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Evaluation of a TaqMan Array Card for Detection of Central Nervous System Infections

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ABSTRACT Infections of the central nervous system (CNS) are often acute, with significant morbidity and mortality. Routine diagnosis of such infections is limited in developing countries and requires modern equipment in advanced laboratories that may be unavailable to a number of patients in sub-Saharan Africa. We developed a TaqMan array card (TAC) that detects multiple pathogens simultaneously from cerebrospinal fluid. The 21-pathogen CNS multiple-pathogen TAC (CNS-TAC) assay includes two parasites (*Balamuthia mandrillaris* and *Acanthamoeba*), six bacterial pathogens (*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Mycoplasma pneumoniae*, *Mycobacterium tuberculosis*, and *Bartonella*), and 13 viruses (parechovirus, dengue virus, Nipah virus, varicella-zoster virus, mumps virus, measles virus, lyssavirus, herpes simplex viruses 1 and 2, Epstein-Barr virus, enterovirus, cytomegalovirus, and chikungunya virus). The card also includes human RNase P as a nucleic acid extraction control and an internal manufacturer control, GAPDH (glyceraldehyde-3-phosphate dehydrogenase). This CNS-TAC assay can test up to eight samples for all 21 agents within 2.5 h following nucleic acid extraction. The assay was validated for linearity, limit of detection, sensitivity, and specificity by using either live viruses (dengue, mumps, and measles viruses) or nucleic acid material (Nipah and chikungunya viruses). Of 120 samples tested by individual real-time PCR, 35 were positive for eight different targets, whereas the CNS-TAC assay detected 37 positive samples across nine different targets. The CNS-TAC assays showed 85.6% sensitivity and 96.7% specificity. Therefore, the CNS-TAC assay may be useful for outbreak investigation and surveillance of suspected neurological disease.

KEYWORDS central nervous system, TaqMan PCR, meningitis, encephalitis

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Infections of the central nervous system (CNS) such as meningitis, encephalitis, or meningoencephalitis may present as an acute illness with significant mortality and extended sequelae (1). Because these infections are often difficult to diagnose in the laboratory, clinical diagnoses often rely upon modern noninvasive techniques, including computerized tomography scans and in some cases magnetic resonance imaging. Access to these advanced clinical diagnostic techniques is limited to large metropolitan hospitals rarely found in developing countries. Even with neuroimaging, the determination of etiologic agents causing meningitis and encephalitis remains complicated, requiring confirmation using other laboratory tests (2, 3). Laboratory tests can identify

a number of etiological agents responsible for bacterial and viral meningitis. Timely identification of these agents continues to be challenging in developing countries, where physicians frequently resort to empirical treatment with little or no benefit.

Advances in molecular diagnostic technology have fostered the development of multiple pathogen detection systems based on PCR. The advent of real-time PCR and multiplexing technologies has facilitated the detection of multiple targets from a single clinical sample (4–7). Although multiplex PCR is susceptible to reduced efficiency and sensitivity due to competition for PCR reagents by the different targets, specificity may also be affected if closely related targets are not selected and validated carefully. These problems can be overcome by using the TaqMan array card (TAC), which utilizes microfluidic technology and single-plex PCRs configured in a 384-well array format. The TAC has previously been used to detect pathogens responsible for respiratory (8, 9), enteric (10), and neonatal (11) infections as well as other acute febrile illnesses (12). Even though a number of multiplex pathogen detection assays, e.g., BioFire, SeeGene, and Fast Track diagnostics, are commercially available, there are numerous advantages to the TAC, including ease of use, low risk of contamination attributable to the sealed format, the ability to modify or replace individual targets without additional optimization, and a small sample volume requirement compared to using multiple single agent real-time PCR assays (13, 14).

In this study, we evaluated a CNS multiple-pathogen TAC (CNS-TAC) assay for 21 etiologies and validated the CNS-TAC assay results alongside individual real-time PCR (IRTP) assays for nine pathogens (four viruses, four bacteria, and one parasite). The purpose of this evaluation was to determine the sensitivity and specificity of the CNS-TAC assay compared to IRTP assays in detecting multiple pathogens from clinical samples. We likewise propose the use of this tool in outbreak settings, providing reduced turnaround times resulting in timely and agent appropriate interventions. In addition, the method will be used to improve our understanding of the epidemiology of the CNS.

