

Development and Implementation of Multiplex TaqMan Array Cards for Specimen Testing at Child Health and Mortality Prevention Surveillance Site Laboratories

Maureen H. Diaz,¹ Jessica L. Waller,¹ M. Jordan Theodore,² Nishi Patel,² Bernard J. Wolff,¹ Alvaro J. Benitez,¹ Timothy Morris,³ Pratima L. Raghunathan,⁴ Robert F. Breiman,⁵ Cynthia G. Whitney,¹ Dianna M. Blau,⁴ and Jonas M. Winchell¹

¹Respiratory Diseases Branch, Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA; ²IHRC, Inc., Atlanta, Georgia, USA; ³Public Health Informatics Institute, The Task Force for Global Health, Atlanta, Georgia, USA; ⁴Center for Global Health, Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and ⁵Emory Global Health Institute, Emory University, Atlanta, Georgia, USA

Child Health and Mortality Prevention Surveillance (CHAMPS) laboratories are employing a variety of laboratory methods to identify infectious agents contributing to deaths of children <5 years old and stillbirths in sub-Saharan Africa and South Asia. In support of this long-term objective, our team developed TaqMan Array Cards (TACs) for testing postmortem specimens (blood, cerebrospinal fluid, lung tissue, respiratory tract swabs, and rectal swabs) for >100 real-time polymerase chain reaction (PCR) targets in total (30–45 per card depending on configuration). Multipathogen panels were configured by syndrome and customized to include pathogens of significance in young children within the regions where CHAMPS is conducted, including bacteria (57 targets covering 30 genera), viruses (48 targets covering 40 viruses), parasites (8 targets covering 8 organisms), and fungi (3 targets covering 3 organisms). The development and application of multiplex real-time PCR reactions to the TAC microfluidic platform increased the number of targets in each panel while maintaining assay efficiency and replicates for heightened sensitivity. These advances represent a substantial improvement in the utility of this technology for infectious disease diagnostics and surveillance. We optimized all aspects of the CHAMPS molecular laboratory testing workflow including nucleic acid extraction, quality assurance, and data management to ensure comprehensive molecular testing of specimens and high-quality data. Here we describe the development and implementation of multiplex TACs and associated laboratory protocols for specimen processing, testing, and data management at CHAMPS site laboratories.

Keywords. TaqMan Array Card; surveillance; multipathogen diagnostics; multiplex real-time PCR.

The array of pathogens responsible for the disproportionate number of childhood deaths that occur in sub-Saharan Africa and South Asia is not fully defined. The Child Health and Mortality Prevention Surveillance (CHAMPS) network is implementing innovative laboratory methods at partner institutes in these regions to fill this information gap. Dramatic reductions in childhood morbidity and mortality associated with individual organisms, including polio, tetanus, measles, human immunodeficiency virus, and malaria [1], have been achieved through successful targeted public health intervention strategies. Identifying and assessing the burden of other specific etiologic agents is a key step to continue progress in reducing childhood mortality. Such information can be used to strengthen existing public health measures such as vaccination, antimicrobial treatment guidelines, and appropriate infection control practices, and to prioritize the development of new tools and strategies.

Molecular testing methods are ideal for infectious disease surveillance in that they can be performed relatively quickly on-site by technically trained staff. Among these methods, nucleic acid amplification techniques, especially real-time polymerase chain reaction (PCR), are most commonly used. However, these methods can be expensive and availability may be limited, particularly in low-resource settings. In addition, molecular testing is extremely sensitive and even small amounts of contamination may lead to inaccurate results. Thus, adequate separation of work areas and strict adherence to good laboratory practices are absolutely required for high-quality data. In addition, these methods require adequate laboratory infrastructure, sophisticated equipment, and trained laboratorians. Nonetheless, molecular methods have become widely used in clinical laboratories, including in low-resource settings.

In recent years a variety of multipathogen detection methods have become commercially available, some of which have been evaluated and used in multisite surveillance studies [2, 3]. A number of platforms were considered for CHAMPS; each has both positive and negative attributes for use with CHAMPS specimen types and implementation in resource-limited settings. Biofire Film Array (bioMérieux), approved by the US Food and Drug Administration for clinical use, is increasingly

Correspondence: J. M. Winchell, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, MS G-03, Atlanta, GA 30329 (jwinchell@cdc.gov)

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common in clinical laboratories in the United States, particularly for respiratory and enteric pathogen detection. The entire testing procedure occurs within a single instrument without a separate step for nucleic acid extraction. Only a single sample can be tested at one time, making throughput a major drawback for implementation of this technology in surveillance studies. In addition, the panels are limited to a defined set of pathogen targets and cannot be customized to allow screening for pathogens specific to a geographic region or patient population. Fast Track Diagnostics (FTD) syndromic panels are commercially available multiplex real-time PCR panels amenable to surveillance applications. FTD respiratory panel 21 was recently used in a large multisite study for respiratory pathogens [4], and diagnostic kits for a range of syndromes are available from this manufacturer. However, assay setup and analysis is complex and prone to operator error, in particular due to the open nature of the system in which the potential for cross-contamination is high. Lack of replicate reactions introduces further potential for unidentified erroneous results. Finally, technical support and performance verification for these products is limited, and panels are not customizable to include pathogens specific to the population under surveillance.

TaqMan Array Card (TAC) is a microfluidic array of reaction vessels containing lyophilized oligonucleotides for amplification of target nucleic acid using 5'-hydrolysis (TaqMan) chemistry. The array can be customized to create a multipathogen panel with a variable number of unique assays or replicate reactions. The closed system is simple to use, requiring minimal pipetting steps, which limits potential for cross-contamination or other operator error that may lead to inaccurate results. The TAC platform was selected for multipathogen detection in CHAMPS due to its flexibility to customize broad syndromic panels, including assays for pathogens relevant to the populations of interest (geographic and age considerations) and the availability of subject matter expertise and technical support of this technology from the Centers for Disease Control and Prevention (CDC, Atlanta, Georgia). TAC has been used for numerous large-scale surveillance studies, most of which are limited to a single syndrome such as diarrheal disease [5, 6], pneumonia [7], sepsis [3, 8], meningitis/encephalitis [9], or acute febrile illness [10–12]. Many of these studies were limited in geographic scope to a single country or region and had limited or no postmortem sampling. However, CDC previously developed and optimized TAC for testing respiratory swabs, cerebrospinal fluid (CSF), and whole blood specimens in Aetiology of Neonatal Infections in South Asia (ANISA), a multicountry study to investigate causes of neonatal sepsis in Bangladesh, India, and Pakistan [3, 13], and has supported use of custom TACs for various large-scale, population-based surveillance studies.

