

Development of a TaqMan Array Card for Acute-Febrile-Illness Outbreak Investigation and Surveillance of Emerging Pathogens, Including Ebola Virus

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Acute febrile illness (AFI) is associated with substantial morbidity and mortality worldwide, yet an etiologic agent is often not identified. Convalescent-phase serology is impractical, blood culture is slow, and many pathogens are fastidious or impossible to cultivate. We developed a real-time PCR-based TaqMan array card (TAC) that can test six to eight samples within 2.5 h from sample to results and can simultaneously detect 26 AFI-associated organisms, including 15 viruses (chikungunya, Crimean-Congo hemorrhagic fever [CCHF] virus, dengue, Ebola virus, Bundibugyo virus, Sudan virus, hantaviruses [Hantaan and Seoul], hepatitis E, Marburg, Nipah virus, o'nyong-nyong virus, Rift Valley fever virus, West Nile virus, and yellow fever virus), 8 bacteria (*Bartonella* spp., *Brucella* spp., *Coxiella burnetii*, *Leptospira* spp., *Rickettsia* spp., *Salmonella enterica* and *Salmonella enterica* serovar Typhi, and *Yersinia pestis*), and 3 protozoa (*Leishmania* spp., *Plasmodium* spp., and *Trypanosoma brucei*). Two extrinsic controls (phocine herpesvirus 1 and bacteriophage MS2) were included to ensure extraction and amplification efficiency. Analytical validation was performed on spiked specimens for linearity, intra-assay precision, interassay precision, limit of detection, and specificity. The performance of the card on clinical specimens was evaluated with 1,050 blood samples by comparison to the individual real-time PCR assays, and the TAC exhibited an overall 88% (278/315; 95% confidence interval [CI], 84% to 92%) sensitivity and a 99% (5,261/5,326, 98% to 99%) specificity. This TaqMan array card can be used in field settings as a rapid screen for outbreak investigation or for the surveillance of pathogens, including Ebola virus.

Fever is a symptom common to a wide variety of infectious diseases, including some of the leading causes of death in sub-Saharan Africa (SSA). Many etiologic studies have been performed for respiratory infections, diarrheal illness, and meningitis (1, 2). However, the incidence and etiology of undifferentiated fever are less clear (3). Most research has examined individual agents such as *Plasmodium*, *Salmonella*, and specific zoonotic or arboviral pathogens (4–6) by utilizing blood culture (7) or a complex mixture of rapid, serologic, culture, and molecular assays and algorithms to determine an etiologic agent (8).

We describe our initial development and validation of a TaqMan array card (TAC) that uses quantitative reverse transcription-PCR (qRT-PCR) for the simultaneous detection of 15 viruses, 8 bacteria, and 3 protozoa of particular relevance to SSA (5, 9–13), with the intended use for outbreak investigation and acute febrile illness (AFI) surveillance. Previous TAC assays have been developed for respiratory diseases, enteric diseases, and etiologies of neonatal sepsis (14–16), and we have shown their robust and comparable performance across several countries (17). Once developed, TaqMan array cards are stable at 4°C for 2 years, can be shipped at ambient temperature, and minimize several cumbersome

steps in the field, such that they are as easy to perform as individual quantitative PCR (qPCR) assays.

This work was primarily a development exercise since clinical

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validation was limited for many rare pathogens. However, we propose that this diagnostic tool can potentially screen for Ebola virus disease (18) or indicate other etiologies of fever such as malaria, dengue, or typhoid. Indeed the slow recognition of the Ebola outbreak in Guinea was largely due to delays in laboratory diagnosis and confirmation and to the inability to perform such testing in the region (9).

MATERIALS AND METHODS

TAC design. Primers and probes for 26 pathogens and 2 extrinsic controls were included on the TaqMan array card. Primer and probe sequences were adapted from published assays whenever possible (Table 1). The assays were evaluated *in silico* with BLAST, Clustal, and Primer Express (Life Technologies, Carlsbad, CA). When needed, slight modifications were made to primer or probe length to optimize performance under universal cycling conditions, and modified versions were tested in parallel with the original assays. Most assays were performed in duplicate to maximize sensitivity as laid out in Fig. 1. Assays were validated on plates using the TaqMan array card universal formula, which is a final primer concentration of 900 nM and a probe concentration of 250 nM. This custom TaqMan array card was manufactured by Life Technologies.