RESULTS

Analytical performance (plasmid controls). All assays exhibited a linear relationship between threshold cycle (C_T) values and the concentrations of nucleic acids. CNS-TAC assays demonstrated linearity with R^2 values ranging between 0.987 and 0.998, except for the measles assay which had a R^2 value of 0.920. The PCR efficiency for detection of all targets in the plasmid ranged from 98.7 to 99.7%, whereas measles virus had an efficiency of 92.0% (Table 1).

The lower limit of detection (LOD) for all targets was 1.6×10^{-7} ng/well, an equivalent of 54 copies per well (Fig. 1). At a concentration of 1.6×10^{-2} ng/well, the assay sensitivity ranged from 80 to 100% for the plasmid targets and was 55% for the measles virus nucleic material. The specificity for all of the positive controls was 100%. The assays showed an accuracy range of 96.7 to 100% at a concentration of 1.6×10^{-2} ng/well. The variation in reproducibility of the C_T values for the 21 targets ranged from 0.9 to 2.2% for the high-concentration control, and it was 8.6% for the measles assay. The variation in the reproducibility of the low-concentration control material ranged from 1.0 to 7.5% for the plasmid targets, and it was 6.7% for the measles assay (Table 1).

Clinical performance. We tested by CNS-TAC assay 120 specimens, 35 of which were initially positive upon IRTP analysis to validate the assays. The specimens were positive for nine targets: 4% (5/120) cytomegalovirus (CMV), 4% (5/120) *Neisseria meningitidis*, 8% (10/120) Epstein-Barr virus (EBV), 3% (3/120) varicella-zoster virus (VZV), 5% (6/120) *Streptococcus pneumoniae*, 2% (2/120) mumps virus, 6% (7/120) *Mycobacterium tuberculosis*, 2% (2/120) *Acanthamoeba*, and 2% (2/120) *Haemophilus influenzae*. The specificity for all nine targets across 120 samples ranged from 87.5 to 100%. *Streptococcus pneumoniae* had the lowest specificity (87.5%), whereas CMV, VZV, mumps virus, and *Haemophilus influenzae* all had specificities of 100%. Compared to IRTP, the overall sensitivity of the CNS-TAC assay ranged from 33.3% for VZV to 100%

TABLE 1 Analytical performance of the CNS-TAC assay[a]

Target	Linearity		Accuracy (sensitivity %)		Reproducibility (CV%)	
	Linearity (R^2)	Efficiency (%)	High concn	Low concn	High concn	Low concn
Bartonella	0.994	99.4	100	80.0	1.6	2.3
Chikungunya 1 virus	0.991	99.1	100	100	1.3	5.7
Chikungunya 2 virus	0.994	99.4	100	100	1.8	5.5
CMV	0.994	99.4	100	100	1.5	4.1
EBV	0.987	98.7	100	100	1.5	6.8
HSV 1	0.987	98.7	96.7	100	2.3	4
HSV 2	0.993	99.3	100	100	0.9	4
Measles virus	0.92	92.0	100	55.0	8.6	6.7
Mumps virus	0.997	99.7	100	100	1.1	2
Mycobacterium tuberculosis	0.993	99.3	96.7	100	1.0	5.1
VZV	0.993	99.3	100	95.0	2.4	2.7
Mycoplasma pneumoniae	0.993	99.3	100	100	1.0	2.9
Nipah virus	0.980	98.0	100	100	1.4	1
Neisseria meningitidis	0.994	99.4	100	100	1.8	4
Pan-dengue virus	0.993	99.3	100	100	2.1	1.7
Haemophilus influenza	0.993	99.3	100	100	1.2	5.2
Parechovirus	0.995	99.5	96.6	85.0	1.9	5.6
Streptococcus pneumoniae	0.992	99.2	100	95.0	1.2	7.4
RNase P	0.993	99.3	100	95.0	1.8	1.6
Acanthamoeba	0.990	99.0	100	100	1.8	4.1
Balamuthia mandrillaris	0.990	99.0	100	100	1.7	5.4

[a]High concentration = 1.6×10^{-2} ng/well or 5.3×10^{6} copies per well; low concentration = 1.6×10^{-7} ng/well or 54 copies per well. CV, coefficient of variance.

for *Neisseria meningitidis* (Table 2). Low sensitivities (<50%) were observed in cases where there were small sample sizes of positive targets as seen with the mumps virus and VZV assays (Table 2). Although *Acanthamoeba* was not detected by IRTP in any of the samples, the CNS-TAC assay detected this target in two samples. Further attempts to detect this by IRTP failed to yield positive results.