CHAMPS aims to identify the specific pathogens causing and contributing to childhood deaths in the developing world through evaluation of postmortem tissue and nontissue

specimens, using pathology methods (see Martines et al in this supplement), blood and CSF culture, and molecular testing for a broad array of microorganisms in multiple specimen types. Specimens are also archived and data made available for future study. Compared to previous surveillance programs, TAC implementation for CHAMPS involves a substantial increase in the number of pathogen-specific assays and card configurations, each requiring development and performance evaluation to cover the broad array of organisms of interest in CHAMPS sites. Here we describe the development and implementation process for custom multiplex TACs for CHAMPS with an emphasis on assay research and development and the novel aspects of implementing this approach for cause of death determination.

METHODS AND RESULTS

Overview of Assay Design and TAC Development

Assay Design and Validation

The stages of TAC development, validation, and implementation at site laboratories are detailed in Figure 1. CHAMPS TAC panels were designed to cover the most important pathogens in newborns, infants, and young children in CHAMPS sites based upon available information and discussions with child health and infectious disease experts. Overall, 116 pathogen targets were designed or modified from existing formats and validated for use on TAC for detection of bacteria (57 targets covering 30 genera), viruses (48 targets covering 40 viruses), parasites (8 targets with 1 target per organism), and fungi (3 targets with 1 target per organism) (Table 1). When needed, >1 assay was developed to differentiate species or identify potentially distinguishing or clinically meaningful features such as toxins (eg, pertussis toxin, diphtheria toxin).

Panels were initially organized into 5 separate TAC configurations (respiratory, enteric, blood/CSF tier 1, blood/CSF tier 2, and neonatal) but after an initial period of testing (approximately 1 year) were reorganized into 4 unique configurations by consolidating the neonatal-specific assays into blood/CSF tier 1 and tier 2 designs (Supplementary Figures 1–4). This change simplified the workflow at each site laboratory performing CHAMPS TAC testing such that specimens from each patient are tested on the same set of 4 TACs (respiratory, enteric, blood/CSF tier 1, and blood/CSF tier 2) regardless of patient age. Each card configuration was designed to allow testing of 6 specimens along with negative and positive controls in each run to ensure data quality and integrity.

Several factors were considered in determining the configuration of assays within each card design. We empirically evaluated assay performance in singleplex and multiplex reaction formats to ensure sensitivity and specificity were not negatively impacted by multiplexing. We also considered the optimal number of replicates; in brief, assays were spotted in 2 replicates on respiratory and enteric configurations and 4 replicates in

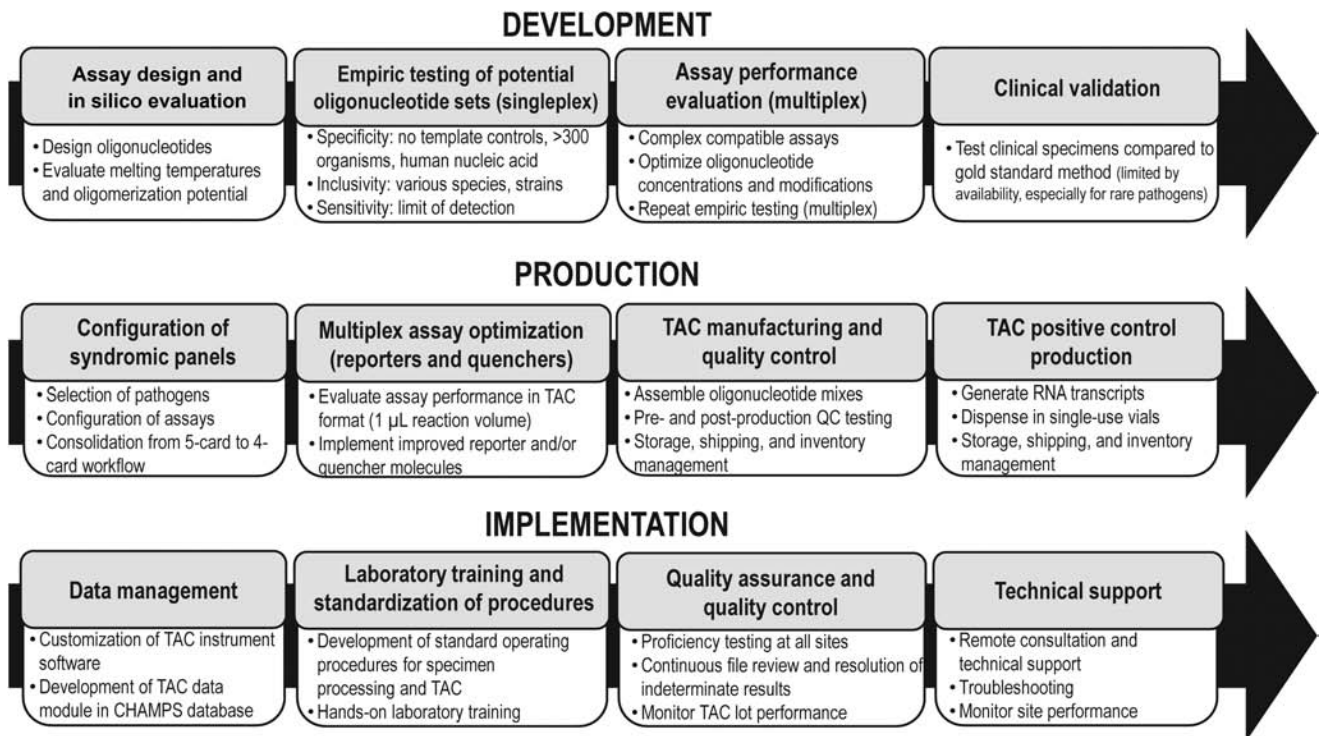


Figure 1. TaqMan Array Card (TAC) development, production, and implementation workflow. Summary of stages of development, production, and implementation of custom TAC configurations for testing minimally invasive tissue sampling (MITS) specimens at Child Health and Mortality Prevention Surveillance (CHAMPS) network site laboratories. Development of novel pathogen-specific assays and configuration and optimization of custom TACs was performed at the Centers for Disease Control and Prevention (CDC). TAC was implemented at all CHAMPS site laboratories for in-country testing of all MITS specimens with ongoing technical support and quality assurance (QA)/quality control (QC) provided by CDC. Abbreviations: CHAMPS, Child Health and Mortality Prevention Surveillance; TAC, TaqMan Array Card.

most cases for blood and CSF configurations due to improved sensitivity with higher replicates for testing of whole blood specimens observed during earlier work [13]. Another consideration for organization of assays on blood tier 1 and tier 2 configurations was ensuring assays related by multitarget interpretation algorithms were included on the same card to simplify data management. In addition, a few targets previously restricted to other card configurations (*Acinetobacter baumannii*, respiratory; *Enterococcus faecalis* and *Enterococcus faecium*, enteric) were added to the blood/CSF TAC configurations to examine the impact of postmortem dissemination of organisms from respiratory or enteric tract niches.