Specimens. Analytical specimens included genomic materials, cultured organisms, and *in vitro* transcripts for several RNA targets, as indicated in Table S1 in the supplemental material. In addition, a total of 1,050 clinical samples were collected through several studies conducted across Africa (Table 2), including 362 archived samples selected based on their likelihood for being positive for some of the pathogens on the TaqMan array card and 688 blood specimens from disease surveillance representing the AFI cases in relevant regions in Tanzania. These samples were frozen at -70°C prior to testing. Briefly, the archived samples included the following: (i) 105 serum samples (166 μl extracted) from outbreak investigations and previous studies at the U.S. Centers for Disease Control and Prevention (CDC)-Kenya from 2008 to 2014, (ii) 49 RNA samples (100 μl extracted) obtained from the ELWA III hospital laboratory in Monrovia, Liberia, during the Ebola virus outbreak in 2014, (iii) 186 whole blood samples (1 to 2.5 ml extracted) obtained at two clinical sites from patients presenting with moderate to severe febrile illness in Kilombero, Tanzania, in 2014, (iv) 16 whole blood specimens (2.5 ml extracted) obtained from inpatients at Kilimanjaro Christian Medical Centre and Mawenzi Regional Hospital, Moshi, Tanzania (10), and (v) 4 whole blood samples (1 ml extracted) known to be positive for *Salmonella enterica* by culture obtained from adult patients with severe sepsis enrolled in a fluid resuscitation study in Uganda and 2 whole blood samples (2.5 ml extracted) that were blood culture positive for *Salmonella enterica* from the clinical laboratory at the University of Virginia. All of the blood samples were collected in Vacutainer EDTA tubes. All tests were performed with institutional approvals as follows: KEMRI ERC-SSC 1899 (SSC 932), Duke University Health System Institutional Review Board protocol 8400-06-4R0, Kilimanjaro Christian Medical Centre Research Ethics Committee protocol 136, Tanzania National Institutes for Medical Research National Research Ethics Coordinating Committee protocol NIMR/HQ/R.8a/Vol. IX/439 and 1735, Uganda National Council for Science and Technology (UNCST HS 419), Centers for Disease Control and Prevention Institutional Review Board protocol 6567, and the University of Virginia HSR 13393 and 17391, respectively.

Combined positive controls. Two combined positive controls, one for DNA targets and one for RNA targets, were designed as previously described (16, 19). Plasmids were synthesized by GeneWiz (South Plainfield, NJ) and were used directly as DNA or *in vitro* transcribed as RNA.

Nucleic acid extraction from blood samples. For the archived CDC-Kenya clinical samples in the first study (Table 2), 166 μl of each sample was processed in a KingFisher ML extraction platform (Thermo Scientific, Waltham, MA) using a MagMAX nucleic acid isolation kit (Life Technologies, Carlsbad, CA). Briefly, 166 μl of sample was mixed with 433 μl of

lysis-binding solution and was then washed once with 600 μl wash solution 1 and twice with 450 μl wash solution 2 and was eventually eluted in 200 μl elution buffer. For the samples from the second study, viral RNA was extracted from 100 μl of blood and was eluted with 90 μl of elution buffer on the MagMAX Express-96 deep-well magnetic particle processor (Life Technologies) using the MagMAX pathogen RNA/DNA kit following the manufacturer's instructions. For the analytical specimens and clinical specimens from the third, fourth, fifth, and sixth studies, total nucleic acid (TNA) was extracted using a High Pure viral nucleic acid large volume kit (Roche) by following the manufacturer's instructions. Extrinsic controls, 10^6 copies of phocine herpesvirus (PhHV) and 10^7 MS2 bacteriophage (ATCC, Manassas, VA), were added to each sample during the lysate preparation to evaluate extraction and amplification efficiency. The total nucleic acid was eluted in 100 or 200 μl of elution buffer to accommodate the sample need of running individual real-time PCR (IRTP) for all 26 targets (Table 2). One extraction blank was included with each batch of extraction to monitor lab contamination, and if it was positive for a given target, then the positive results for this target in the entire batch were considered invalid (this only happened once, invalidating the *Salmonella* results of 5 clinical samples).

PCR using TaqMan array cards. All analytical specimens and 1,050 clinical samples were tested with TaqMan array cards. We mixed 46 or 75 μl of total nucleic acid extract with AgPath one-step RT-PCR reagents or with TaqMan fast virus one-step master mix (Life Technologies), respectively, in a 100- μl reaction mixture, as shown in Table 2, and then pipetted the mixture into the inlet port of each channel. Cards were centrifuged (1 min at 1,200 rpm twice) and sealed, and the inlet ports were removed following the manufacturer's instructions. Cards were run on the ViiA 7 real-time PCR system (Life Technologies) using PCR cycling conditions comprising 10 min at 50°C and 20 s at 95°C followed by 45 two-step cycles of 3 s at 95°C and 30 s at 60°C . A sample is called positive when any of the duplicate reactions yield amplification (quantification cycle [C_q] < 45).

Performance on analytical specimens. Linearity was tested with a 10-fold serial dilution of combined positive controls. For limit of detection (LOD), intra-assay precision, and interassay precision, positive materials (either genomic material, organisms, or *in vitro* transcripts as indicated in Table S1 in the supplemental material) were spiked into lysis buffer and added to blood samples from healthy donors (2.5 ml blood per sample). Nucleic acid was extracted and assayed in the card, as described above, with an elution volume of 100 μl and a sample volume of 75 μl . Intra-assay precision was tested with eight repeats on one single TAC with pooled extracts from 8 identical 2.5-ml spiked samples. Interassay precision was tested with 10 identically spiked samples that were extracted and assayed over 5 days. Limit of detection was estimated as the lowest concentration at which the target could be detected in all 10 spiked samples. Matrix inhibition was evaluated with extrinsic controls spiked into each clinical sample type (serum, whole blood, and plasma) in the Tanzania surveillance study, where 688 samples were in various forms, including 55 whole blood samples (0.7 to 2.5 ml), 419 serum samples (0.25 to 1.5 ml), and 214 plasma samples (1 ml). For genus-specific bacterial and protozoan assays, multiple species were assayed as listed in Table S2 in the supplemental material. A panel of commonly found bloodstream pathogens were also spiked at a concentration of 10^7 copies per extraction and were tested to evaluate the specificity of each assay, including *Acinetobacter baumannii*, *Cryptococcus neoformans*, cytomegalovirus, *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Toxoplasma gondii*.

