identification of *S. aureus* and Gram-negative bacteria were used because there were high Cq values for the *S. aureus* target at 10,000 cells/mL and because amplifications occurred in no-template control reactions with Gram-negative assays in the training set. Both are necessity driven to avoid false-positive results when testing coded samples. This strategy was successful based on results presented in Table 4.

The performance of the BBPP OpenArray platform with characterized residual donor specimens was indicative of its potential for further development as a blood-screening device in the future. The limited diversity of the donor panel that was tested did not evaluate the extensive discriminatory capability of the platform. In the case of discriminating HIV-1 group M, subtype B from subtype G, the results followed the decision tree that was derived from laboratory specimens. However, the proportion of subtype G we identified in this pool of 24 HIV-1-infected donors (the licensed testing did not include discrimination of subtypes) was high (8 of 24, 33%) and exceeds the proportion of all non-B subtype donors combined of 2.5%.^{39,40} Therefore, the sensitivity for HIV-1 detection is demonstrated; however, subtyping will require more validation before it can be applied to donor screening in the future. Optimizing an ability to accurately identify HIV-1 subtypes is important because projecting pathogenesis,⁴⁴ drug resistance,⁴⁵ and performing molecular epidemiology⁴⁶ can depend on the infecting viral subtype.

There are a few multiplex PCR-based tests approved by the US FDA for screening blood donors, and those allow simultaneous detection and discrimination of three viruses (HIV, HCV, and HBV). The inclusion in this study of 92 coded samples tested positive with FDA-licensed molecular tests provided a true comparison of BBPP performance with tests approved by the regulatory agency and used by the blood industry. Analytical performance of the developed multiplex device was established with samples spiked with organisms at levels similar to the lower limits for existing FDA-approved tests, and little loss of sensitivity or specificity was detected at this high level of assay multiplexing. Although assay optimization is often required to avoid cross-reactivity or interference for primer mixes using single-reaction primers and probes that have been pooled into multiplex PCR, the utilization of singleplex assays on spatially isolated individual PCR reaction chambers is the essential feature of the OpenArray platform, which allows achieving a high level of multiplexing without adverse effects on sensitivity and specificity. The utility and flexibility of the OpenArray platform for different applications had been demonstrated earlier.^{5,47,48} Thus, results of testing 92 coded donors' samples on BBPP proved that this device

can accurately identify contaminating pathogens in clinical samples (Table 5), and its performance is comparable with existing FDA-approved tests. Although the sample size is much smaller than what is required and used in FDA-approved applications, the small percentage of false-positive and false-negative results detected for each target suggests acceptable performance in a trial for future regulatory approval of a medical device.

Although the threat of HIV, HCV, and HBV infections through blood transfusion has been largely eliminated by effective development and utilization of molecular tests, in the past few years the focus has shifted to timely detection of emerging pathogens (Ebola, Zika, chikungunya, and dengue viruses) in the donor blood supply. The strategy for identification of emerging infections requires a rapid response from industry to develop novel molecular tests and from regulatory agencies to assess test safety and efficacy for utilization in the highly regulated environment of the blood bank industry. Situations such as the looming Zika virus crisis call for the approval of screening devices that can be rapidly adapted to new agents with the potential for a short approval time. From the data presented in this study, a multiplex device such as BBPP can be easily adaptable for research to detect any emerging pathogen threatening the blood supply. This study provides evidence that, in addition to being a highly multiplexed device, the OpenArray platform has flexibility, allowing manufacturers to include any novel assay with minor device modifications. The fact that real-time PCR assays are spatially separated and performed in individual reaction wells minimizes any assay optimization time and does not affect analytical performance of the device. Application of novel technologies, such as BBPP, offers the prospect for future development of the next generation of molecular diagnostics devices for detection of increasing number of transfusion-transmissible agents in multiplex format with superior analytical performance, which is required to maintain the safety of blood products.

Acknowledgments

We thank Zhiping Ye (Center for Biologics Evaluation and Research, FDA, Silver Spring, MD) for providing influenza virus stocks, Indira Hewlett (Center for Biologics Evaluation and Research, FDA, Silver Spring, MD) for providing HIV stocks, Craig Cummings (Genentech, South San Francisco, CA) for help with assay design, and Ranadhir Dey and Rana Nagarkatti (Center for Biologics Evaluation and Research, FDA, Silver Spring, MD) for reviewing the manuscript.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.jmoldx.2017.03.004>.