DISCUSSION

We describe our evaluation of an in-house-developed CNS-TAC assay that can be used to test cerebrospinal fluid (CSF) for infections associated with meningitis and encephalitis. Infections of the CNS comprise a number of serious and often fatal infections, and yet such infections often pose challenges in diagnosis (15). Many of the pathogens associated with CNS infections are detected by culture, microscopy, or

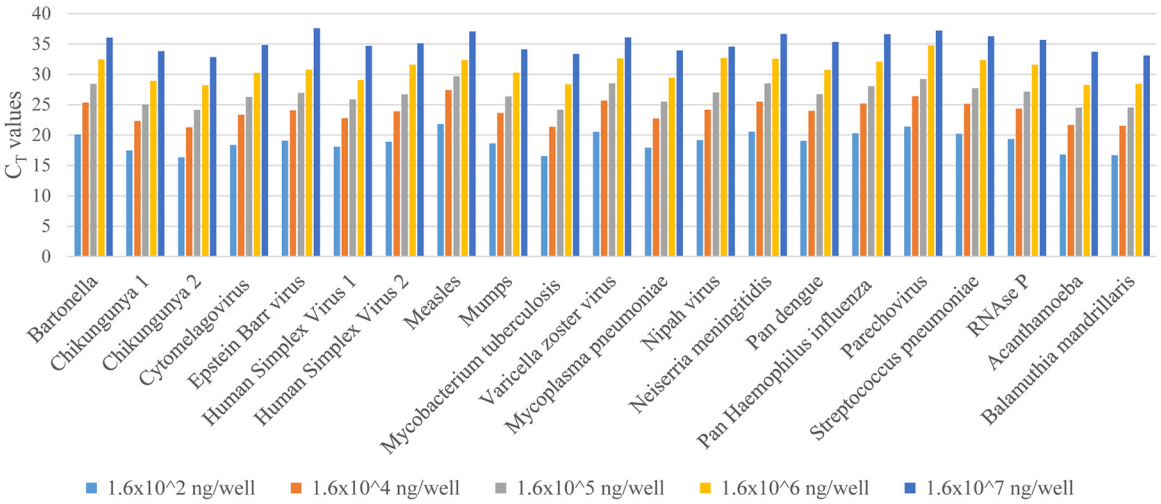

FIG 1 Test of linearity for all targets in the TAC. Dilutions from 1.6×10^{-2} to 1.6×10^{-7} are shown as bars on the *x* axis.

TABLE 2 Sensitivity of CNS-TAC assays compared to IRTP assays using a C_T of 40 as a cutoff[a]

Target and parameter	IRTP assay	TAC assay	% Sensitivity (95% CI)
CMV			
No. positive	7	5	100 (59.0–100)
Mean $C_T \pm$ SD	35.5 ± 2.8	33.0 ± 2.3	
Median C_T (range)	36 (30.7–38.9)	33.2 (29.9–36)	
Neisseria meningitidis			
No. positive	5	5	100 (47.8–100)
Mean $C_T \pm$ SD	24.2 ± 1.9	23.16 ± 1.6	
Median C_T (range)	24.4 (21.5–26.7)	23 (21.1–24.9)	
EBV			
No. positive	8	10	80.0 (44.4–97.5)
Mean $C_T \pm$ SD	34.9 ± 2.0	32.2 ± 1.1	
Median C_T (range)	35.5 (31.5–37.0)	32.1 (29.8–34.0)	
VZV			
No. positive	1	3	33.3 (0.8–90.6)
Mean $C_T \pm$ SD	30.6	28.3 ± 3.5	
Median C_T (range)		29.0 (24.4–31.3)	
Streptococcus pneumoniae			
No. positive	5	6	83.3 (35.9–99.6)
Mean $C_T \pm$ SD	23.0 ± 3.2	22.4 ± 4.0	
Median C_T (range)	22.8 (18.8–27.5)	22.3 (17.6–27.6)	
Mumps virus			
No. positive	1	2	50.0 (1.3–98.7)
Mean $C_T \pm$ SD	31.8	27.3 ± 6.2	
Median C_T (range)		27.3 (22.9–31.8)	
Mycobacterium tuberculosis			
No. positive	6	7	85.7 (42.1–99.6)
Mean $C_T \pm$ SD	33.4 ± 2.5	32.2 ± 4.0	
Median C_T (range)	33.1 (29.9–37.7)	34.0 (24.8–36.8)	
Haemophilus influenzae			
No. positive	2	2	100 (47.8–100)
Mean $C_T \pm$ SD	22.1 ± 6.6	23.2 ± 5.6	
Median C_T (range)	22.1 (17.4–26.8)	23.2 (19.2–27.2)	
Acanthamoeba			
No. positive	0	2	
Mean $C_T \pm$ SD		34.9 ± 3.2	
Median C_T (range)		34.1 (33.9–38.4)	