Performance on clinical specimens. Comparison of the TAC was made against individual real-time PCR (IRTP), with the cognate assays tested on plates with 2 to 5 μl of nucleic acid, the same PCR master mix, and the same PCR conditions. The number of samples that were tested for each pathogen was listed in Table 2. All samples that tested positive on TAC for a given target were evaluated for the corresponding targets on

TABLE 1 Primer and probe sequences for the TaqMan array card assays

Pathogen	Target	Sequence (5'–3') ^a	Reference
Viruses			
Chikungunya	NSP4	F: TCACTCCCTGYTGGACTTGTATAGA R: TTGACGAACAGAGTTAGGAACATACC P: AGGTACGCGCTTCAAGTTCGGCG	Modified (34)
CCHF	NP	F: CAAAGAAACACGTGCCGCTT R: ATTCACCTCGATTTTGTTCAT P: ACGCCACAGTGTCTCTGAGTGTAGCA	(35)
Dengue	3'NC	F: GGATAGACCAGAGATCCTGTGT R: CATTCCATTTCTGGCGTTC R: CAATCCATCTTGC GGCGCTC P: CAGCATCATTCCAGGCACAG	(28)
Ebola Zaire	NP	F: TGGAAAAACATTAAGAGAACACTTGC R: AGGAGAGAACTGACCGCAT P: CATGCCGGAAGAGGAGACA ACTGAAGC	(28)
Bundibugyo	VP40	F: MGCATCRTAYACCATCACTCA R: SCCAGGACCAAGTCGRITGA P: TTTGGCAAAACCTCMAATCC	(13)
Sudan	NP	F: GCCATGGITTCAGGTTTGTAG R: GGTIACATTGGGCAACAATTCA P: ACGGTGCACATTCTCCTTTCTCGGA	(13)
Hantavirus	NP	F: CATGGCWTCHAAGACWGTGGG R: TTKCCCCAGGCAACCAT P: CAATCAATGGGRATACA ACTGG	Modified (36)
Hepatitis E	ORF3	F: GGTGGTTTCTGGGGTGAC R: AGGGTTGGTTGGATGAA P: TGATTCTCAGCCCTTCGC	(37)
Marburg	VP40	F: GGACCACTGCTGGCCATATC R: GAGAACATITCGGCAGGAAG P: AAAGTCCCAGAGAAGACA	Modified (20)
Nipah	NP	F: CTGGTCTCTGCAGTTATCACCATCGA R: ACGTACTTAGCCATCTTCTAGTTTCA P: CAGCTCCCGACACTGCCGAGGAT	(38)
ONNV	E1	F: GCAGGGAGGCCAGGACAGT R: GCCCCTTTTTCYTTGAGCCAGTA P: TGTATTGCTCCTGCCGCTGG	Modified (39)
Rift Valley fever	L	F: TGAAAATTCTGAGACACATGG R: ACTTCCTTGCATCATCTGATG P: CACAAGTCCACACAGGCCCTTACAT	Modified (40)
West Nile	3'NC	F: CAGACCACGCTACGGCG R: CTAGGGCCGCGTGGG P: TCTGCGGAGAGTGCAGTCTGCGAT	(41)
Yellow fever	RdRp	F: GGGAAAACCTCAGGAGGAGGA F: GGGAGAATTCRGGGGGAGGA R: AAGGTCTGCCTCTGTGATGC P: TCAGAGACCTGGCTGCAATGGATGGT	Modified (42)
Bacteria			
<i>Bartonella</i> spp.	ssrA	F: GGCTAAATIAGTAGTTGCAAAYGACA R: GCTTCTGTTGCCAGGTG P: ACCCCGCTTAAACCTGCGACG	Modified (43)
<i>Brucella</i> spp.	IS711	F: GCTTGAAGCTTGC GGACAGT R: GGCTACCGCTGCGAAT P: AAGCCAACACCCGGCCATTATGGT	(44)
<i>Coxiella burnetii</i>	IS1111	F: CCGATCATTGGGGCGCT R: CGGCGGTGTTAGGC P: TTAACACGCCAAGAAACGTATCGCTGTG	(45)
<i>Leptospira</i> spp.	LipL32	F: CCCTAIGGATCTGTRATCAACTA R: GAACTCCCATTTCAGCGATT P: AAAGCCAGGACAAGCGCCG	Modified (46)
<i>Rickettsia</i> spp.	23S	F: AGCTTGCTTTTGGATCATTTGG R: TTCCTTGCTTTTCATACATCTAGT P: CCTGCTTCTATTTGCTTGC	Modified (47)