References

1. Alter HJ, Stramer SL, Dodd RY: Emerging infectious diseases that threaten the blood supply. *Semin Hematol* 2007, 44:32–41
2. Gurukumar KR, Priyadarshini D, Patil JA, Bhagat A, Singh A, Shah PS, Cecilia D: Development of real time PCR for detection and quantitation of Dengue Viruses. *Viol J* 2009, 6:10
3. Bloch EM, Lee TH, Krause PJ, Telford SR III, Montalvo L, Chafets D, Usmani-Brown S, Lepore TJ, Busch MP: Development of a real-time polymerase chain reaction assay for sensitive detection and quantitation of Babesia microti infection. *Transfusion* 2013, 53: 2299–2306
4. Duncan R, Kourout M, Grigorenko E, Fisher C, Dong M: Advances in multiplex nucleic acid diagnostics for blood-borne pathogens: promises and pitfalls. *Expert Rev Mol Diagn* 2016, 16:83–95
5. Grigorenko E, Fisher C, Patel S, Chancey C, Rios M, Nakhasi HL, Duncan RC: Multiplex screening for blood-borne viral, bacterial, and protozoan parasites using an OpenArray platform. *J Mol Diagn* 2014, 16:136–144
6. Davis C, Heath A, Best S, Hewlett I, Lelie N, Schuurman R, Holmes H: Calibration of HIV-1 working reagents for nucleic acid amplification techniques against the 1st international standard for HIV-1 RNA. *J Virol Methods* 2003, 107:37–44
7. Lee S, Wood O, Taffs RE, Hu J, Machuca A, Vallejo A, Hewlett I: Development and evaluation of HIV-1 subtype RNA panels for the standardization of HIV-1 NAT assays. *J Virol Methods* 2006, 137: 287–291
8. Damond F, Collin G, Descamps D, Matheron S, Pueyo S, Taieb A, Campa P, Benard A, Chene G, Brun-Vezinet F: Improved sensitivity of human immunodeficiency virus type 2 subtype B plasma viral load assay. *J Clin Microbiol* 2005, 43:4234–4236
9. Saldanha J, Heath A, Lelie N, Pisani G, Nubling M, Yu M: Calibration of HCV working reagents for NAT assays against the HCV international standard. The Collaborative Study Group. *Vox Sang* 2000, 78: 217–224
10. Duan H, Struble E, Zhong L, Mihalik K, Major M, Zhang P, Feinstone S, Feigelstock D: Hepatitis C virus with a naturally occurring single amino-acid substitution in the E2 envelope protein escapes neutralization by naturally-induced and vaccine-induced antibodies. *Vaccine* 2010, 28:4138–4144
11. Grinev A, Daniel S, Laassri M, Chumakov K, Chizhikov V, Rios M: Microarray-based assay for the detection of genetic variations of structural genes of West Nile virus. *J Virol Methods* 2008, 154:27–40
12. Dong M, Fisher C, Anez G, Rios M, Nakhasi HL, Hobson JP, Beanan M, Hockman D, Grigorenko E, Duncan R: Standardized methods to generate mock (spiked) clinical specimens by spiking blood or plasma with cultured pathogens. *J Appl Microbiol* 2016, 120: 1119–1129
13. Zhou H, Yu Z, Hu Y, Tu J, Zou W, Peng Y, Zhu J, Li Y, Zhang A, Yu Z, Ye Z, Chen H, Jin M: The special neuraminidase stalk-motif responsible for increased virulence and pathogenesis of H5N1 influenza A virus. *PLoS One* 2009, 4:e6277
14. Rios M, Zhang MJ, Grinev A, Srinivasan K, Daniel S, Wood O, Hewlett IK, Dayton AI: Monocytes-macrophages are a potential target in human infection with West Nile virus through blood transfusion. *Transfusion* 2006, 46:659–667
15. Rios M, Daniel S, Chancey C, Hewlett IK, Stramer SL: West Nile virus adheres to human red blood cells in whole blood. *Clin Infect Dis* 2007, 45:181–186
16. Rios M, Daniel S, Dayton AI, Wood O, Hewlett IK, Epstein JS, Caglioti S, Stramer SL: In vitro evaluation of the protective role of human antibodies to West Nile virus (WNV) produced during natural WNV infection. *J Infect Dis* 2008, 198:1300–1308
17. Hockman D, Dong M, Zheng H, Kumar S, Huff MD, Grigorenko E, Beanan M, Duncan R: Comparison of multiplex PCR hybridization-based and singleplex real-time PCR-based assays for detection of