[a]IRTP, individual real-time PCR; TAC, TaqMan array card; CI, confidence interval.

antigen detection techniques. PCR is generally more reliable at detecting pathogens in the CSF, with substantially higher sensitivity than other diagnostic methods such as culture and enzyme-linked immunosorbent assay if the samples are collected at the appropriate time during the infection (16–18). PCR-based approaches for detecting multiple pathogens in a single array not only increase the number of pathogens that can be detected but also reduce the overall amount of time needed to rule out multiple pathogens. Therefore, we utilized previously published real-time PCR assays incorporated into a CNS-TAC assay. With improvements in sensitivity and ease of use, such multipathogen TAC assays have been used in the detection of both human respiratory and enteric pathogens (8–10). All CNS-TAC assays had LODs similar to what has been described elsewhere for respiratory and enteric pathogens (8, 10). Although this CNS-TAC method was designed for East Africa, many of the pathogens evaluated here cause CNS infections worldwide, and therefore the card is suitable for broader use. In this study, we designed and evaluated a CNS-TAC method that was able to detect 13 viruses, 6 bacteria, and 2 parasites. Clinical evaluation was against 120 patient samples;

35 of these specimens were found to be positive for eight pathogens using IRTP. The patient samples used in clinical validation were collected from subjects presenting at either Mbagathi District Hospital or Siaya District Hospital for patient care.

Analysis of the clinical validation demonstrated an average sensitivity of 79% across the TAC. This excluded the two *Acanthamoeba* positive specimens that failed to amplify by IRTP, suggesting that these were false-positive reactions. However, this average sensitivity was skewed by the VZV assay, which had a suboptimal sensitivity of 33.3%; eliminating these results from the calculation yields an average sensitivity of 85.6%. The low sensitivity for VZV and mumps virus may be attributed to the low numbers of positive samples tested. A parasite with global distribution, *Balamuthia mandrillaris*, was not detected by the assay in this card. This could have been due to the small sample size tested or to the absence of this pathogen in patients from the two geographical regions sampled in Kenya. Additional positive samples for these targets are needed to accurately determine the sensitivity of these assays. Alternatively, we advise the exclusion of these targets with low sensitivities from cards designed for future studies. *Acanthamoeba* spp. and *Balamuthia mandrillaris* are free-living amoebas that can potentially cause infections in humans and have been implicated in CNS infections worldwide (19). However, the prevalence of these pathogens is not well documented in sub-Saharan Africa. On the other hand, *Plasmodium falciparum* infection can present in a severe form of cerebral malaria, with a mortality rate of 10 to 25%, and is most common in sub-Saharan Africa (20, 21). Similarly, several studies have implicated cryptococcal meningitis as the major cause of meningitis among HIV-infected individuals in sub-Saharan Africa (22–24). Therefore, we recommend replacement of the *Acanthamoeba* and *Balamuthia mandrillaris* targets with *Plasmodium falciparum* and cryptococcal meningitis, which are more prevalent in sub-Saharan Africa, in future versions of these cards. The average specificity for CNS-TAC assay for the eight targets was 96.7%. A subset of samples failed to amplify RNase P in CNS-TAC (13%) and IRTP (6%) analyses. Usually, this would suggest inappropriate specimen collection, sample degradation, or the complete absence of human DNA in some CSF samples. However, the concentration of RNase P should reflect the concentration of white blood cells in the specimen, since CSF is usually free of human DNA. This suggests that a different marker should be used in the future as a control for specimen integrity for CSF.