(Continued on following page)

TABLE 1 (Continued)

Pathogen	Target	Sequence (5'-3')	Reference
<i>Salmonella enterica</i>	ttr	F: CTCACCAGGAGATTACAACATGG R: AGCTCAGACCAAAAGTGACCATC P: CACCGACGGCGAGACCGACTTT	(48)
<i>Salmonella</i> Typhi	STY0201	F: CGCGAAGTCAGAGTCGACATAG R: AAGACCTCAACGCCGATCAC P: CAGCCTGCTCCAGAACA	Modified (49)
<i>Yersinia pestis</i>	CafI	F: CCACTGCAACGGCAACTCTT R: TGTAATTGGAGCGCCTTCTT P: TTGAACCAAGCCCGCATCACTCTTACA	
Protozoa			
<i>Leishmania</i> spp.	18S	F: AAGTGCTTTCCCATCGCAACT R: GACGACTAAACCCCTCAA P: CGGTTCCGGTGTGTGGCGCC	Modified (50)
<i>Plasmodium</i> spp.	18S	F: GCTCTTTCTTGATTCTTGGATG R: AGCAGGTTAAGATCTCGTTCC P: CACGAACTAAAACGGCCAT	Modified (51)
<i>Trypanosoma brucei</i>	18S	F: CGCCAAGCTAATACATGAACCAA R: TAATTTTCATTTCGCTGGACG P: CTTGTGTTACGCACTTGTC	Modified (52)

^a F, forward primer; R, reverse primer; P, TaqMan MGB probe.

96-well or 384-well plates, and a subset of negative samples (to the extent that specimen was available) was also retested on plates. Positive samples from studies 4, 5, and 6 (Table 2) were further confirmed by amplicon sequencing with the assays listed in Table S3 in the supplemental material.

Statistics. Quantification cycle (C_q) values were compared with the Mann-Whitney U test between sequence-confirmed and -unconfirmed samples and between IRTP-confirmed and -unconfirmed samples, among different sample types. C_q values obtained from the TAC and IRTP

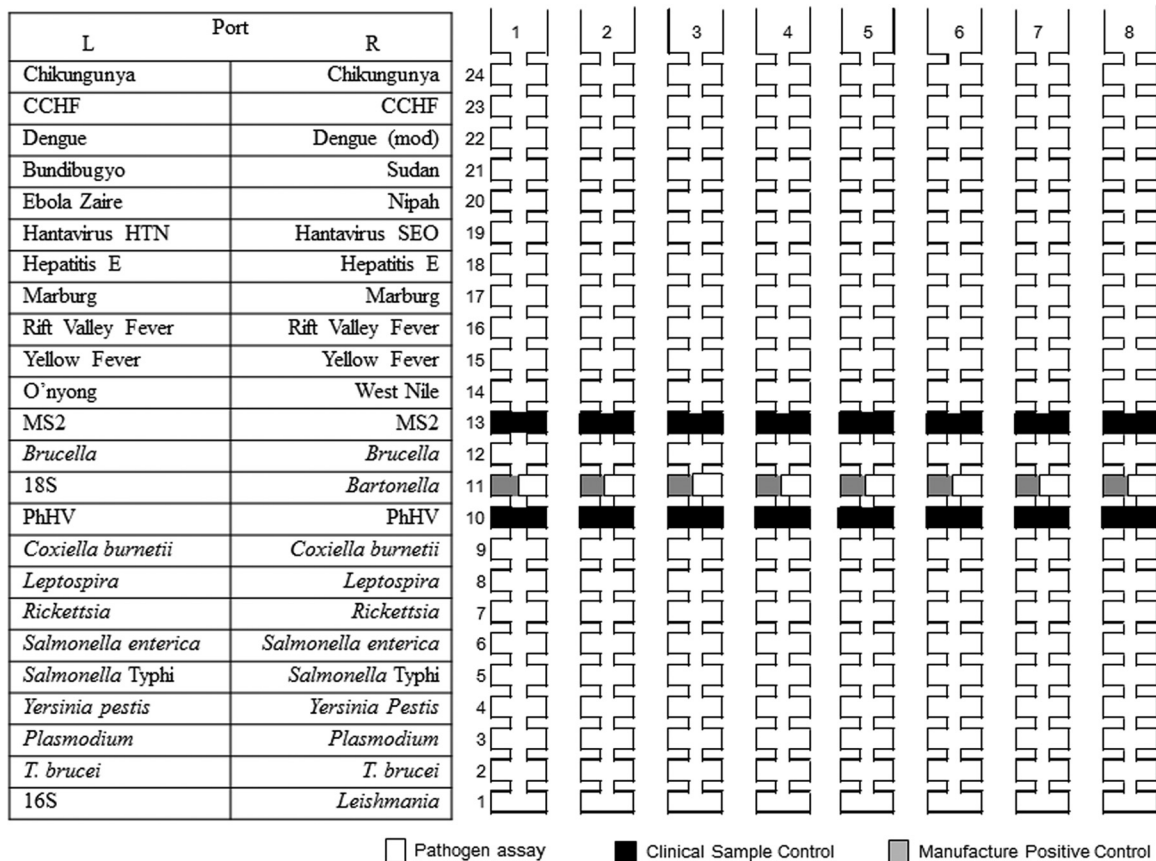


FIG 1 Configuration of the TaqMan array card for detection of the agents causing acute febrile illness.

