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Title: Multi-Echelon Diagnostics (MEDx): Platform Performance in an Austere Environment, Bo, Sierra Leone

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EXECUTIVE SUMMARY

Early detection and identification of pathogens (e.g., influenza, malaria, or hemorrhagic fever viruses) is essential for the protection of civilian populations and US forces. In January 2018, the Center for Bio/Molecular Science and Engineering at the Naval Research Laboratory (NRL) and Mercy Hospital began a collaborative effort to test custom Multi-Echelon Diagnostic (MEDx) platforms under austere conditions of use. The goal was to provide situational awareness of disease prevalence in West Africa while also determining the performance characteristics of the MEDx tests. Urine and blood samples collected from 535 human volunteers were subjected to up to 10 different tests for malaria, chikungunya, dengue, and melioidosis. Based on both molecular and immunological tests, the overall prevalence of dengue and melioidosis were low. Antibody-based tests indicated that approximately 25% of participants had malaria, while PCR indicated a much higher prevalence (approximately 50%). Results observed with custom MEDx tests demonstrate that additional improvements are required to attain similar sensitivities and specificities of commercially available platforms. In addition to generating valuable performance data on a sponsor-provided diagnostic platform, the current study also provided timely epidemiological data from febrile individuals. The latter information can improve situational awareness and mitigate the potential for unexpected outbreaks of infectious diseases.

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MULTI-ECHELON DIAGNOSTICS: PLATFORM PERFORMANCE IN AN AUSTERE ENVIRONMENT, BO, SIERRA LEONE

1. INTRODUCTION

Historically, infectious disease has played a significant role in the success or failure of military campaigns and represents a considerable burden for deployed military personnel throughout the world. The widespread practice of self-diagnosis, prevalence of poor quality or misused therapeutics, and dearth of commercially available, inexpensive, and reliable diagnostic tests for common infectious diseases all contribute to an overall lack of basic infectious disease surveillance capabilities in developing countries (1–4). The resulting ignorance of temporal and geographic patterns of infectious diseases may allow emerging pathogens to circulate undetected for significant periods prior to discovery, leading to surprise outbreaks such as the 2012 cholera and 2014 Ebola outbreaks in West Africa. With limited situational awareness of these and other circulating diseases, US forces deployed to these regions are likely to encounter unexpected pathogens without supporting medical diagnostic or therapeutic infrastructure. For this reason, far-forward deployment of rapid and reliable diagnostics is critical to ensure the health of US forces deployed abroad.

The Defense Threat Reduction Agency (DTRA) has implemented the Multi-Echelon Diagnostics (MEDx) program to demonstrate that rapid diagnostic tests (RDTs) and more labor-intensive lab-based tests can be rapidly developed, manufactured, and performance-tested for forward deployment when needed. The Center for Bio/Molecular Science and Engineering at the Naval Research Laboratory (NRL) has supported previous biosurveillance and diagnostic testing efforts in collaboration with George Mason University (GMU) and Mercy Hospital Research Laboratory (MHRL) in Kulanda Town, Bo, Sierra Leone (7.96°N, 11.74°W) (5,6). The NRL/GMU/MHRL team has developed a municipal map of Bo using a combination of satellite imagery and Participatory Geographic Information System methods, combining this with household surveys to determine populations for the different municipal sections of Bo to map Mercy Hospital catchment areas (7–10). Furthermore, the MHRL facilities have been equipped with diagnostic testing capabilities that rival those found only in specialized regional hospitals. Recent studies supporting DTRA and other surveillance efforts in West Africa have included testing for chikungunya, Ebola, hepatitis B infection, HIV, plague, dengue, melioidosis, and malaria (11–17).

Here, the joint NRL/MHRL team built on our previous biosurveillance efforts in support of DTRA's MEDx program. Our specific objectives were:

(1) To quantify performance characteristics of DTRA-developed RDTs for diagnosis of disease caused by chikungunya virus (CHIKV), dengue virus (DENV), *Burkholderia pseudomallei* (BURK), and various *Plasmodium* species under austere conditions for point-of-need applications

(2) To provide clinical and testing datasets in real time to a cloud-based database for shared data analysis

(3) To report on the presence of malaria and other pathogens causing febrile illness in Sierra Leone that may have importance to DTRA and USAFRICOM for future planning.

Manuscript approved May 6, 2020

2. MATERIALS AND METHODS

2.1 Participant enrollment, clinical sample collection and processing

Protocol-trained staff recruited subjects ages 5 and over presenting to MHRL with self-reported or clinically confirmed fever with onset within the previous ten days. Detailed symptomatic information and clinical histories were obtained for each participant in a private setting. Informed consent was obtained from all patients or from the parents of minor children. The complete research protocol was approved by the institutional review boards of GMU and NRL and by the Sierra Leone Ethics and Scientific Review Committee.

Three types of clinical samples were collected from each informed participant: urine, venous blood (collected in two Vacutainers), and capillary blood (Fig. 1). Vacutainers and Microtainers used for blood collection were obtained from Becton Dickinson (Franklin Lakes, NJ, USA). Capillary blood was obtained by fingerstick using spring-loaded lancets and was collected in heparin-containing Microtainers. Venous blood was obtained using standard butterfly collection sets and was collected into two types of Vacutainers: (1) citrate Vacutainers for commercial RDT testing, malaria smears, DNA extraction, and preservation on Whatman FTA blood sample cards (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA), and (2) serum separator (SST) Vacutainers for RNA processing. Serum was prepared in SST Vacutainers according to manufacturer's instructions. Urine was collected in 150 mL sterile plastic collection vials. Urine, capillary and venous blood, and serum were used within 2h of collection.