Real-time PCR assays using 5' hydrolysis probe (TaqMan) chemistry were designed for each microorganism of interest (Table 1). Subject matter experts from various divisions within CDC or other institutes were consulted to determine the best genetic targets to optimize sensitivity and specificity of detection of the desired microorganism(s). Oligonucleotides were designed using Primer Express version 3.0.1 (Thermo Fisher Scientific, Waltham, Massachusetts), PrimerQuest Tool (Integrated DNA Technologies [IDT], Coralville, Iowa; www.idtdna.com/primerquest), or manually via sequence alignment of gene or target region using Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo). In some cases, whole genome multiple sequence

alignments were performed using the progressiveMauve algorithm [14] to confirm presence of target regions in targeted organisms and absence in closely related species. Each set of oligonucleotides was evaluated to ensure optimal melting temperature for the thermocycling conditions as well as to identify potential self-dimerization or intermolecular dimerization between selected oligonucleotide combinations. All oligonucleotides were assessed in silico by Basic Local Alignment Search Tool analysis against the GenBank *nt* database (www.ncbi.nlm.nih.gov) to assess specificity and inclusivity of selected target regions for detection of the appropriate group of organisms (genus, species, subtype, etc). When necessary, degenerate bases were included to ensure primer/probe binding in slightly variable target sequence regions. Probes incorporating a Minor Groove Binder moiety were used in some assays to increase the melting temperature of the probe when only a short stretch of nucleotides was identified to have sufficient sequence identity across variants available within public databases [15]. Oligonucleotides were manufactured by IDT, Thermo Fisher Scientific, or the Biotechnology Core Facility at CDC.

In addition to these in silico analyses, all assays underwent empirical performance evaluation, including analytical sensitivity, specificity, and inclusivity. Specificity was assessed by extensive testing of no-template controls (water), human nucleic acid, and

Table 1. Pathogen Targets (N = 116) Validated for Use on Child Health and Mortality Prevention Surveillance TaqMan Array Cards

| Bacteria (n = 57) | | |
|---|--|--|
| <i>Acinetobacter baumannii</i> (R, B2) | <i>Enterococcus faecalis</i> (B2, E) | <i>Neisseria gonorrhoeae</i> (B2) |
| <i>Aeromonas</i> spp (E) | <i>Enterococcus faecium</i> (B2, E) | <i>Neisseria meningitidis</i> (B1) |
| <i>Bartonella</i> spp (B2) | Enteroaggregative <i>Escherichia coli</i> aaiC (E) | <i>Orientia tsutsugamushi</i> (B1) |
| <i>Bordetella parapertussis</i> /B. <i>bronchiseptica</i> (pIS1001) (R) | Enteroaggregative <i>E. coli</i> aatA (E) | <i>Rickettsia</i> spp (B1) |
| <i>Bordetella pertussis</i> /B. <i>holmesii</i> (IS481) (R) | Enteroinvasive <i>E. coli</i> / <i>Shigella</i> spp ipaH (E) | <i>Salmonella enterica</i> Paratyphi A (B1) |
| <i>B. pertussis</i> /B. <i>parapertussis</i> pertussis toxin <i>ptxS1</i> (R) | Enteropathogenic <i>E. coli</i> bfpA (E) | <i>S. enterica</i> Typhi (B1) |
| <i>Brucella</i> spp (B2) | Enteropathogenic <i>E. coli</i> eae (E) | <i>S. enterica</i> /bongori (B1, E) |
| <i>Burkholderia pseudomallei</i> (R, B2) | Enterotoxigenic <i>E. coli</i> heat-labile toxin <i>eltA</i> (E) | Shiga toxin <i>stx1</i> (E) |
| <i>Campylobacter coli</i> (E) | Enterotoxigenic <i>E. coli</i> heat-stable toxin STH <i>estA</i> (E) | Shiga toxin <i>stx2</i> (E) |
| <i>Campylobacter jejuni</i> (E) | Enterotoxigenic <i>E. coli</i> heat-stable toxin STp <i>estA</i> (E) | <i>Staphylococcus aureus</i> (R, B1) |
| <i>Chlamydia pneumoniae</i> (R) | <i>E. coli</i> / <i>Shigella</i> spp <i>uidA</i> (B1) | <i>Streptococcus agalactiae</i> (GBS) (R, B1) |
| <i>Chlamydia trachomatis</i> (R) | <i>Haemophilus influenzae</i> (R, B1) | <i>Streptococcus pneumoniae</i> (R, B1) |
| <i>Clostridioides difficile</i> , nontoxigenic (E) | <i>H. influenzae</i> type b ^a (R, B1) | <i>Streptococcus pyogenes</i> (GAS) (R, B1) |
| <i>C. difficile</i> toxin A <i>tcdA</i> (E) | <i>Klebsiella pneumoniae</i> ^b (R, B1) | <i>Streptococcus suis</i> (B2) |
| <i>C. difficile</i> toxin B <i>tcdB</i> (E) | <i>Leptospira</i> (B2) | <i>Treponema pallidum</i> (B1) |
| <i>Corynebacterium diphtheria</i> (R) | <i>Listeria monocytogenes</i> (B1) | <i>Ureaplasma urealyticum</i> /parvum (B2) |
| <i>Corynebacterium pseudotuberculosis</i> /C. <i>ulcerans</i> (R) | <i>Moraxella catarrhalis</i> (R, B2) | <i>Yersinia</i> spp (B2) |
| <i>C. diphtheriae</i> /C. <i>pseudotuberculosis</i> / C. <i>ulcerans</i> diphtheria toxin <i>tox</i> (R) | <i>Mycobacterium tuberculosis</i> (R, B2, E) | <i>Vibrio cholerae</i> cholera toxin <i>ctxA</i> (E) |
| <i>Coxiella burnetii</i> (B2) | <i>Mycoplasma pneumoniae</i> (R) | <i>V. cholerae</i> (E) |
| Viruses (n = 48) | | |
| Adenovirus (R, B1, E) | Human parainfluenza virus 1 (R) | Norovirus genogroup GII (E) |
| Adenovirus serotype 40/41 (E) | Human parainfluenza virus 2 (R) | Parvovirus B19 (B2) |
| Astrovirus (E) | Human parainfluenza virus 3 (R) | Respiratory syncytial virus (R) |
| Chikungunya virus (B1) | Human parainfluenza virus 4 (R) | Rhinovirus (R) |
| Crimean-Congo hemorrhagic fever virus (B2) | Human parechovirus (B1) | Rift Valley fever virus (B2) |
| Cytomegalovirus (B2) | Influenza A virus (R) | Rotavirus A (E) |
| Dengue virus (B1) | Influenza B virus (R) | Rotavirus B (E) |
| Enterovirus (R, B1, E) | Japanese encephalitis virus (B2) | Rotavirus C (E) |
| Hepatitis E virus (B2) | Lassa fever virus ^c (B2) | Rotavirus, nontypeable (E) |
| Human coronavirus 229E (R) | Lassa fever virus lineage 1/2/4 (B2) | Rubella virus (R, B1) |
| Human coronavirus NL63 (R) | Measles virus (R, B2) | Sapovirus I/II/IV (E) |
| Human coronavirus OC43 (R) | MERS coronavirus N (R) | Sapovirus V (E) |
| Human coronavirus HKU1 (R) | MERS coronavirus upE (R) | Varicella zoster virus (R, B2) |
| Human herpesvirus 1 (B2) | Mumps virus (B2) | West Nile virus (B2) |
| Human herpesvirus 2 (B2) | Nipah virus (B2) | Yellow fever virus (B2) |
| Human metapneumovirus (R) | Norovirus genogroup GI (E) | Zika virus (B1) |
| Parasites (n = 8) | | |
| <i>Ascaris lumbricoides</i> (E) | <i>Giardia intestinalis</i> (<i>lamblia</i>) (E) | <i>Toxoplasma gondii</i> (B1) |
| <i>Cryptosporidium parvum</i> (E) | <i>Plasmodium falciparum</i> (B1) | <i>Trichuris trichiura</i> (E) |
| <i>Entamoeba histolytica</i> (E) | <i>Plasmodium vivax</i> (B1) | |
| Fungi (n = 3) | | |
| <i>Candida albicans</i> (B2) | <i>Cryptococcus neoformans/gattii</i> (B1) | <i>Pneumocystis jirovecii</i> (R) |