The CNS-TAC assay detected seven targets that were not detected by the IRTP assays. These additional detections failed to yield positive results using IRTP despite numerous attempts, and as such, are possibly false positives that would negatively impact the specificity of the TAC assays since the IRTP method was considered the gold standard for these comparisons. These discrepancies could possibly be explained by additional freeze-thaw cycles negatively impacting nucleic acid integrity for the IRTP assay. However, the observed mean C_T of 30.8 $\pm$ 4.2 indicates significant amplification and would argue against this possibility.

In our protocol, two of eight lanes were occupied on the first TAC: one of the eight lanes of the card was designated a no-template negative control, and another was designated the combined positive control. Subsequent cards tested would hold a negative control and seven specimens on a card. Up to three cards were tested per day in one ViiA-7 machine, which allowed for 20 specimens to be tested for 21 pathogens each per day. This greatly reduces the turnaround time for specimen testing compared to IRTP assays. Despite the discrepancies observed between TAC and IRTP, we think there is added value in the use of CNS-TAC as a screening assay in outbreak settings. Indeed, samples with positive IRTP or TAC results require further investigations, including gene sequencing, among other confirmatory tests. Our future plans are therefore to confirm all the TAC positives by sequencing, as well as to further validate the CNS-TAC assay using a larger sample size from patients presenting with CNS infections from other geographical sites within Kenya. This will help us better understand the utility of CNS-TAC in outbreak investigations.

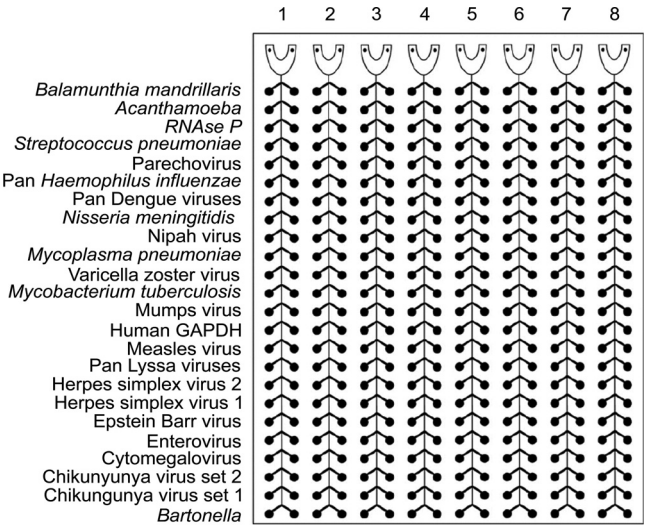

FIG 2 CNS-TAC layout with 22 encephalitis targets, as well two human DNA/RNA controls, GAPDH and RNase P. PCRs for all the targets, including intrinsic controls, were customized for testing in duplicates.

MATERIALS AND METHODS

CNS-TAC design. The CNS card includes assays for the detection of six bacterial pathogens, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Mycoplasma pneumoniae*, *Mycobacterium tuberculosis*, and *Bartonella* (genus specific); 13 viruses, parechovirus, pan-dengue virus (detects all four serotypes), Nipah virus, VZV, mumps virus, measles virus, pan-Lyssa virus, HSV-1 and -2, EBV, enterovirus, CMV, and chikungunya virus (detection is based upon two different gene targets for all three genotypes); and two parasites, *Balamuthia mandrillaris* and *Acanthamoeba* (Fig. 2). All primers and probes were adapted from previously published assays, except for the *M. tuberculosis* assay, which is described for the first time here. Primers and probes for the targets were titrated individually by real-time PCR using genomic DNA, plasmid DNA, or RNA on a Bio-Rad CFX 96 platform and AgPath-ID One-Step RT-PCR master mix (Life Technologies, United Kingdom). Once titrated, the primers and probe for each assay were preloaded and dried by a ViiA7 Applied Biosystems (Foster City, CA) instrument in duplicate wells on the TAC, which included three intrinsic controls: (i) an extraction control, RNase P; (ii) measles virus RNA acting as both a target control and a RNA control; and (iii) an internal manufacturer control, glyceraldehyde-3-phosphate (GAPDH) (Table 3). All of the assays in this card apart from lyssavirus and pan-dengue virus assays were developed at the Centers for Disease Control and Prevention (CDC) and are used routinely for clinical diagnosis in the United States. The other two assays were developed at the University of Pretoria, South Africa, and the Bernhard Nocht Institute, Germany, respectively.