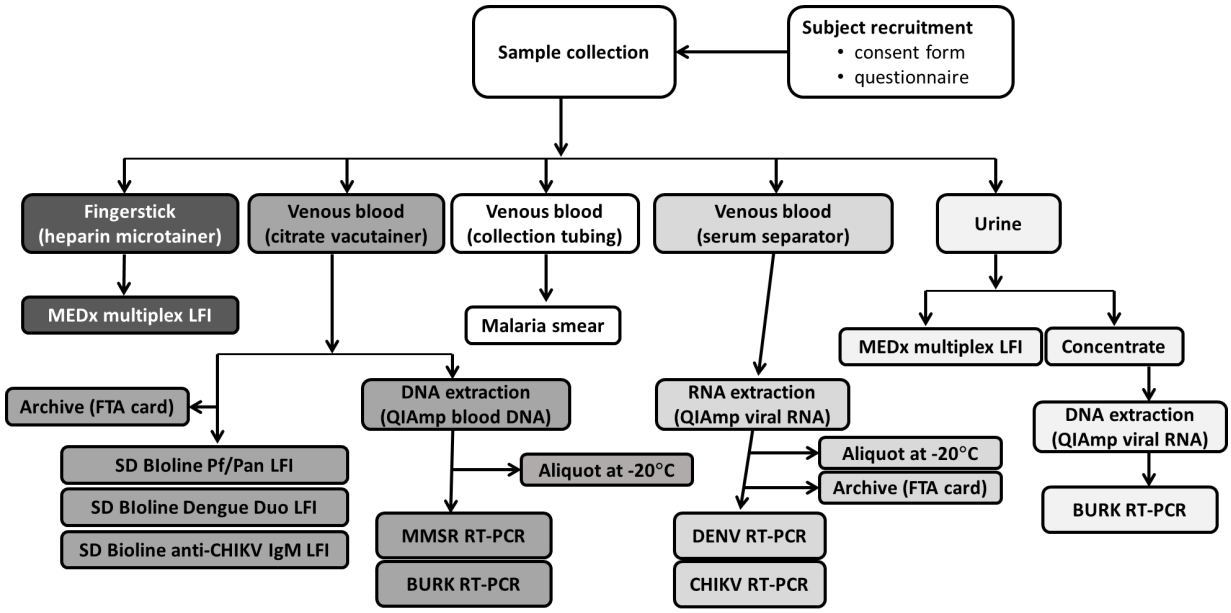


Fig. 1—Collection, processing, and archiving pathway for samples collected from each participant

A packet of datasheets accompanied each participant's sample(s) through all steps to track the processing and analyses performed. At each step, the technician noted the time, date, and any additional descriptions or issues encountered. All test results associated with these samples were noted on the appropriate page. File names and sample locations (e.g., during PCR analyses) were also noted on the appropriate pages to allow remote analysts to quantify results more accurately. After all tests were

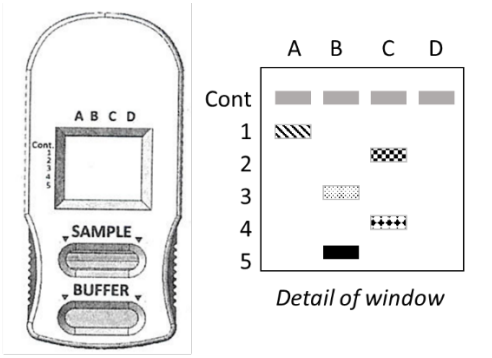
performed and results noted, all data (including metadata) were transcribed into electronic format and uploaded to a cloud-based database.

2.2 Antibody-Based Tests

Commercial off-the-shelf (COTS) tests for malaria (Malaria Ag Pf/Pan), anti-CHIKV IgM (Chikungunya IgM), and dengue (Dengue Duo IgG/IgM/NS1) were purchased from SD Bioline (Gyeonggi-do, Republic of Korea). Fifty microliters of citrated whole blood were used for each of the COTS tests. The presence or absence of each target was noted by the appearance of a line in the appropriate spot; tests were marked as inconclusive (invalid) if the control line was not observed.

DTRA-provided MEDx multiplex RDTs were manufactured by InBios International, Inc. (Seattle, WA, USA). Two MEDx tests were used for each participant: one test used capillary blood to test for dengue, malaria, and chikungunya; and one test used urine to test for melioidosis.

The MEDx tests were designed in a multiplexed, parallel-lane format for simultaneous detection of NS1 protein from DENV, CPS antigen from BURK, histidine-rich protein II (HRP II) from *P. falciparum*, and both IgG and IgM antibodies directed against CHIKV (Fig. 2). The 2-dimensional array was intended to detect one or more target in each of three parallel lanes (Lanes A–C), and a blank lane (Lane D) was included for future integration of additional tests. One hundred microliters of heparinized capillary blood or urine were applied to the sample port of the MEDx device, and the entire contents of the Chase Buffer vial was applied to the Buffer port within 30 sec of sample application. After 20 min incubation, stripes would appear in specific loci in each lane, with the identity of each stripe dictated by patterns on the device surface. Melioidosis results were recorded only for tests using urine, whereas results for malaria, chikungunya, and dengue were recorded for tests using capillary blood.