Abbreviations: B1, blood/cerebrospinal fluid tier 1 (Supplementary Figure 2); B2, blood/CSF tier 2 (Supplementary Figure 3); E, enteric (Supplementary Figure 4); GAS, group A *Streptococcus*; GBS, group B *Streptococcus*; MERS, Middle East respiratory syndrome; R, respiratory (Supplementary Figure 1).

^aMay cross-react with some *Haemophilus influenzae* type a isolates.

^bMay cross-react with *Klebsiella varicola* and *Klebsiella quasipneumoniae*.

^cMay cross-react with lymphocytic choriomeningitis virus.

an extensive list of other microorganisms (~300 isolates of bacteria, viruses, and protozoa representing 36 genera and 143 species) as previously described [13]. This testing was performed to ensure no false amplification due to oligomerization of oligonucleotides, non-specific priming events, or lack of specificity for target sequence within targeted genus, species, or strain type. Analytical sensitivity (determination of lower limit of detection) was independently

determined for each assay in both singleplex and, when applicable, multiplex format by testing at least 8 replicates each of a 10-fold dilution series of specific total nucleic acid ranging from 0.1 fg/μL to 1 ng/μL. Inclusivity was assessed by testing representative isolates, including various subspecies, serotypes, or clonal groups, as appropriate. Final reaction concentrations varied from 200 to 1000 nM for primers and 100 to 300 nM for probes.

All individual and multiplex real-time PCR reactions for assay development, validation, and TAC production quality control (QC) were performed on the Applied Biosystems 7500 real-time PCR system (Thermo Fisher Scientific) with the following cycling conditions: 45°C for 10 minutes, 94°C for 10 minutes, 45 cycles of 94°C for 30 seconds, and 60°C for 60 seconds. Data acquisition occurred in FAM, VIC, ROX, Cy5, and/or ABY channels as appropriate based on fluorophores included in each multiplex reaction. Each 25- μ L reaction consisted of 1 \times qScript XLT 1-step reverse-transcription quantitative PCR (RT-qPCR) ToughMix, low ROX (Quanta Biosciences, Gaithersburg, Maryland), forward and reverse primers and hydrolysis probe (with 5' FAM, HEX, VIC, Cy5, or ABY and corresponding 3' quencher molecule), and 5 μ L of total nucleic acid template.

All TAC runs for assay development, validation, and QC conducted at CDC and for specimen testing at site laboratories were performed as previously described using either Applied Biosystems ViiA7 or QuantStudio 7 Flex real-time PCR system [13]. In brief, mastermix for each TAC consisted of 50 μ L 2 \times qScript XLT 1-step RT-qPCR ToughMix, low ROX, and 50 μ L nucleic acid template. A no-template control and positive control consisting of combined RNA transcripts (described below) were included on each TAC.

Performance Optimization of Multiplex Reactions

Multiple real-time PCR assays were complexed together into duplex or triplex reactions using different 5' reporter fluorophores (FAM, VIC or HEX, Cy5, or ABY) in order to allow detection of up to 3 individual targets in a single reaction well. Combinations of assays were determined based on both in silico analysis for potential oligonucleotide interactions and empiric testing with no template. Assays were also challenged with combinations of high and low template amounts to ensure equivalent sensitivity in multiplex reactions in the situation of potential competition for components required for amplification, such as polymerase or dNTPs.

Performance characteristics for select duplex and triplex reactions were confirmed using a small batch of TACs manufactured for proof-of-concept testing of multiplex reactions on TAC. In particular, these TACs were used to assess the impact of reduced reaction volume (25 \times smaller on TAC compared to 96-well plate format used for assay development), lyophilization of oligonucleotides on microfluidic array, and performance and compatibility of differentially labeled oligonucleotide probes. Initially, triplex reactions were evaluated with probes differentially labeled at the 5' position with FAM, HEX, and Cy5, all with 3' modification with a Black Hole Quencher (BHQ) fluorescent quencher molecule. Despite successful performance in 96-well plate format, Cy5-labeled probes performed poorly on TACs; in some cases these assays were unable to be interpreted. We evaluated a newer reporter dye (ABY, Thermo

Fisher Scientific) to replace Cy5. ABY-labeled probes were modified with a different quencher molecule (QSY) and exhibited superior performance compared to 5'Cy5/3'BHQ both in 96-well and TAC microfluidic card formats. However, ABY-labeled probes were incompatible with HEX-labeled probes in multiplex format due to emission spectral overlap of the 2 fluorophores and inadequate quenching of signal using BHQ compared to QSY. Assay performance was improved by replacing 5'HEX/3'BHQ probes with 5'VIC/3'QSY versions, eliminating spectral cross-talk for multiplex reaction formats on TAC. We also evaluated probe concentrations using the optimal probe quencher modifications, including QSY and ZEN double-quenched probes (IDT). The amplification signal was substantially improved with both 5'FAM/3'QSY and 5'FAM/ZEN/3'IBQ probes compared to 5'FAM/3'BHQ probes; the intensity of FAM fluorescence was approximately 10-fold higher for QSY-quenched compared to BHQ-quenched probes (data not shown). Use of these quenchers allowed for a lower final concentration of probe to be used in multiplex reactions when using 5'FAM/3'QSY or 5'FAM/ZEN/3'IBQ, saving on cost. In addition to the evaluation in 96-well plate format, a batch of TACs was manufactured with varying permutations of probe modifications (reporters, quenchers, and reaction concentrations) to compare performance of 5'FAM/3'QSY, 5'FAM/ZEN/3'IBQ, and 5'FAM/3'BHQ in both singleplex and multiplex reaction formats on the TAC microfluidic system. For most assays, 5'HEX/3'BHQ was replaced with 5'VIC/3'QSY unless the assay required an internal quencher, in which case 5'HEX/ZEN/3'IBQ was used. Overall, the use of QSY and/or ZEN/IBQ quenchers and FAM, VIC, and ABY reporter dyes was identified as the optimal combination for reducing/removing fluorescence cross-talk between detection channels on TAC.