Design of combined positive control. Customized combined positive controls were designed and synthesized in two different plasmids. The design and orientation of the positive-control plasmid maps are similar to that described by Kodani and Winchell (25). The forward primer sequence was placed downstream of the plasmid pUC57 T7 sequence, followed by the probe sequence and finally the reverse primer sequence. The plasmid comprised these concatenated sequences for all targets. The two plasmids were designated A and B. Plasmid A contained sequences for *Bartonella*, CMV, EBV, HSV-1 and -2, mumps virus, *Mycobacterium tuberculosis*, VZV, *Mycoplasma pneumoniae*, Nipah virus, *Neisseria meningitidis*, pan-dengue virus, *Haemophilus influenzae*, parechovirus, *Streptococcus pneumoniae*, and RNase P. Plasmid B contained sequences for chikungunya virus targets 1 and 2, *Acanthamoeba*, and *Balamuthia mandrillaris*. Measles virus RNA was spiked into plasmid B preparation as an exogenous control for the virus, as well as an RNA control. Positive-control extracts were titrated following a 10-fold dilution to determine the LOD.

Analytical validation. The LOD, linearity, repeatability, and reproducibility were determined using 10-fold dilutions of the positive-control material using infection-free CSF as the diluent. The positive-control materials were derived from nucleic acid materials from the respective targets. Nucleic acid material from RNA viruses was transcribed into cDNA and prepared for gene cloning. Repeatability was tested using eight repeats on a single card, whereas reproducibility was tested with 10 serial dilutions of each plasmid and assayed over 5 days. The lower LOD was defined as the lowest concentration at which the target could be detected in all of the diluted samples. Analytical validation of these assays was performed at the National Center for Emerging and Zoonotic Infectious Diseases at the CDC.

Testing of CSF. CSF samples were obtained from patients in either Mbagathi District Hospital in Nairobi or Siaya District Hospital in rural Western Kenya. Children older than 6 weeks and adults of all ages were eligible for lumbar puncture if they presented with two or more signs and symptoms of CNS infection, such as fever (≥38°C) and/or history of reported fever in the last 3 days, neck stiffness and/or

TABLE 3 Oligonucleotide sequences for CNS-TAC assays[a]