Disease	Marker	Locus
Dengue	DENV NS1	A-1
Malaria	*P. falciparum* HRP II	B-3
Melioidosis	*B. pseudomallei* CPS	B-5
Chikungunya	Anti-CHIKV IgM	C-2
	Anti-CHIKV IgG	C-4

Fig. 2—MEDx test for multiplex diagnosis of dengue, malaria, melioidosis, and chikungunya.

MEDx devices were imaged both before and after test performance using a Deki reader v200 (Fio Corporation, Toronto, ON, Canada). Images were uploaded to a secure web-based database upon

assay completion; participant number, technician name, and other metadata were also entered into the Deki reader before assays were started. After the timer on the Deki reader indicated that 20 min had elapsed and a final image had been taken, the location of lines was written on the datasheet and transposed to the Deki reader screen. The images, associated metadata, and manually-entered results were then uploaded to a proprietary Fio-managed remote database.

2.3 Nucleic Acid Extraction and Molecular Tests

DNA was extracted from citrated venous blood and RNA for serum using QIAamp DNA Blood Mini Kits or QIAamp Viral RNA Mini Kits, respectively, according to manufacturer's instructions (QIAGEN, Germantown, MD, USA). Aliquots of each were frozen at -20ºC and aliquots of extracted RNA were spotted onto FTA cards for archiving.

Multiplex Malaria Sample Ready (MMSR) PCR was purchased from BioGX (Birmingham, AL, USA). This multiplex TaqMan based real-time PCR assay—provided in lyophilized form—is designed to detect and distinguish between *P. falciparum*, *P. vivax*, and *Plasmodium* spp. based on real-time amplification curves (18, 19); an RNase P control was included in each reaction to confirm adequate nucleic acid extraction from clinical samples. The facile protocol entailed simply rehydrating the lyophilized reagent with water (5 µL per tube), and then adding 1 µL sample, water (negative control), or control DNA before loading into the thermal cycler. Amplification was performed using a QuantStudio5 instrument measuring the appropriate fluorophores (18). A small subset of samples (n = 15) was analyzed for species identification using a panel of SYBR green real-time PCRs; targets included a pan-*Plasmodium* 18S rRNA target and five non-18S rRNA gene targets specific to *P. vivax*, *P. falciparum*, *P. ovale*, *P. malariae*, and *P. knowlesi* (20); pan-*Plasmodium* 18S rRNA amplicons were sequenced to confirm the identity of the detected species (Eurofins Genomics, Louisville, KY, USA).

Protocols for TaqMan based real-time PCR detection of DENV serotypes 1–4 and CHIKV were provided by the US Naval Medical Research Unit-6 Peru (NAMRU-6, Lima, Peru). Two versions of the NAMRU-6 DENV assays were performed: v1 used separate reactions for detection of DENV serotypes 1/3/4 and DENV serotype 2, whereas v2 combined all DENV primers and probes into a single multiplex reaction (DENV 1/2/3/4). Amplification of RNase P was performed for each sample as an extraction control.

Due to reagent shortages, a COTS multiplex test for CHIKV and DENV was used in place of the NAMRU-6 PCR for the final ~140 samples (TaqMan Zika Virus Triplex kit; ThermoFisher, Rockland IL). This kit contains controls for human cyclophilin A (PPIA) as a substitute for the RNase P extraction control.

Menzies School of Health Research (Darwin, Northern Territory, Australia) provided the protocol for real-time PCR of BURK, using DNA extracted from whole blood. As MMSR assays were performed on the same samples as the BURK PCRs, the RNase P results in the MMSR assays were treated as the extraction control for the BURK assays. For the first 40 samples, BURK PCR assays were also performed on DNA extracted from urine using QIAGEN QIAamp viral RNA mini kit (extracted per the manufacturer's instructions); this latter set of BURK assays did not have RNase P extraction controls.

Primers, probes, positive controls, and cycling conditions are shown in Table 1.

Table 1—Primers, Probes, and Positive Controls Used in Molecular Assays

Assay	Primers, Probes, Controls	Cycling
MMSR	Primers/probes listed in (18); all components purchased from BioGX Positive controls: *Plasmodium* spp., *P. falciparum*, and *P. vivax* control templates provided with tests	2 min at 95°C; 45 cycles of 95°C for 10 sec, 59°C for 60 sec
DENV	Primers and probes sequences listed in (20); fluorophore/quencher pairs modified as follows: DENV-3 probe—CalRd610/BHQ1; DENV-4 probe—Quas670/BHQ1 Positive controls: Synthetic RNA preparations from ATCC: VR-3228SD™ (type 1), VR-3229SD™ (type 2), VR-3230SD™ (type 3), VR-3231SD™ (type 4)	5 min at 50°C, 20 sec at 95°C; 40 cycles of 95°C for 15 sec, 60°C for 30 sec
CHIKV	Primers, probes ("3855 set") found in (21) Positive control: Quantitative synthetic chikungunya RNA (VR-3246SD™) from ATCC	5 min at 50°C, 20 sec at 95°C; 40 cycles of 95°C for 15 sec, 60°C for 30 sec
TaqMan Zika Virus Triplex Kit (Zika/CHIKV/DENV)	ThermoFisher A31747; proprietary primers, probes	20 min at 50°C, 2 min at 95°C; 40 cycles of 95°C for 15 sec, 60°C for 1 min
RNase P (extraction control)	RP-F primer: CCAAGTGTGAGGGCTGAAAAG RP-R primer: TGTTGTGGCTGATGAACTATAAAAGG RP-probe: FAM/CCCCAGTCTCTGTCAGCACTCCCTTC/BHQ1	Same conditions as DENV and CHIKV targets, above
BURK	Primers and probes found in (22) Positive controls: Whole genome amplicon from *B. pseudomallei* strain 1710a, NR-8218 from BEI Resources*	2 min at 50°C, 10 min at 95°C; 40 cycles of 95°C for 15 sec, 60°C for 1 min

*Whole genome amplicons were generated by amplifying 10 ng genomic DNA with GenomiPhi HY (ThermoFisher, Rockland, IL, USA) and were used without purification.