Positive Control Production

For QC testing of oligonucleotide stocks and reaction mixes for TAC production, individual plasmids containing concatenated forward primer, probe, and reverse primer (reverse complement) sequences for an individual target within the multiple cloning site of a standard cloning vector plasmid were ordered from IDT or Thermo Fisher Scientific. Stocks were diluted to the appropriate concentration to yield positive amplification with crossing threshold (Ct) values between 25 and 35 when adding 5 μ L to a 25- μ L reaction well in 96-well plate format or 50 μ L to the loading portal of a TAC.

To control for assay performance on each TAC run, we engineered a combined positive control by expanding an existing pool of plasmids containing concatenated forward primer, probe, and reverse primer (reverse complement) sequences for each assay included on any TAC configuration as previously described [16]. The inserts were spread across multiple plasmids due to limitations of insert size and transcript length. In vitro transcription was performed using MegaScript high-yield

transcription kit (Applied Biosystems) as previously described [16]. Transcripts were pooled and titrated such that amplification occurred with Ct < 30 for each assay. Single-use aliquots were made from bulk transcript stock at the Division of Scientific Resources at CDC, stored at < -70°C, and shipped to site laboratories on dry ice.

TAC Production and Quality Control Testing

Each set of 3 oligonucleotides (individual assays) were combined at 60× final reaction concentration. These assay working stocks were combined and/or diluted as appropriate to generate singleplex, duplex, or triplex assay stocks at 20× final concentration for each assay in the reaction mix. Quality control testing was performed by testing each multiplex assay 20× mix using individual plasmid containing concatenated forward primer, probe, and reverse primer (reverse complement) sequences for each assay in the reaction mix and no template control reactions in triplicate. All 20× reaction mixes were shipped to Thermo Fisher Scientific (Pleasanton, California). Oligonucleotides were diluted and shipped to 3M (Milford, Ohio) for spotting onto plastic microfluidic arrays. TACs were selected from the beginning, middle, and end of the production lot for QC testing. Upon receipt of each lot at CDC, selected TACs were used to confirm spotting of each assay in the designated reaction wells by using the same plasmid material from preproduction QC as template in individual lanes. All TACs were stored at 2°C–8°C and shipped to site laboratories in insulated shipping boxes with cold packs. Each production lot was assigned a unique 5-character lot number, and each TAC was assigned a unique barcode identification number consisting of the lot number followed by a 4-digit unique identifier.

Specimen Processing and Nucleic Acid Extraction

Extraction Protocol Development and Optimization

Development and validation of specimen processing and nucleic acid extraction protocols was performed at the Pneumonia Response and Surveillance Laboratory at CDC using mock panels or clinical specimens (spiked healthy donor blood, universal transport media, lung tissue, and stool) and available archived clinical specimens using the MagNA Pure Compact instrument (Roche Diagnostics, Indianapolis, Indiana) with Nucleic Acid Isolation Kit I and Total NA Plasma External Lysis protocol. This platform and programmed protocol were selected because of the ability to accommodate a large specimen volume input (≤700 µL) and an off-board lysis procedure to ensure inactivation of pathogenic microbes and optimal release of nucleic acid from difficult-to-lyse microorganisms (eg, gram-positive bacteria, fungi, and parasites), and specimen types requiring homogenization (eg, lung tissue). MagNA Pure LC Total Nucleic Acid Isolation Kit–Lysis/Binding Buffer Refill (MagNA Pure Bacteria Lysis Buffer [BLB]) (Roche Diagnostics) was selected for specimen inactivation because of compatibility

with the Roche MagNA Pure platforms and the ability to effectively lyse eukaryotic and prokaryotic cells and to inactivate nucleases and other degradative enzymes. Other buffers were also considered and tested; where necessary, modifications were made to protocols to accommodate use of other buffers consistent with available extraction platforms at each site.

Optimization of mechanical disruption methods, including testing beads of various materials (ceramic, glass, stainless steel) and/or sizes was performed using spiked whole blood and frozen lung tissue specimens (Figure 2). The final protocols for specimen processing and nucleic acid extraction were determined by selecting the combination of reagents and methods allowing detection of the lowest amount of difficult-to-lyse pathogenic organisms as measured by real-time PCR (Supplementary Materials). In the final laboratory protocols, all clinical specimens are immediately treated with BLB for specimen inactivation and undergo additional lysis procedures prior to extraction depending on the specimen type (Figure 2). To ensure comparable performance of extraction platforms at each site, proficiency testing panels were prepared, consisting of universal transport media, whole blood, or stool spiked with quantified culture stocks. Specimens were extracted and tested at each site using the appropriate syndromic TAC. Results were analyzed at the site by trained laboratory staff and sent to the Pneumonia Response and Surveillance Laboratory at CDC for evaluation. Results were used to inform modifications to extraction procedures specific to each extraction platform.

Implementation of TAC at Site Laboratories

TAC Implementation

Each ViiA7 or QS7 instrument was installed and calibrated by field service technicians. Standard dye (FAM, VIC, ROX) and custom dye (Cy5, ABY) calibrations were performed on each instrument. Routine background and dye calibration is performed by site laboratory staff according to the manufacturer's recommendations. Each TAC run is initiated from a standardized run template file (.edt) for the appropriate card configuration and version. The cycling parameters listed above were set in the run template to ensure consistency between runs and between sites for TAC testing of minimally invasive tissue sampling (MITS) specimens. Use of the run template also ensured each assay was assigned to the proper well positions and reporter dyes for accurate results. Run templates utilized codes for each assay consisting of a unique 4 letter code followed by a version number to indicate the analytes used in production of a given card lot. The unique card barcode is entered by scanning or manual entry into the designated field in the software during run setup to ensure the proper run template is used for each specimen type. Standard operating procedures for TAC setup also specify standardized file naming conventions for compatibility with the custom database, which specify card configuration, site, date of run, and operator, as well as file status (unanalyzed, analyzed by site, reviewed by CDC).