- low prevalence pathogens in spiked samples. *J Microbiol Methods* 2016, 132:76–82
18. Kourout M, Fisher C, Purkayastha A, Tibbetts C, Winkelman V, Williamson P, Nakhasi HL, Duncan R: Multiplex detection and identification of viral, bacterial, and protozoan pathogens in human blood and plasma using a high-density resequencing pathogen microarray platform. *Transfusion* 2016, 56:1537–1547
 19. Moitra P, Zheng H, Anantharaman V, Banerjee R, Takeda K, Kozakai Y, Lepore T, Krause PJ, Aravind L, Kumar S: Expression, purification, and biological characterization of Babesia microti apical membrane antigen 1. *Infect Immun* 2015, 83:3890–3901
 20. Debrabant A, Joshi MB, Pimenta PFP, Dwyer DM: Generation of Leishmania donovani axenic amastigotes: their growth and biological characteristics. *Int J Parasitol* 2004, 34:205–217
 21. Selvapandian A, Duncan R, Mendez J, Kumar R, Salotra P, Cardo LJ, Nakhasi HL: A Leishmania minicircle DNA footprint assay for sensitive detection and rapid speciation of clinical isolates. *Transfusion* 2008, 48:1787–1798
 22. Mahajan B, Zheng H, Pham PT, Sedegah MY, Majam VF, Akolkar N, Rios M, Ankrah I, Madjitey P, Amoah G, Addison E, Quakyi IA, Kumar S: Polymerase chain reaction-based tests for pan-species and species-specific detection of human Plasmodium parasites. *Transfusion* 2012, 52:1949–1956
 23. Collins WE, Warren M, Huong AY, Skinner JC, Sutton BB, Stanfill PS: Studies of comparative infectivity of fifteen strains of Plasmodium vivax to laboratory-reared anopheline mosquitoes, with special reference to Anopheles culicifacies. *J Parasitol* 1986, 72:521–524
 24. McDaniel JP, Dvorak JA: Identification, isolation, and characterization of naturally-occurring Trypanosoma cruzi variants. *Mol Biochem Parasitol* 1993, 57:213–222
 25. Lanciotti RS, Kosoy OL, Laven JJ, Panella AJ, Velez JO, Lambert AJ, Campbell GL: Chikungunya virus in US travelers returning from India, 2006. *Emerg Infect Dis* 2007, 13:764–767
 26. Nicolas L, Prina E, Lang T, Milton G: Real-time PCR for detection and quantitation of leishmania in mouse tissues. *J Clin Microbiol* 2002, 40:1666–1669
 27. Selvapandian A, Dey R, Nylen S, Duncan R, Sacks D, Nakhasi HL: Intracellular replication-deficient Leishmania donovani induces long lasting protective immunity against visceral leishmaniasis. *J Immunol* 2009, 183:1813–1820
 28. Michelin ADF, Perri SH, De Lima VM: Evaluation of TNF-alpha, IL-4, and IL-10 and parasite density in spleen and liver of L. (L.) chagasi naturally infected dogs. *Ann Trop Med Parasitol* 2011, 105:373–383
 29. Wortmann G, Hochberg L, Houng HH, Sweeney C, Zapor M, Aronson N, Weina P, Ockenhouse CF: Rapid identification of Leishmania complexes by a real-time PCR assay. *Am J Trop Med Hyg* 2005, 73:999–1004
 30. Piron M, Fisa R, Casamitjana N, Lopez-Chejade P, Puig L, Verges M, Gascon J, Gomez i Prat J, Portus M, Sauleda S: Development of a real-time PCR assay for Trypanosoma cruzi detection in blood samples. *Acta Trop* 2007, 103:195–200
 31. Ohmori S, Kawai A, Takada N, Saito-Ito A: Development of real-time PCR assay for differential detection and quantification for multiple Babesia microti genotypes. *Parasitol Int* 2011, 60:403–409
 32. Morrison T, Hurley J, Garcia J, Yoder K, Katz A, Roberts D, Cho J, Kanigan T, Ilyin SE, Horowitz D, Dixon JM, Brenan CJ: Nanoliter high throughput quantitative PCR. *Nucleic Acids Res* 2006, 34:e123
 33. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT: The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009, 55:611–622