3. RESULTS AND DISCUSSION

3.1 Enrollment and overall disease prevalence by age, gender

For the duration of this project (5 Feb to 6 Dec 2018), a total of 535 participants were enrolled. Thirty-nine percent of the subjects were male. Participant ages ranged from 5 to 92 years of age, with a median age of 27. From this population, complete sample sets (capillary blood, whole blood, serum, urine) were collected from 533 participants, and the full complement of 10 tests was performed on samples from 436 subjects: MEDx LFI on urine; MEDx LFI on capillary blood; COTS RDTs for malaria, dengue, and anti-CHIKV IgM LFIs; MMSR; CHIKV, DENV, and BURK PCRs; and malaria smear.

Table 2 presents the overall and age-categorized prevalence of dengue, malaria, melioidosis, and chikungunya based on the diagnostic platform yielding the highest number of positives. Malaria results (representing *Plasmodium* spp.) were obtained by PCR. Dengue results represent anti-DENV IgG and were determined by COTS RDT. Chikungunya results were determined by MEDx and represent anti-CHIKV IgG. Data presented for melioidosis were determined by PCR (blood) and MEDx (urine). As observed worldwide (23), there was a significantly higher burden of malaria borne by children than by persons of other age brackets ($p < 0.001$); no other age- or gender-related differences were noted.

Table 2—Overall Diagnoses for Dengue, Malaria, Melioidosis, and Chikungunya based on the Test with Highest Number of Positives

Infection	Population	# Tested	# Positive	% Positive	p-Value
Dengue (anti-DENV IgG, COTS RDT)	All	533	14	2.6%	--
	Male	210	6	2.9%	0.793
	Female	322	8	2.5%	
	Age 5–14	72	1	1.4%	0.873
	Age 15–29	229	6	2.6%	
	Age 30–44	118	4	3.4%	
	Age 45+	109	3	2.8%	
Malaria (MMSR data)	All	527	261	49.5%	--
	Male	206	106	51.4%	0.692
	Female	318	158	49.7%	
	Age 5–14	72	52	72.2%	<0.001
	Age 15–29	227	126	55.5%	
	Age 30–44	117	46	39.3%	
	Age 45+	105	39	37.1%	
Melioidosis	All (PCR)	485	0	0%	--
	All (MEDx, urine)	526	0	0%	
Chikungunya (anti-CHIKV IgG, MEDx RDT)	All	533	137	25.7%	--
	Male	209	52	24.8%	0.472
	Female	325	90	27.7%	
	Age 5–14	72	14	27.5%	0.420
	Age 15–29	229	63	23.1%	
	Age 30–44	119	30	25.2%	
	Age 45+	108	33	30.6%	

3.2 Antibody-based Tests

3.2.1 MEDx Test Results (On-site Visual Interpretation)

Table 3 shows the total number of MEDx tests performed, the number of tests with control line present, and the number of positive results observed amongst tests deemed valid, as visually determined by the user. Automated results determined by the Deki reader are considered in Section 3.2.4.

Though other platforms detected a greater number of positives for three targets, MEDx results followed the general trends observed. Low rates of DENV and BURK were observed, while a high prevalence of *P. falciparum*-positives was noted. In contrast to our previous observations (12, 24), a relatively low rate of positives for anti-CHIKV IgM was observed, which may be related to a current lull in CHIKV infection, as detailed in Table 3.

Table 3—Urine and Capillary Blood Test Results from MEDx Tests (On-site Visual Inspection)

<table>
<tr><td rowspan="2">Urine</td><td colspan="4">Total # Test Results Available</td><td>526</td></tr>
<tr><td colspan="2">#Tests Shown as Valid</td><td rowspan="2"># Tests Positive (%)</td><td rowspan="2">BURK (lane B)</td><td rowspan="2">0 (0%)</td></tr>
<tr><td>Lane B</td><td>526</td></tr>
<tr><td rowspan="6">Finger stick</td><td colspan="4">Total # Test Results Available</td><td>533</td></tr>
<tr><td colspan="2">#Tests Shown as Valid</td><td rowspan="5"># Tests Positive (%):</td><td colspan="2"></td></tr>
<tr><td>Lane A</td><td>533</td><td>Dengue NS1 (lane A)</td><td>1 (0.2%)</td></tr>
<tr><td>Lane B</td><td>533</td><td>P. falciparum (lane B)</td><td>86 (16.1%)</td></tr>
<tr><td>Lane C</td><td>533</td><td>Anti-CHIK IgM (lane C)</td><td>27 (5.1%)</td></tr>
<tr><td>Lane D</td><td>533</td><td>Anti-CHIK IgG (lane C)</td><td>137 (25.7%)</td></tr>
</table>

Confounding interpretation of the anti-CHIKV antibody results was the presence of multiple stripes in Lane C (Fig. 3, left), designated for detection of anti-CHIKV IgG and IgM. Non-relevant stripes in lane C were present in almost half (71 of 147) of the capillary blood samples positive for either antibody. If all samples reporting non-relevant bands in Lane C are removed from calculations of anti-CHIKV IgG and IgM positives, the values of prevalence are 23% (106/462) and 2.3% (12/462), respectively (down from 25.7% and 5.1%).