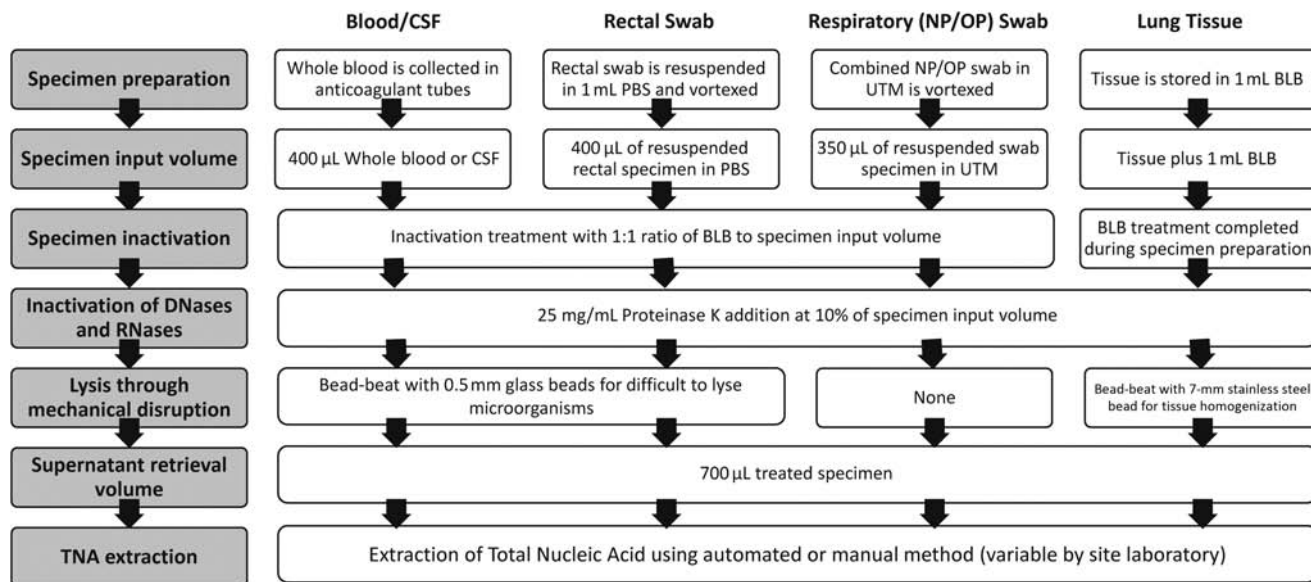


Figure 2. Nucleic acid extraction protocol for clinical specimen types. Specimen types include whole blood, cerebrospinal fluid, rectal swabs, nasopharyngeal and oropharyngeal combined respiratory swabs, and lung tissue. Roche MagNA Pure Compact instrument used for development of extraction protocols at the Centers for Disease Control and Prevention. Automated or manual extraction platforms used at Child Health and Mortality Prevention Surveillance (CHAMPS) network site laboratories varied (Supplementary Materials). Abbreviations: BLB, bacteria lysis buffer; CSF, cerebrospinal fluid; NP/OP, nasopharyngeal/oropharyngeal; PBS, phosphate-buffered saline; TNA, total nucleic acid; UTM, universal transport media.

On-site laboratory training was completed at 3 sites in 2016 (Respiratory and Meningeal Pathogens Research Unit, Soweto, South Africa; Kenya Medical Research Institute, Kisumu, Kenya; Manhica Health Research Centre, Manhica, Mozambique) and 2 sites in 2019 (Haramaya University, Harar, Ethiopia and Makeni General Hospital, Makeni, Sierra Leone). Training was conducted at CDC for staff from 2 sites (icddr, Dhaka, Bangladesh and Centre for Vaccine Development–Mali, Bamako, Mali) in 2017. Hands-on laboratory training included specimen processing (inactivation, beadbeating, etc), nucleic acid extraction using site-specific platforms or kits, TAC setup and performance, ViiA7 or QS7 instrument operation, and TAC results analysis and interpretation. Fundamental principles of multiplex real-time PCR as well as program-specific procedures are emphasized during each training session, and technical support and retraining is available as needed. Training was also provided by informatics staff to laboratory and/or data management staff at each site for uploading of data to the CHAMPS data portal.

Data Management

The ultimate objectives of the CHAMPS TAC data management solution are to generate a comprehensive report of all TAC result interpretations for a single CHAMPS case subject within a packet of information compiled with other case data, and to provide this case packet to a panel of experts for determination of cause of death. The TAC report must distill the complex instrument output into a format that is easily

understood by users from a variety of health-related disciplines. Thus, the CHAMPS TAC data management solution needed to support all steps in the analysis lifecycle that lead to the final report.

The analysis process using ViiA7 and QS7 instrument software creates multiple file types including an analysis run file (.eds) and a Microsoft Excel file (.xlsx) containing multiple tabs of data from the run. The .eds file is generated in real time as the amplification process proceeds and is analyzed by laboratory staff to generate positive, negative or indeterminate results for each target replicate, internal control, positive control and no-template control on the card. Assay results may be considered indeterminate based upon performance of assay or specimen control reactions, quality of amplification curves, or assay-specific limitations. The .xlsx file is an exported file containing results for each replicate, associated cycle threshold (Ct) values, and other data associated with the run. The CHAMPS protocol for TAC analysis specifies that the analyst retain an unanalyzed.eds file and an analyzed.eds file, in addition to the .xlsx file, for QC, reanalysis (if required), and documentation of results.

The exported data file (.xlsx) contains a significant amount of data, with each card generating between 700 and 1000 rows of results depending on the number of targets, replicates of each target, and associated controls. The dataset includes either 2 or 4 replicates per target, depending on card type, that must be interpreted to arrive at a single target interpretation for each specimen. Additionally, some pathogens may have multiple

single target interpretations that must be interpreted as a group to generate a multitarget result, typically representing the subspecies or other characteristics identified below the pathogen level (Supplementary Table 1). While analysis and generation of final results from the .xlsx file can be done manually, the process is tedious and potentially error prone. With the CHAMPS program projected to analyze thousands of TAC files with millions of row-level results, there was a need to generate a data management solution that would provide automation in the interpretation, storage, and presentation of results.

The solution was a new subcomponent of the overall CHAMPS data management web portal where CHAMPS sites upload output used for the analysis and interpretation of data. Upon upload, submitted files are validated for file naming conventions, structure, content, and matching of specimen identifiers to existing CHAMPS cases. Validation errors result in rejection of the file with an email notification sent to the submitter with all validation errors. Once the card passes initial validation checks, the data from the card is processed by an algorithm that generates single-target replicate and multitarget interpretations and stores all replicate data and interpretations in a relational database. Single-target replicates are interpreted using the same pattern for all targets and all cards. If ≥ 1 positive replicate for a target exists, the interpretation is positive for that target. Targets with negative results from all replicates produce a negative interpretation; any combination of negative and indeterminate results from related replicates yields an indeterminate interpretation.