TABLE 2 Clinical specimens used in this study and the corresponding sample processing methods^a

Study ID	Selection rationale	No. of samples	Region	Sample type (no.)	Extraction		TAC	
					Method	Elution vol (μl)	Enzyme	Sample vol (μl)
1	Outbreak investigation	105	Kenya (various)	Serum	MagMAX	200	AgPath	46
2	Ebola virus outbreak	49	Monrovia, Liberia	Whole blood	MagMAX	90	AgPath	46
3	Hospitalized AFI patients	186	Kilombero, Tanzania	Whole blood	High Pure	200	AgPath	46
4	Hospitalized AFI patients	16	Moshi, Tanzania	Whole blood	High Pure	100	TaqMan fast virus kit	75
5	Patients presenting fever with blood culture positive for <i>Salmonella</i>	6	Uganda (4), Virginia (2)	Whole blood	High Pure	100	TaqMan fast virus kit	75
6	AFI cases	688	Tanzania (various)	Serum (419), plasma (214), whole blood (55)	High Pure	100	TaqMan fast virus kit	75

^a No difference in detection of external controls (MS2 and PhHV) was observed with the deviations in extraction methods and PCR reagents (data not shown).

for the same samples were compared with the Wilcoxon signed-rank test. Receiver operating characteristic (ROC) analysis was used to derive C_q cutoffs based on sequencing results as the gold standard. Mean and standard deviation were shown. Correlation was tested by regression analysis using the analysis of variance (ANOVA) test. Two-tailed P values were calculated, and values of <0.05 were considered statistically significant. All analyses were performed using SPSS version 22.

RESULTS

Performance on analytical specimens. Each TAC assay detected the relevant species or serovar with 100% specificity using the specificity panel. Assays exhibited a linear relationship between C_q values and quantity (R^2 from 0.994 to 1), high PCR efficiency ($91\% \pm 5\%$), and robust intra-assay precision and interassay precision. The lower limit of detection was estimated to be 10^4 copies/ml of blood for viral targets, 10^3 copies/ml of blood for bacterial targets, and 10^2 copies/ml of blood for parasitic targets, equivalent to 2 to 200 copies (prior to extraction) per 1-μl reaction mixture. The extrinsic controls MS2 and PhHV were used to assess matrix inhibition and were detected in 91% of the whole blood samples, in $>99\%$ of the serum samples, and in $>97\%$ of the plasma samples. Surprisingly, whole blood samples yielded lower C_q values for the extrinsic controls than those for serum and plasma but were within 2 C_q s (data not shown). Considering that some of the pathogens interrogated are intracellular, we would recommend using whole blood.

Performance on clinical specimens. We then proceeded to the clinical specimens, sourcing material from several studies in attempts to obtain blood samples that may be PCR positive for dengue, Ebola virus, hepatitis E, Rift Valley fever virus, *Bartonella* spp., *Brucella* spp., *Coxiella burnetii*, *Leptospira* spp., *Rickettsia* spp., *Plasmodium* spp., and *Salmonella enterica*, including serovar Typhi. Among duplicate reactions on the TAC, most (75%) were positive in the two wells while the rest were positive in only one well (usually at higher C_q , averaging 38.7 ± 3.3 versus 25.3 ± 5.3 for duplicate positives; $P < 0.001$). Either was considered positive by TAC. All positive TAC results were tested with plate-based PCR using the cognate assays (Table 3), and negative TAC results were retested to the extent that sample quantity allowed. Compared to the plate-based assays, the overall sensitivity and specificity of TAC were 88% (95% confidence interval, 84% to 92%) and 99% (98% to 99%), respectively. Discrepancies were generally observed at late C_q values. For example, the TAC-positive/IRTP-positive samples for *Brucella* yielded a TAC C_q of 34.2 ± 0.6 while

the TAC-positive/IRTP-negative samples yielded 38.3 ± 4.0 ($P < 0.05$) and the TAC-negative/IRTP-positive samples yielded 40.3 ± 2.3 ($P < 0.05$). Likewise, the dengue TAC-positive/IRTP-positive samples yielded a TAC C_q of 24.3 ± 5.6 versus the TAC C_q of TAC-positive/IRTP-negative samples of 38.0 ± 2.3 ($P < 0.05$). In contrast, no difference in C_q was observed between the TAC-positive/IRTP-positive and the TAC-negative/IRTP-positive samples for hepatitis E. Overall, TAC C_q s were higher than the corresponding C_q s obtained from IRTP by an average ΔC_q of 1.8 ± 4.1 ($P < 0.05$) (Fig. 2), which corresponds to approximately 1 log loss in sensitivity.