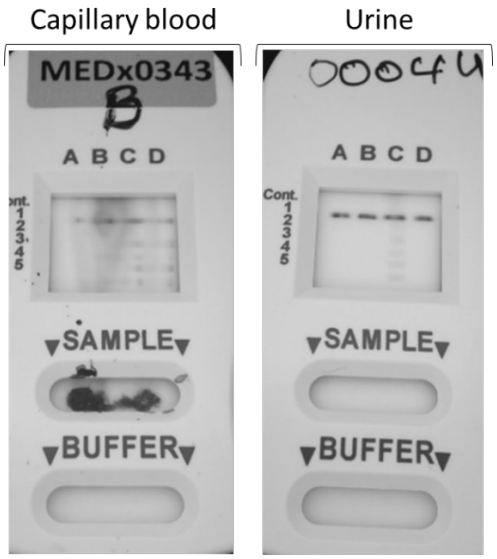


Fig. 3—Images of MEDx tests of capillary blood (left) and urine samples (right) showing multiple non-specific stripes in Lane C. The test for MEDx0343(blood), left, also shows stripes from Lane C bleeding over into other lanes.

In the subset of blood samples without additional stripes in Lane C (n = 462 samples), four samples were positive for anti-CHIKV IgM alone. The presence of IgM alone is generally indicative of a current or very recent infection, whereas the presence of IgG (+/- IgM) is indicative of a previous infection. The high numbers of IgG observed here (106/462) may well be due to a previous infection with CHIKV or a similar alphavirus in previous years. We previously observed large numbers of participants within the MHRL catchment area who were anti-CHIKV IgM-positive, indicating a potential outbreak in 2012–2013 (12, 15); the prevalence of anti-CHIKV IgG may be reflective of persons exposed during 2012–2013. Anti-CHIKV-specific antibodies may persist in affected patients over an extended period (25, 26).

Non-specific binding was also particularly pronounced in urine samples (Fig. 3, right), where 237 samples showed stripes in at least one non-specific Lane C locus (45%); this non-specific binding may also have confounded interpretation of results for BURK and *P. falciparum* by automated analysis, as described in Section 3.2.2.

3.2.2 Remote, automated analysis of MEDx LFIs

DTRA provided MHRL with several automated Deki readers to image the MEDx tests, quantify the test lines, and provide qualitative output based on pre-determined thresholds calculated from the line-specific and localized signals (17). Data were then uploaded to a Fio-hosted cloud-based database where the images and qualitative results could be retrieved by approved users; spreadsheets with sample information and individual positive/negative calls for each lane could also be generated. The ultimate goal of having a remote, real-time diagnostic capability is to provide more timely and effective therapeutic decisions.

Throughout the project's period of performance, we encountered problems with uploading data from the readers. Data from only 430 of the 526 urine MEDx tests (81.7%) and 440 of the 533 blood MEDx tests (82.6%) were found on the Fio website. Failure to accrue data was tracked over time (Fig. 4) and, for several months, could be traced to use of one or two specific readers (Panel B); amounts of data lost from reader #FH21112 were particularly high during the project's first four months. While MHRL personnel

were eventually able to update the malfunctioning instrument software and restore activity to the readers when required, any data previously sent was never recovered.

The ability to view images of MEDx tests on the Fio website was also severely compromised (Fig. 4, panels C and D). Images could be viewed for only 666 of the 870 tests (76.5%) for which data were available on the Fio website. As with the data uploads, the imaging problems were periodic, with over half of the images unavailable for viewing during May and June 2018. Therefore, information available in the Fio database represents only a portion of the data actually generated.