Target	Gene	ID	Sequence (5′–3′)	Reference
CMV	UL55	For	AGG TCT TCA AGG AAC TCA GCA AGA	26
		Rev	CGG CAA TCG GTT TGT TGT AAA	
		Pr	FAM-ACC CCG TCA GCC ATT CTC TCG GC-BHQ 1	
EBV	BNRF1	For	GGA ACC TGG TCA TCC TTT GC	27
		Rev	ACG TGC ATG GAC CGG TTA AT	
		Pr	FAM-CGC AGG CAC TCG TAC TGC TCG CT-BHQ 1	
Mycobacterium tuberculosis	IS*6110*	For	CCT ACT ACG ACC ACA TCA	—[b]
		Rev	CCG TAA ACA CCG TAG TTG	
		Pr	FAM-ATG TGC TCC TTG AGT TCG CCA T-BHQ 1	
Neisseria meningitidis	*sodC*	For	CCA CCC GTG TGG ATC ATA ATA GA	28
		Rev	GCA CAC TTA GGT GAT TTA CCT GCA T	
		Pr	FAM-CA TGA TGG CAC AGC AAC AAA TCC TGT TT-BHQ 1	
Streptococcus pneumoniae	*lytA*	For	ACG CAA TCT AGC AGA TGA AGC A	29
		Rev	TCG TGC GTT TTA ATT CCA GCT	
		Pr	FAM-TG CCG AAA ACG CTT GAT ACA GGG AG-BHQ 1	
VZV	ORF29	For	CAC GTA TTT TCA GTC CTC TTC AAG TG	30
		Rev	TTAGACGTGGAGTTGACATCGTTT	
		Pr	FAM-TACCGCCCGTGGAGCGCG-BHQ 1	
Chikungunya virus 1	NSP1	For	AAAGGGCAAACTCAGCTTCAC	31
		Rev	GCCTGGGCTCATCGTTATTC	
		Pr	FAM-CTGTGATACAGTGGTTTCGTGTG-BHQ 1	
Chikungunya virus 2	NSP4	For	TCACTCCCTGTTGGACTTGATAGA	31
		Rev	TTGACGAACAGAGTTAGGAACATACC	
		Pr	FAM-AGGTACGCGCTTCAAGTTCGGCG-BHQ1	
Enterovirus	5′ UTR	For	GGC CCC TGA ATG CGG CTA ATC C	32
		Rev	GCG ATT GTC ACC ATWA GCA GYC A	
		Pr	FAM-CC GAC TAC TTT GGG WGT CCG TGT-BHQ1	
Mycoplasma pneumoniae	CARDS toxin	For	TTT GGT AGC TGG TTA CGG GAA T	33
		Rev	GGT CGG CAC GAA TTT CAT ATA AG	
		Pr	FAM-TG TAC CAG AGC ACC CCA GAA GGG CT-BHQ1	
HSV 1	US4	For	TAT TGG TGC GAT GGC GAC AC	34
		Rev	CTT TCC GCA TGT GGG CTC TC	
		Pr	FAM-CCC CGC CCC ATA CCC TAC CCG C-BHQ1	
HSV 2	US6	For	AGC ATC CCG ATC ACT GTG TAC TA	34
		Rev	GCG ATG GTC AGG TTG TAC GT	
		Pr	FAM-CAG TGC TGG AAC GTG CCT GCC GC-BHQ 1	
Measles virus	N	For	TGG CAT CTG AAC TCG GTA TCA C	35
		Rev	TGT CCT CAG TAG TAT GCA TTG CAA	
		Pr	FAM-CCGAG GAT GCA AGG CTT GTT TCA GA-BHQ1	
Mumps virus	NP	For	GTA TGA CAG CGT ACG ACC AAC CT	36
		Rev	GCG ACC TTG CTG CTG GTA TT	
		Pr	FAM-CC GGG TCT GCT GAT CGG CGA T-BHQ 1	
Parechovirus	5′ UTR	For	GTAACASWWGCCTCTGGGSCCAAAAG	37
		Rev	GGCCCCWGRTCAGATCCAYAGT	
		Pr	FAM-CCTRYGGGTACCTYCWGGGCATCCTT-BHQ 1	
Bartonella	*ssrA*	For	GCTATGGTAATAAATGGACAATGAAATAA	38
		Rev	GCTTCTGTTGCCAGGTG	
		Pr	FAM-ACCCCGCTTAAACCTGCGACG-BHQ1	
Haemophilus influenzae	*bexA*	For	TGCGGTAGTGTTAGAAAATGGTATTATG	39
		Rev	GGACAAACATCACAAGCGGTTA	
		Pr	FAM-ACAAAGCGTATCAATACTACAACGAGACGCAAAAA-BHQ1	
Acanthamoeba	18S rRNA	For	CCCAGATCGTTTACCGTGAA	40
		Rev	TAAATATTAATGCCCCCAACTATCC	
		Pr	FAM-CTGCCACCGAATACATTAGCATGG-BHQ1	
Lyssavirus	N	For	GTRCTCCARTTAGCRCACAT	41
		Rev	CACMGSNAAYTAYAARACNAA	
		Pr	FAM-CATCACACCTTGATGACAACTCACAA-BHQ1	
Balamunthia mandrillaris	18S rRNA	For	TAA CCT GCT AAA TAG TCA TGC CAA T	40
		Rev	CAA ACT TCC CTC GGC TAA TCA	
		Pr	FAM-AG TAC TTC TAC CAA TCC AAC CGC CA- BHQ1	
Pan-dengue virus	3′ NCR	For	GGA TAG ACC AGA GAT CCT GCT GT	42
		Rev 1	CAT TCC ATT TTC TGG CGT TC	
		Rev 2	CAA TCC ATC TTG CGG CGC TC	
		Pr	FAM CA GCA TCA TTC CAG GCA CAG-BHQ1	

(Continued on next page)

TABLE 3 (Continued)

Target	Gene	ID	Sequence (5′–3′)	Reference
Nipah virus	N	For	CTG GTC TCT GCA GTT ATC ACC ATC GA	43
		Rev	ACG TAC TTA GCC CAT CTT CTA GTT TCA	
		Pr	FAM-CAG CTC CCG ACA CTG CCG AGG AT-BHQ	
RNase P	RPP30	For	AGA TTT GGA CCT GCG AGC G	44
		Rev	GAG CGG CTG TCT CCA CAA GT	
		Pr	FAM-TTC TGA CCT GAA GGC TCT GCG CG-BHQ	