Sequence confirmation. In order to further confirm the positive TAC results, amplicons of a subset of samples were sent for sequencing. Since TaqMan assays require short amplicons that are not suitable for direct sequencing, primers flanking the targeted regions were designed or adapted from publications to generate longer amplicons (for primers, see Table S3 in the supplemental material). The amplification results with these confirmatory assays correlated well with those of the original TAC assays run on a plate (data not shown). As expected, the C_q values of the samples that were sequence confirmable were significantly lower than the values of those that could not be sequenced. ROC analysis revealed that C_q values of 34.3, 32.8, 35.7, 34.9, and 35.0 or less for dengue, *Plasmodium*, *Rickettsia*, *Brucella*, and *Salmonella enterica* maximized the likelihood of sequence confirmation (Fig. 3); therefore, in our view results below these C_q s can be trusted.

Comparison of TAC results with initial testing. The clinical samples were collected from diverse sites across time and were stored in a range of conditions with limited clinical microbiology; therefore, clinical performance versus culture or other non-PCR method is uncertain. Furthermore, we documented obvious degradation of the specimens by comparing the PCR C_q values of the samples when tested upon initial collection with identical real-time PCR assays (data not shown). That said, we do know that seven whole blood samples were initially positive for *Salmonella enterica* by blood culture, of which TAC identified five (after extraction of various volumes of blood from 166 μl to 2.5 ml; sensitivity, 71%). There were also 30 positive *Salmonella enterica* culture samples that were stored in clot or serum form, of which none were positive by TAC. There were 2 samples originally positive for *Leishmania* by rapid test (rK39), and they were both negative by TAC but positive by IRTP at late C_q s (38 and 42). The only mi-

TABLE 3 Sensitivity and specificity of TAC assays on clinical specimens compared to individual real-time PCR assays^a

Pathogen	No. of samples				% TAC sensitivity (95% CI)	% TAC specificity (95% CI)	Source(s) of the positives ^b
	IRTP positive and TAC positive	IRTP positive and TAC negative	IRTP negative and TAC positive	IRTP negative and TAC negative			
Chikungunya	0	0	2	216	NA ^c	99 (97–100)	NA
CCHF	0	0	0	218	NA	100 (98–100)	NA
Dengue	37	9	8	203	80 (66–91)	96 (93–98)	Studies 1 and 6
Ebola	49	0	0	266	100 (93–100)	100 (99–100)	Study 2
Bundibugyo	0	0	0	208	NA	100 (98–100)	NA
Sudan	0	0	0	217	NA	100 (98–100)	NA
Hantavirus	0	0	0	209	NA	100 (98–100)	NA
Hepatitis E	32	9	0	206	78 (62–89)	100 (98–100)	Study 1
Marburg	0	0	0	213	NA	100 (98–100)	NA
Nipah	0	0	0	217	NA	100 (98–100)	NA
ONNV	0	0	0	218	NA	100 (98–100)	NA
Rift Valley fever	7	1	0	215	88 (47–100)	100 (98–100)	Study 1
West Nile	0	0	0	180	NA	100 (98–100)	NA
Yellow fever	0	0	0	219	NA	100 (98–100)	NA
<i>Bartonella</i> spp.	1	0	0	217	100 (3–100)	100 (98–100)	Study 6
<i>Brucella</i> spp.	8	6	11	205	57 (29–82)	95 (91–97)	Studies 1 and 6
<i>Coxiella burnetii</i>	4	0	6	216	100 (40–100)	97 (94–99)	Study 6
<i>Leptospira</i> spp.	3	0	11	204	100 (29–100)	95 (91–97)	Studies 3 and 4
<i>Rickettsia</i> spp.	24	2	8	211	92 (75–99)	96 (93–98)	Studies 4 and 6
<i>Salmonella enterica</i>	7	1	5	223	88 (47–100)	98 (95–99)	Studies 1, 5, and 6
<i>Salmonella</i> Typhi	4	1	2	225	80 (28–99)	99 (97–100)	Studies 1, 5, and 6
<i>Yersinia pestis</i>	0	0	0	218	NA	100 (98–100)	NA
<i>Leishmania</i> spp.	0	2	0	215	0 (0–84)	100 (98–100)	Study 1
<i>Plasmodium</i> spp.	102	6	12	143	94 (88–98)	92 (87–96)	Studies 1, 3, 5, and 6
<i>Trypanosoma brucei</i>	0	0	0	179	NA	100 (98–100)	NA
Total	278	37	65	5261	88 (84–92)	99 (98–99)	

^a No cutoff was applied, and the two tests were run for 45 cycles. A sample was called positive when any of the duplicate reactions yielded amplification ($C_q < 45$).

^b Studies 1 to 6 are listed in Table 2.

^c NA, not applicable.

croscopy data we have show that eight samples were positive for *Plasmodium* by microscopy, and all 8 were detected by TAC and IRTP.

DISCUSSION

This work details the development of an integrated TaqMan array card that can be used on blood samples to screen for several infectious etiologies of acute febrile illness. Given that there may be multiple agents contributing to fever, this card may be an efficient tool for pathogen detection using a single reaction.