After initial processing by the algorithm the card is queued for QC by the CHAMPS Program Office (PO) TAC Team. The QC review process involves several steps, including review and potential reanalysis of the initial card submission, upload of the reanalyzed .eds and .xlsx files, manual determination of invalid results, and manual interpretation of multitarget indeterminate results (Figure 3). After each step of the review process, the interpretation algorithm is initiated to reinterpret the results based on the reanalyzed card uploaded or manually marked results. To initiate review, the PO team downloads the files submitted by the site and may choose to accept the results without submitting reanalyzed files or upload .eds and .xlsx files from their analysis. When reanalyzed files are uploaded, the PO team analyst has an option to proceed to manual review of single target replicates, typically for results that are invalid due to internal control or other issues. These cards reappear in the queue with a status of “invalid review.” Any cards with indeterminate multitarget interpretations remaining after automated and manual single target review appear in the queue with a status of “multitarget review.” After all steps have been completed, the card is set to a status of “review complete.”

Additional alternative processing scenarios were also implemented in the TAC interpretation algorithm including resubmission of the same target/specimen combination on another

card either due to an extraction failure or some other issue on the first submission or the same target specimen combination may exist on another card type. In either case the replicates from both cards having the same target/specimen combination are “stacked” and all replicates are interpreted together to generate a new interpretation. If invalid replicates are included in the “stack,” these replicates are ignored by the interpretation algorithm.

The system can generate a report for a specific case at any point in time. The complete set of results for a case will span multiple card types, multiple target/specimen combinations, and potentially multiple cards of the same target/specimen combination. A case manager reviews TAC results to ensure completeness prior to generating the case packet for the expert panel review. Additional reports and queries are provided to PO and site case managers to track progress of TAC result completion prior to case packet generation.

Quality Assurance

Laboratory staff competency for performance and analysis of TAC is assessed using proficiency panels generated by CDC as described above. Two panels (whole blood and nasopharyngeal/oropharyngeal swabs in transport media) consisting of 6 mock specimens each were extracted and tested at each site using the appropriate syndromic TAC. Trained laboratory staff analyzed run results and sent run files to the Pneumonia Response and Surveillance Laboratory at CDC for evaluation. Competency was assessed initially for each laboratorian prior to beginning testing of CHAMPS specimens and offered thereafter on an as-needed basis based on performance assessment through ongoing review of analyzed TAC files.

TAC data for CHAMPS specimens tested at all sites are reviewed by CDC laboratorians by uploading unanalyzed and analyzed TAC run files (.eds) and exported data files (.xlsx) to the CHAMPS data portal for a site validation period of variable duration. After confirming satisfactory performance of testing and data analysis during this period, files are only reviewed to (1) resolve indeterminate results for individual targets or specimens; (2) review unexpected results for negative or positive controls; or (3) resolve interpretations for multitarget algorithms resulting from indeterminate interpretations. This review process enables rapid identification of any aberrant results or unexpected performance issues related to operator error, reagent quality, or other aspects of the laboratory workflow that may require investigation and corrective action.

In general, interpretive thresholds were not set such that amplification at any Ct value is considered a positive result as long as the curve shape and fluorescence level are deemed appropriate, with few exceptions. Proper interpretation of amplification curves and other fundamental principles of real-time PCR are emphasized during hands-on laboratory training. For the *Escherichia coli*/*Shigella* assay, a Ct cutoff value of 30

TAC Manual Review Include complete cards

| File Barcode | Specimen IDs | Site | Date Uploaded | Card Type | Number of Indeterminates | Status | Actions |
|--------------|--|------------|---------------|--------------------|--------------------------|--------|---------|
| B35850097 | MO1116 026, MO1310 026, MO1312 026, MO1300 026, MO1133 026, MO1317 026 | Bangladesh | 25-05-2019 | Diarrheal TAC | 0 | New | |
| B33491202 | MO1311 018, MO1002 018, MO1307 018, MO1048 018 | Bangladesh | 28-05-2019 | BloodCSF Tier 2 V2 | 0 | New | |
| B33491204 | MO1310 010, MO1310 018, MO1317 010, MO1300 018, MO1300 010 | Bangladesh | 28-05-2019 | BloodCSF Tier 2 V2 | 1 | New | |
| B33491207 | MO1133 010, MO1118 018, MO1312 010, MO1312 018, MO1118 010 | Bangladesh | 28-05-2019 | BloodCSF Tier 2 V2 | 0 | New | |
| B33490409 | MO1027 018, MO1018 018, MO1012 018, MO1116 018, MO1322 018 | Bangladesh | 28-05-2019 | BloodCSF Tier 2 V2 | 0 | New | |
| B35850094 | MO1296 026, MO1289 026, MO1283 026, MO1230 026, MO1231 026, MO1553 026 | Kenya | 29-05-2019 | Diarrheal TAC | 4 | New | |
| B35850086 | MO1557 026, MO1569 026, MO1558 026, MO1566 026, MO1552 026, MO1570 026 | Kenya | 29-05-2019 | Diarrheal TAC | 0 | New | |
| B35850087 | MO1560 026, MO1554 026, MO1559 026, MO1551 026, MO1556 026, MO1555 026 | Kenya | 29-05-2019 | Diarrheal TAC | 0 | New | |
| B35000094 | MO1272 040, MO1281 040, MO1282 040, MO1284 040, MO1293 040, MO1229 040 | Kenya | 29-05-2019 | Respiratory TAC V2 | 0 | New | |
| B35000095 | MO1570 040, MO1552 040, MO1553 040, MO1283 040, MO1569 040, MO1566 040 | Kenya | 29-05-2019 | Respiratory TAC V2 | 0 | New | |

101 - 110 of 230 items

Figure 3. View of portal review widget from the Child Health and Mortality Prevention Surveillance custom database. TaqMan Array Card run files (.eds) and exported data files (.xlsx) are downloaded for manual review and modified files are uploaded. Files can be sorted by unique identifier (barcode ID), specimen IDs, site, date uploaded, card type, number of indeterminate results, or status. Abbreviations: CSF, cerebrospinal fluid; ID, identifier; TAC, TaqMan Array Card.

was implemented at all sites due to potential false-positive results arising from residual *E. coli* nucleic acid in the enzyme mastermix. Extensive validation of this assay supported the use of this threshold value. Although not initially indicated based on assay validation performed in 96-well plate format during the development phase, interpretive thresholds were implemented for several additional assays after assessment of assay performance on TAC based on review of files from each site during the early validation period, including hepatitis E virus (considered indeterminate if Ct > 30) and astrovirus, chikungunya virus, dengue virus, Middle East respiratory syndrome coronavirus, Rift Valley fever virus, rubella virus, *Salmonella enterica* Paratyphi A, and Zika virus (considered indeterminate if Ct > 35).