[a]ID, oligonucleotide identity; For, forward; Rev, reverse; Pr, probe; CMV, cytomegalovirus; EBV, Epstein-Barr virus; VZV, varicella-zoster virus; HSV, herpes simplex virus; UTR, untranslated region; NCR, noncoding region; ORF, open reading frame.
[b]—, J. Posey, unpublished data.

bulging fontanel, headache, reduced level of consciousness, or new-onset seizures. A total of 120 samples, including 35 that were positive for any of the CDC in-house IRTP assays, were also tested using the CNS-TAC method. CDC in-house assays were designed to include targets for CMV, EBV, mumps virus, *Mycobacterium tuberculosis*, VZV, *Neisseria meningitidis*, *Haemophilus influenzae*, *Acanthamoeba*, and *Streptococcus pneumoniae*. In addition, 85 randomly selected samples, determined to be negative in IRTP assays, were tested by the CNS-TAC assay to determine specificity.

Nucleic acids were extracted from CSF specimens using the KingFisher ML extraction platform (Thermo Scientific, Waltham, MA) and MagMax nucleic isolation kit (Life Technologies, Carlsbad, CA). Portions (100 μl) of CSF specimens were mixed with 260 μl of lysis binding solution and added to the columns. The column was washed once with 600 μl of wash solution 1 and then twice with 450 μl of wash solution 2 according to the manufacturer's recommendations. After the wash steps, the nucleic acids were eluted with 60 μl of elution buffer. An additional 166 μl of previously PCR-positive samples was reextracted for IRTP testing using the same platform and kit. We used 433 μl of lysis binding solution to adjust for the increased sample volume. The samples were then eluted in 100 μl of elution buffer. An increased extraction volume was required for the eight IRTP assays, and this increased volume did not alter the sensitivity of the assays. The CNS-TAC assays were compared to the cognate IRTP assays on 96-well plates under the same thermocycling conditions using the same PCR master mix and 5 μl of nucleic acids as the template. Samples with a C_T of $\geq$40 were interpreted as negative, and those with a C_T of 35 to 40 were classified as indeterminate and retested. If these C_T values remained within the range of 35 to 40, they were ultimately classified as weak positives.

The CNS-TAC assays were run on a ViiA-7 real-time PCR system using an AgPath-ID One-Step real-time PCR kit (Applied Biosystems, Foster City, CA). The PCR master mix for each card included 1× RT-PCR buffer, RT-PCR enzyme in a final 100-μl reaction volume. A 46-μl portion of nucleic acid extract was added to the master mix. Each run consisted of a negative control and a positive control for the first card of the day to be tested. A minimum of three cards were tested per day, with thermal cycling conditions as follows: 45°C for 10 min, 94°C for 10 min, and then 45 cycles of 94°C for 30 s and 60°C for 1 min. These clinical analyses were performed at the Centre for Global Health Research of the Kenya Medical Research Institute (KEMRI) in western Kenya.

Data analysis. Receiver operating characteristic analysis was used to derive C_T cutoffs. The C_T values for CNS-TAC and IRTP assays were compared using a *t* test, whereas dichotomous measures of the presence or absence of extrinsic controls were compared using a Fisher exact test. Linearity was tested by fitting linear regression models of C_T values against the concentrations of nucleic acids and interpreting the R^2. The sensitivity of the CNS-TAC assay was also calculated against the gold standard: IRTP for nine targets. All analyses were performed using STATA v13 (StataCorp).

Ethical approval. This study was covered under an investigational protocol reviewed by human subject review experts from the institutional review boards at the CDC (protocol 6092) and KEMRI (SSC protocol 1948). Informed written consent for survey participation, and CSF collection was obtained from all adult participants 18 years of age and older and from mature minors 13 to 17 years old. Verbal assent from minors (children 13 to 17 years old) and written consent from parents or guardians of those minors were obtained, and written consent from parents was obtained for children $\leq$13 years old. If a patient of any age was unable to provide consent or assent because of altered mental status, consent was obtained from the patient's responsible family member or guardian.

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