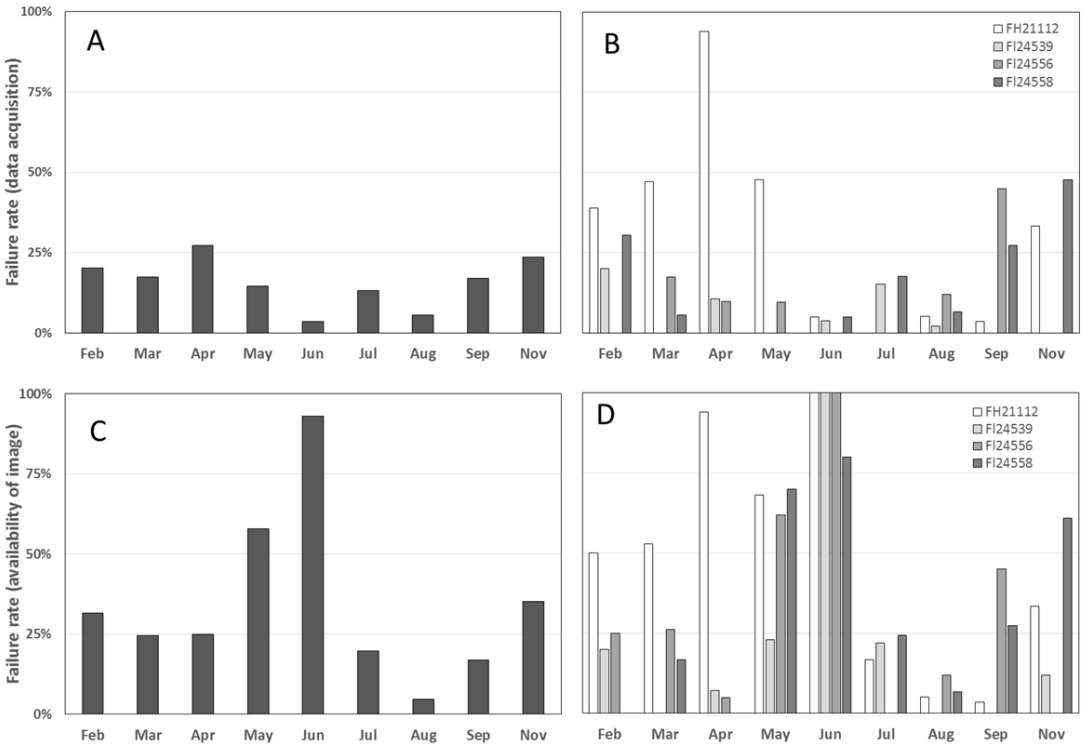


Fig. 4—Monthly failure rates for data transmission from Deki readers to the Fio-hosted website (panels A and B) and availability of images to view/download (panels C and D). Panels B and D show failure rates for individual readers.

A promising aspect of using the Deki readers was the ability to provide an automated positive/negative result for five targets for each sample. Of the 428 urine samples for which Deki results were available, device-based calls were made for 412 (96%). The number of device-based calls was much lower for blood tests (295 calls/435 samples, 67.8%). Table 4 compares results obtained by visual inspection of the MEDx platform and the automated data analysis.

Table 4—Comparison of Positive/Negative Calls Made by Visual Inspection and Automated Analysis

		On-site Visual Inspection			Concordance
		Positive	Negative	Total	
DENV (locus A1)					
Automated data calling (Fio)	Positive	1[a]	2[a,b]	3	99.3%
	Negative	0	289	289	
	Total	1	291	292	
Malaria (locus B3)					
Automated data calling (Fio)	Positive	39	7[c]	46	94.9%
	Negative	8[d]	239	247	
	Total	47	246	293	
BURK (urine, locus B5)					
Automated data calling (Fio)	Positive	0	55[e]	55	86.8%
	Negative	0	362	362	
	Total	0	417	417	
Anti-CHIKV IgM (locus C2)					
Automated data calling (Fio)	Positive	5	8[f]	13	93.5%
	Negative	10[g]	271	281	
	Total	15	280	295	
Anti-CHIKV IgG (locus C4)					
Automated data calling (Fio)	Positive	62	45[h]	107	82.3%
	Negative	7[i]	180	187	
	Total	69	225	294	

[a]None of these samples were positive for NS1 via the Dengue Duo RDT.
[b]Images for MEDx0091 and MEDx0329 were clearly positive in locus A1, but were entered as negative on the paper result form and into the Deki reader.
[c]MEDx0036 was listed as being positive in locus B2 and four other samples were listed as being positive for loci C2, C3, C4, and/or C5. MEDx0063 was clearly positive from its available image, and was marked positive on the Deki, but was marked as negative on the sample result sheet.
[d]MEDx0062 was marked on its results sheet as being positive for malaria, but was entered into the Deki reader as negative by eye; the image for this sample does not show any line in locus B3. The remainder of samples showed smears or very faint (but positive) spots in locus B3.
[e]Of the 16 samples for which images were available, all 16 showed smears or multiple lines in Lane C.
[f]Four samples were marked positive at locus C4 (but not at locus C2); MEDx0312 and MEDx0428 were marked positive for locus B3.
[g]Six samples were marked positive for multiple bands in lane C, but were entered in the Deki as negative for locus C2. Images for MEDx0108 and MEDx0253 showed faint bands or smears in lane C.
[h]On the hardcopy results sheets, users called 17 samples positive for C3, one positive for C2, one positive for D4, one positive for B4, and two positive for B3.
[i]All samples except MEDx0086 were positive for multiple lines in Lane C.

In all, manual inspection and the automated positive/negative calls agreed 91% of the time. Contrasting calls likely arose from a combination of non-specific binding in Lane C, poor positioning of the nitrocellulose substrate, imprecise sample flow, misreading of the test window by on-site technicians, and the thresholds used for positive/negative discrimination by the Deki unit. These disparate calls were most apparent with BURK assays in urine and anti-CHIKV IgG assays in blood.

In 52 of the 55 urine samples called positive for BURK (locus B5) by the Deki, a series of positive stripes in neighboring Lane C were detected, suggesting that anomalous migration of these stripes may have been responsible for the discrepancy between Deki and user calls for BURK. Figure 5 shows three examples of urine tests called positive for BURK where entire lanes appear to have shifted from their locations within the test window; all three were called valid by the users, as control stripes were indeed observed.