Additional quality assurance is planned for implementation pending full execution of material transfer agreements between CDC and each site laboratory institute. Ongoing quality assurance testing will consist of nucleic acid extraction from primary specimen and TAC testing for 5% of all specimens from each site at the Pneumonia Response and Surveillance Laboratory at CDC.

DISCUSSION

Development and implementation of multiplex TAC configurations for screening of diverse specimen types allows for

a comprehensive evaluation of infectious causes of death. In CHAMPS, TAC augments pathology analysis on postmortem specimens, clinical data, and other information to specify the microorganisms contributing to death in each case. Attributing death to specific pathogens rather than only a syndrome such as pneumonia, sepsis, or diarrheal disease provides more actionable information to communities to implement potentially life-saving interventions, and large-scale data analysis can help prioritize needs for new vaccines or antimicrobials. Importantly, this technology is performed locally by trained staff. TAC is an ideal platform for multipathogen detection in low-resource settings and has vast potential for expansion beyond CHAMPS.

To ensure the highest quality of data, CHAMPS TAC laboratory protocols were carefully developed using empiric testing whenever possible in addition to literature review and consultation with subject matter experts. Laboratory protocols were modified for each site as needed, and proficiency testing was implemented to ensure proper training and competency of testing personnel as well as to minimize any intersite variation resulting from different nucleic acid extraction platforms. Special effort was made to incorporate an effective pathogen inactivation step to protect laboratory staff due to the potential presence of highly transmissible pathogens capable of causing severe infections. Inactivation also ensures compliance with national and

international laws and guidelines regarding handling of select agents. Ongoing technical support through remote and on-site trainings, personnel competency assessment, quality assurance testing, and data review will continue throughout the duration of CHAMPS testing at each site laboratory.

While there are many advantages to use of TAC in CHAMPS, there are also a substantial number of complex challenges. One limitation of TAC is that the pathogens included in each panel are selected a priori, creating a potential bias toward identifications, although specimens are stored in a central repository and can be tested later for additional pathogens of interest or using newer test methods. The specimen types collected for testing are diverse and complex matrices requiring specialized specimen processing. We addressed this through focused development of specimen processing and nucleic acid extraction protocols, although these efforts were limited by the material available to use for validation, including clinical specimens. In the case of a few assays, we were unable to perform a comprehensive validation using clinical specimens, especially for rare pathogens.

Even greater challenges exist beyond these technical and logistical issues. Aside from the technical challenges, the simple abundance of data presents an inherent challenge. The development of a custom data portal for CHAMPS has enabled management of the vast amount of data generated for each case. In some cases, nucleic acids from a large number of potential pathogens are detected, particularly in nonsterile site specimens. Attributing detected organisms to cause of death is extremely complex as detection of pathogen nucleic acid does not necessarily indicate a contribution to disease state or to death. Many potentially pathogenic organisms may be present in a long-term carriage state, especially in upper respiratory and gastrointestinal specimens, yet it is nearly impossible to distinguish this status using molecular detection methods alone. In addition, microorganisms may disseminate from the original site of infection during the time leading up to and following death. However, TAC results are a highly valuable adjunct to postmortem tissue pathology studies helping to suggest the need for special stains and immunohistochemistry testing (see Martines et al in this supplement). Further evaluation will be performed to investigate the extent of perimortem dissemination to pathogen detection and cause of death determination, including the impact of time from death to completion of the MITS procedure. Evaluation of concordance of pathogen detection using pathology and molecular tests is also planned.

Production of TAC configurations with custom assays requires substantial preproduction steps and QC testing throughout the process. Distribution of TACs and ancillary reagents as well as inventory management are also required. We successfully procured, produced, and distributed CHAMPS-specific TACs and ancillary reagents, including custom positive control material, to each site laboratory. Outside of CHAMPS,

the lack of commercial availability of study-specific TAC configurations is perhaps the greatest barrier to TAC implementation.

Despite these challenges, TAC is very well suited for infectious disease surveillance and outbreak response, and the research and development efforts presented here and elsewhere have expanded the utility through improvements in breadth of pathogen panels and assay performance characteristics. In keeping with the capacity-building goals of CHAMPS and global preparedness and response initiatives of CDC, these custom TACs have been made available on a case-by-case basis for outbreak investigations [17, 18]. In 2017, CHAMPS TACs were employed to rapidly identify *Neisseria meningitidis* as the causative agent of an outbreak in Liberia [19]. Implementation of similar custom TAC configurations is being supported for other large-scale surveillance studies to examine infectious etiologies in children, neonates, and mother–infant pairs.

TAC has proven to be a useful modern tool for global disease surveillance and outbreak response, even in resource-limited settings. However, infectious disease diagnostics rapidly evolve, and continual advancement is inevitable. The dramatic increase in widespread use of next-generation sequencing technology in clinical, academic, and public health laboratories worldwide signals a substantial shift in laboratory capacity. Culture-independent diagnostic tests represent the application of these advanced technologies to clinical diagnostics and disease surveillance. As CHAMPS moves forward, we will continue to pursue emerging advanced technologies for application to MITS specimen testing. Continual improvements in reagents, equipment, and analysis pipelines for targeted resequencing or metagenomics may enable more widespread use of these approaches in resource-limited settings, and CHAMPS sites are interested in pursuing their implementation. Deep sequencing analysis of specimens in which no pathogens are identified using TAC may provide a good starting point for implementation of these methods. However, next-generation sequencing methods are currently cost-prohibitive in many settings. Other technical barriers, such as laboratory infrastructure, equipment technical support, and reagent procurement may be restricting factors. Access to bioinformatics resources and high performance computing capacity is a major obstacle to implementation. Examination of specimens for unique biomarkers may also provide added value to cause of death determination. However, all of these methods require substantial further development prior to implementation with clinical specimens within laboratories in resource-limited settings.

CHAMPS is an unprecedented approach to addressing the global disparity in childhood deaths with many novel aspects, including implementation of advanced laboratory methods on postmortem specimens to attribute specific pathogens to cause of death. TAC is a broad, flexible multipathogen detection platform that is ideal for disease surveillance, outbreak response,

and other uses. The innovation of applying multiplex PCR technology to the microfluidic array vastly increased the number of pathogens that can be screened with a small amount of primary specimen. Application of TAC within the CHAMPS network heralds its potential for wider use in global disease surveillance. Moreover, use of TAC in CHAMPS is the first application of this technology to postmortem specimen testing. The combination of comprehensive development and implementation of the cards and custom data management system, along with ongoing technical support through the CHAMPS program office and technical partners, make this system a model for future global disease surveillance networks.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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