 34. Wilson EB: Probable inference, the law of succession, and statistical inference. *J Am Stat Assoc* 1927, 22:209–212
 35. Dreier J, Stormer M, Kleesiek K: Real-time polymerase chain reaction in transfusion medicine: applications for detection of bacterial contamination in blood products. *Transfus Med Rev* 2007, 21:237–254
 36. Margaritis AR, Brown SM, Seed CR, Kiely P, D'Agostino B, Keller AJ: Comparison of two automated nucleic acid testing systems for simultaneous detection of human immunodeficiency virus and hepatitis C virus RNA and hepatitis B virus DNA. *Transfusion* 2007, 47:1783–1793
 37. Moritz ED, Winton CS, Johnson ST, Krysztow DE, Townsend RL, Foster GA, Devine P, Molloy P, Brissette E, Berardi VP, Stramer SL: Investigational screening for Babesia microti in a large repository of blood donor samples from nonendemic and endemic areas of the United States. *Transfusion* 2014, 54:2226–2236
 38. Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, Turner P, Parkhill J, Loman NJ, Walker AW: Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biol* 2014, 12:87
 39. Brennan CA, Yamaguchi J, Devare SG, Foster GA, Stramer SL: Expanded evaluation of blood donors in the United States for human immunodeficiency virus type 1 non-B subtypes and antiretroviral drug-resistant strains: 2005 through 2007. *Transfusion* 2010, 50:2707–2712
 40. Delwart E, Slikas E, Stramer SL, Kamel H, Kessler D, Krysztow D, Tobler LH, Carrick DM, Steele W, Todd D, Wright DJ, Kleinman SH, Busch MP; NHLBI-REDS-II Study Group: Genetic diversity of recently acquired and prevalent HIV, hepatitis B virus, and hepatitis C virus infections in US blood donors. *J Infect Dis* 2012, 205:875–885
 41. Ivens AC, Peacock CS, Worthey EA, Murphy L, Aggarwal G, Berriman M, et al: The genome of the kinetoplastid parasite, Leishmania major. *Science* 2005, 309:436–442
 42. Peacock CS, Seeger K, Harris D, Murphy L, Ruiz JC, Quail MA, et al: Comparative genomic analysis of three Leishmania species that cause diverse human disease. *Nat Genet* 2007, 39:839–847
 43. Kuehnert MJ, Roth VR, Haley NR, Gregory KR, Elder KV, Schreiber GB, Arduino MJ, Holt SC, Carson LA, Banerjee SN, Jarvis WR: Transfusion-transmitted bacterial infection in the United States, 1998 through 2000. *Transfusion* 2001, 41:1493–1499
 44. Samikkannu T, Rao KV, Salam AA, Atluri VS, Kaftanovskaya EM, Agudelo M, Perez S, Yoo C, Raymond AD, Ding H, Nair MP: HIV subtypes B and C gp120 and methamphetamine interaction: dopaminergic system implicates differential neuronal toxicity. *Sci Rep* 2015, 5:11130
 45. Bhargava M, Cajas JM, Wainberg MA, Klein MB, Pant Pai N: Do HIV-1 non-B subtypes differentially impact resistance mutations and clinical disease progression in treated populations? Evidence from a systematic review. *J Int AIDS Soc* 2014, 17:18944
 46. Tatem AJ, Hemelaar J, Gray RR, Salemi M: Spatial accessibility and the spread of HIV-1 subtypes and recombinants. *AIDS* 2012, 26:2351–2360
 47. Li P, Grigorenko E, Funari V, Enright E, Zhang H, Kim HL: Evaluation of a high-throughput, microfluidics platform for performing TaqMan qPCR using formalin-fixed paraffin-embedded tumors. *Bioanalysis* 2013, 5:1623–1633
 48. Patel SN, Wu Y, Bao Y, Mancebo R, Au-Young J, Grigorenko E: TaqMan(R) OpenArray(R) high-throughput transcriptional analysis of human embryonic and induced pluripotent stem cells. *Methods Mol Biol* 2013, 997:191–201