The pathogens we chose to test on this card were optimized for our intended use of outbreak investigation and of AFI surveillance in Africa (5, 9–11, 13, 20). Depending on demographic and temporal or geographic circumstances, users may prefer to prioritize certain pathogens to be included. Since the format is a modular arrangement of singleplex PCR assays, one could include primer and probe sequences from relevant microbial agents (e.g., *Streptococcus pneumoniae*, *Neisseria meningitidis*, etc.) that have been previously validated in TAC platforms for other syndromes with similar cycling conditions (14–16). In our view, this is an advantage of TAC over conventional multiplex PCR-based platforms (e.g., Biofire's BioThreat panel, FTD Tropical panels). We utilized primer and probe sequences from the published literature wherever possible to leverage the considerable experience of subject

matter experts given the many pathogens assayed. All assays revealed excellent analytical performance, and the limit of detection of the assays was estimated to be 10^2 to 10^4 copies/ml of blood, similar to that of the previously reported enteric or respiratory cards.

This limit of detection would be expected to be adequate to detect dengue, chikungunya, Ebola virus, and *Plasmodium* in most patients since these pathogens are usually present at even higher levels (21–24). Indeed, we were able to evaluate the assay on large numbers of specimens that were PCR positive for Ebola, dengue, hepatitis E, and *Plasmodium*, and we observed, as expected, reasonable sensitivities (100%, 80%, 78%, and 94%, respectively) and high specificities (96% to 100%) (Table 3). For these pathogens, where PCR is generally considered a gold-standard diagnostic test (particularly early in illness) (25–27), the performance of the TAC assay is likely to be acceptable. For Ebola, we deliberately used the exact nucleoprotein (NP) RT-PCR assay frequently used during outbreak investigation (28), and TAC revealed 100% sensitivity and 100% specificity versus those of the individual assay on 49 samples with diverse Ebola viral loads and a tight C_q correlation (RT-PCR C_q values ranging from 16 to 37; $R^2 = 0.87$; $P < 0.001$). Forty-five percent of these samples were also positive for *Plasmodium*, reinforcing the value of a multitarget diagnostic tool.

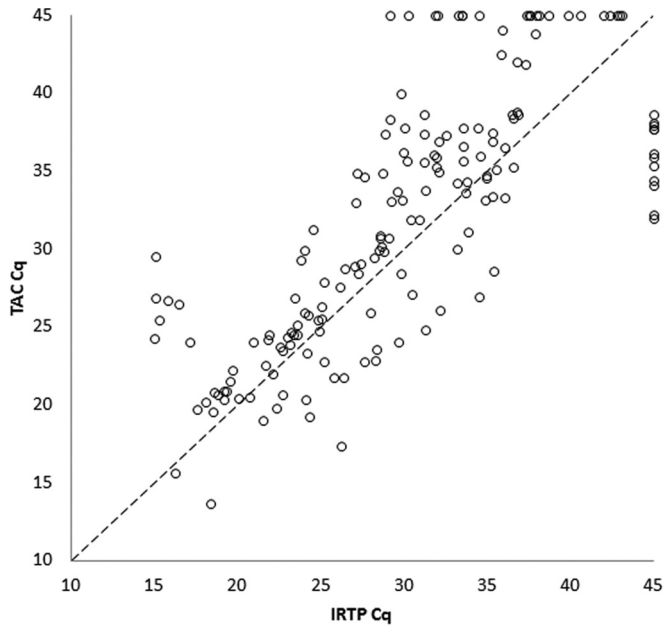


FIG 2 Paired comparison of C_q s from IRTP and TAC reactions. In general, the C_q s exhibited a linear relationship ($R^2 = 0.632$; $P < 0.001$). The results from different targets were pooled and compared with the Wilcoxon signed-rank test for the samples where IRTP and TAC yielded positive results. The difference was within 2 units (IRTP C_q - TAC $C_q = -1.8 \pm 4.1$; $P < 0.001$). The data points at the top and right of the graph represented IRTP-positive/TAC-negative and IRTP-negative/TAC-positive results, respectively. There was no significant difference in the number of samples that were IRTP positive/TAC negative or IRTP negative/TAC positive (chi-square test; $P = 0.205$).

We anticipated that the sensitivity of PCR for bacterial pathogens on a single blood specimen, particularly with TAC and its small sample volumes (up to 0.75 μ l nucleic acid in the 1- μ l reaction mixture), may be mediocre versus that of culture. Meta-analyses of commercial PCR tests for detecting bacteria in blood from sepsis patients reveal sensitivities of only 61% to 80% versus blood culture (29). This is particularly well known for typhoidal and nontyphoidal *Salmonella enterica*, where in one study the median bacterial burden was 0.3 CFU/ml in blood (30, 31), and *Salmonella* Typhi PCR performed directly on blood has often revealed sensitivity of $\sim 50\%$ versus that of culture (32). Our results with TAC were in keeping with these prior studies (71% sensitivity versus that of culture; $>80\%$ sensitivity versus that of IRTP), even though we purposely selected an extraction method that could process up to 2.5 ml of blood to improve sensitivity. Therefore, we acknowledge that this AFI TAC may have limited sensitivity for diagnosis of certain pathogens if it were applied to individual patients using single specimens. However, our main goal was to develop the card for surveillance or outbreak purposes, and in this context one could use TAC to test hundreds of specimens given its high throughput and any limitations in sensitivity could be statistically managed. For example, if one detected by TAC 20 cases of typhoid fever out of 1,000 specimens, then even accepting a 50% sensitivity of PCR versus that of culture, the predicted 95% confidence interval of typhoid prevalence would be 2.8% to 5.4%. In other words, testing larger numbers of specimens for surveillance and outbreak investigation could lead to tight estimates even with imperfect sensitivity.