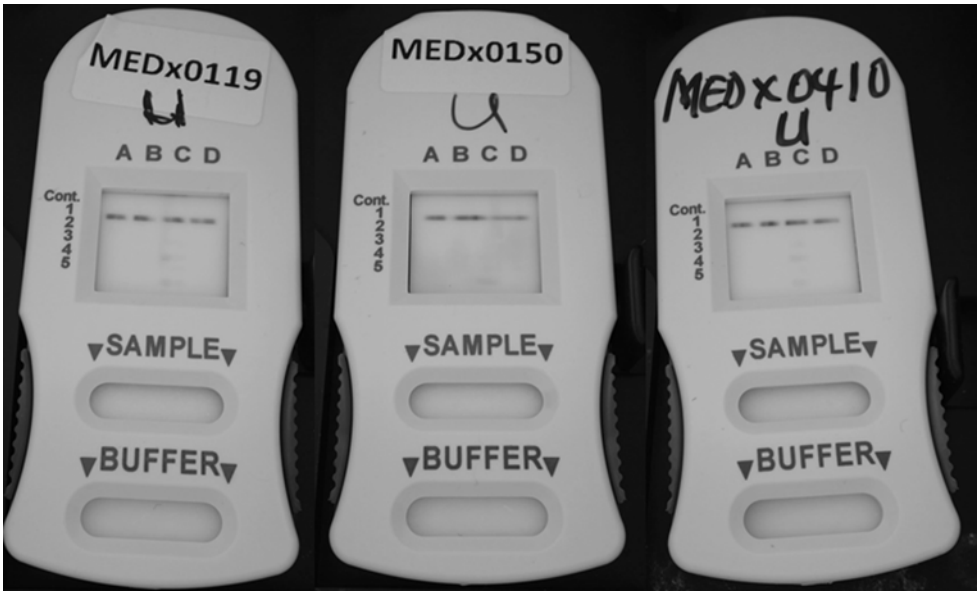


Fig. 5—Examples of MEDx urine tests called positive for BURK by Deki reader, where lanes have shifted within the test window.

The close spacing between stripes 1–5 may also have proven problematic by limiting the users' ability to discriminate between relevant and non-relevant loci, especially in Lane C. Of the 45 blood samples called positive for anti-CHIKV IgG by Deki but negative by users, approximately half were designated as positive for other loci in close proximity to the IgG stripe (22/45; Table 4). A similar situation was observed with anti-CHIKV IgM where 6 of the 8 samples deemed negative by users but positive by Deki had nearby loci designated as positive by the users.

In spite of these shortcomings, the reach-back capability engendered by the Deki readers proved critical in allowing us to assess the data's accuracy. For example, we identified over 35 samples for which sample IDs or reader numbers were incorrectly recorded by the on-site technicians. Perhaps more importantly, it allowed us to document issues associated with test performance, such as smears, lane shifts, missed-calls, and the presence of multiple stripes in Lane C.

3.2.3 COTS lateral flow tests

Microscopy of Giemsa-stained thick and thin blood smears has long been considered as the gold standard in malaria detection, as it is relatively sensitive, quantitative, and allows for species identification. However, many resource-poor environments lack widespread expertise in microscropy and

high quality equipment. For this reason, the World Health Organization (WHO) has recommended the use of RDTs for diagnosis of malaria in these settings (27) and is assessing their use for other diseases (28). Here, we used COTS RDTs as point-of-care complementary assays to determine the potential utility of the MEDx custom diagnostics.

Table 5 shows the numbers of samples analyzed using COTS lateral flow tests and the numbers of positive samples. In most cases, MHRL personnel were careful to write down the overall results of each test. However in some cases, the control line results were not recorded on the written sheets. In this instance, the availability of an image—such as that provided by the Deki readers for the MEDx tests—would have been of tremendous benefit.

Table 5—Standard of Care, Complementary COTS Tests Performed

Complementary Test	# Samples Tested	# Positive (%)	
SD Bioline Dengue Duo	532 (Ig test) 528 (Ag test)	Anti-DENV IgM	2 (0.6%)
		Anti-DENV IgG	14 (2.6%)
		NS1 antigen	0 (0%)
SD Bioline Pf/Pan malaria	532	Pf	132 (24.9)
		Pan	30 (5.6%)
Microscopy (malaria)	530	Parasite-positive	68 (12.8%)
SD Bioline anti-Chikungunya IgM	527	Anti-CHIKV IgM	2 (1%)

A small number of samples were positive for the presence of anti-DENV antibodies or NS1 antigen, in agreement with previous observations (15) and with the MEDx test results for NS1. As previously observed (15), most of the dengue-positive subjects were positive for the presence of anti-DENV IgG, but not anti-DENV IgM or NS1 antigen, though one participant was positive for both IgG and IgM.

Twenty-five percent of the tested participants were positive for malaria, as indicated by results on the Pf/Pan test; all samples testing positive for *Plasmodium* spp. on the SD Bioline test were also positive on the *P. falciparum* line. Microscopy of thick and thin smears identified only about half the number of malaria-positive subjects as the COTS tests.

In general agreement with MEDx results, only a small proportion of samples were positive for anti-CHIKV IgM with the COTS test. Using subject populations with similar recruitment criteria, we and others have previously observed prevalence of anti-CHIKV IgM approaching 40% using the same COTS RDT (12, 15, 29).

3.2.4 Concordance between antibody-based tests

Concordance between COTS and custom MEDx RDTs was determined (Table 6). Of the 500+ samples analyzed by both methods, a high concordance between tests for CHIKV IgM and DENV NS1 was observed, but is primarily due to the large number of samples negative by both tests. There was no overlap in positive determinations for these targets between the commercial and custom tests. Two of the samples positive for anti-CHIKV IgM on the COTS RDT also gave rise to positive results in MEDx tests for anti-CHIKV IgG, but not anti-CHIKV IgM. The cause of this discrepancy remains unclear.