There are other pathogens that are traditionally detected with serology (e.g., *Rickettsia*, *Bartonella*, *Coxiella*, *Leptospira*, *Brucella*, and hantavirus). Paired serologic testing is intrinsically an imperfect comparator because it audits exposure over a broad time pe-

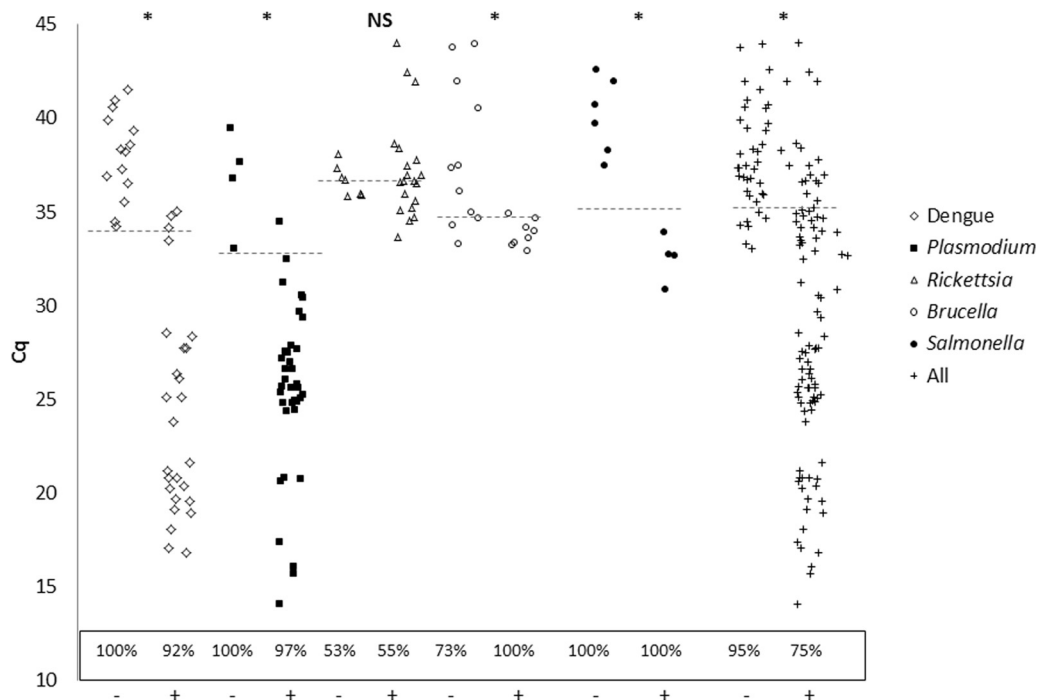


FIG 3 Optimal TAC C_q cutoffs using amplicon sequencing as the reference. The dashed line shows the cutoffs based on ROC analysis. On the x axis, symbols indicate that a sample was confirmed by sequencing (+) or not (-). TAC specificity and sensitivity versus sequencing at these cutoffs are shown in the box.

riod while the TAC tests only a single moment (33). Furthermore, these pathogens cause bacteremia only during certain stages of illness due to different pathogenesises. For example, rickettsemia occurs 5 to 10 days after onset of illness, and leptospiremia usually appears before clinical presentation. Thus, if one is particularly interested in such pathogens, testing of sequential specimens or with additional modalities would be sensible. That said, we were able to source 48 specimens that were IRTP positive for these pathogens, and against this standard the TAC exhibited an 83% sensitivity (range, 57% to 100%) and a 97% specificity.

Finally, there were a large number of rare pathogens on our card (e.g., yellow fever and CCHF), which could not be clinically validated through the current study. We presume all of our specimens were negative for these pathogens. For such entities, clinical validation will always be difficult; however, we did document a high specificity of these tests, such that there were seemingly no false positives. Therefore, operationally we would recommend keeping these assays on the card (if one is interested in screening for them) and confirming any TAC positives for such pathogens with other means.

Discrepancies, namely, the false-negative/positive TAC results, were generally seen with lower burden infections and very late C_{qs} by IRTP or TAC. Whether these IRTP or TAC results are truly positive requires further investigation. We know that many of these would not be confirmable by sequencing. Our future plans are to further validate the assays on newly collected specimens. This will allow us to understand the optimal positioning of TAC in AFI surveillance algorithms and to utilize the assay in the field for outbreak investigations. For the moment, we would advocate using whole blood.

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