Table 6—Concordance between MEDx RDTs and Commercial RDTs

		MEDx			Overall concordance
		Positive	**Negative**	**Total**	
SD Bioline Dengue Duo (NS1 test)	Positive	0	0	0	99.8%
	Negative	1	531	532	
	Total	1	531	532	
SD Bioline Pf/Pan Malaria	Positive	73	57	132	86.3%
	Negative	13	387	401	
	Total	86	447	533	
SD Bioline anti-CHIKV IgM	Positive	0	2	2	94.7%
	Negative	26	498	524	
	Total	26	500	526	

The overall concordance for malaria was lower between the MEDx and COTS RDTs. The COTS test was significantly more sensitive than either MEDx or smears ($p<0.001$); one-thirds of the samples positive for *P. falciparum* by the COTS test were not detected by MEDx, and only about half were detected by smears. Figure 6 shows a Venn diagram illustrating the overlap of samples deemed positive in MEDx, COTs RDT, and thick smears.

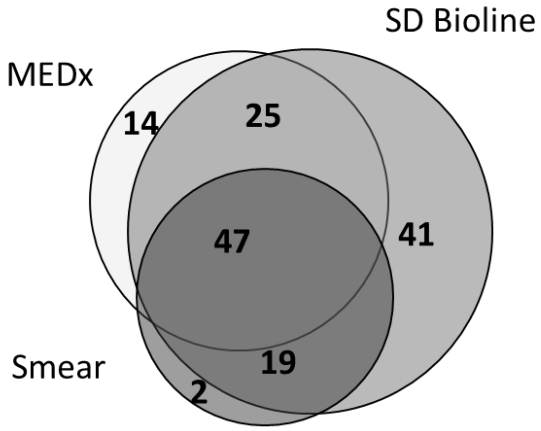


Fig. 6—Venn diagram—Overlap of malaria-positives in RDTs and thick smears. In total, 530 samples were tested with all three methods.

3.3 Nucleic acid-based tests

3.3.1 Overview of PCR results

MHRL personnel performed real-time PCR (rtPCR) for all four targeted pathogens on nucleic acid samples extracted from whole blood (DNA) or serum (RNA) samples. In a small subset of samples (n = 40), BURK-specific PCR was also performed on DNA extracted from urine.

In general, RNase P controls for nucleic acid extractions worked well in both DNA- and RNA-based assays; the PPIA extraction control worked similarly well (100% positive) in the 144 samples analyzed for DENV and CHIKV using the Zika Virus Triplex Kit. For each assay, target-specific positive controls were also analyzed with each batch of PCRs to ensure that the assays were performed correctly and that no reagents had degraded. Although positive controls for DNA targets (BURK, malaria) worked consistently, RNA controls did degrade over time and therefore required both re-optimization and frequent replacement.

For each PCR performed, on-site personnel provided a rapid assessment of the assays' results and also saved the QuantStudio data files in a cloud-based repository for detailed retrospective analysis by NRL personnel. Final designation of any sample as positive or negative was based on amplification curve analysis. Designation as a positive result required both a Ct < 35 and a logarithmic increase in signal with time. Samples with Ct values greater than 35 or irregular curves that crossed the threshold were regarded as negative.

Independent of how the data were analyzed, no positive results were observed with DENV, BURK, and CHIKV PCRs (Table 7). These results are consistent with our previous observations of low prevalence for dengue, chikungunya, and melioidosis within the Mercy Hospital catchment population, as measured by PCR (17). Furthermore, retrospective BLAST searches of the CHIKV primer and probe sequences indicated that the West African genotype of CHIKV is not detected by the PCR used here. Primer and probe sequences for the COTS PCR for CHIKV, DENV, and Zika were not available.

Table 7—Results from Complementary PCRs

Test	**# Samples Run**	**Samples Deemed Positive**	
DENV RT-PCR	219 (219 valid)	DENV1/3/4	0
NAMRU-6 PCR (v1)		DENV2	0
	138 (131 valid)	DENV1/2/3/4	0
NAMRU-6 PCR (v2)	143 (140 valid)	DENV 1-4	0
Zika Triplex PCR			
MMSR (malaria)	533 (526 valid) [1]	On-site user (by eye)	248 (46.5%)
		Retrospective analysis (n = 526)	
		P. falciparum	256 (48.6%)
		P. vivax	0
		Universal *Plasmodium*	204 (38.6%)
BURK PCR	Urine (n = 40)	Urine	0
	Blood (n =485)	Blood	0[1]
CHIKV RT-PCR			
NAMRU-6 PCR	390 (380 valid)[2]	CHIKV1	0
		CHIKV2	0
Zika Triplex PCR	143 (140 valid)[3]	CHIKV (generic)	0

[1]Samples were deemed valid if both RNase P control (extraction control) and target-specific positive control were positive. Exceptions noted in (2) and (3), below

[2]239 samples were run without any control for CHIKV1. One hundred fifty-one samples were run with appropriate controls for CHIKV1 and CHIKV2; the CHIKV1 positive controls worked in all 151 samples tested, while CHIKV2 positive controls worked in 114 of these same samples

[3]The previously obtained CHIKV positive controls did not work with the Zika Triplex primers.

Malaria was detected in nearly half of the samples tests. As previously observed (15), malaria prevalence roughly tracked with the rainy season (Fig. 7), but was poorly correlated with monthly rainfall ($r^2 = 0.129$; (30)).

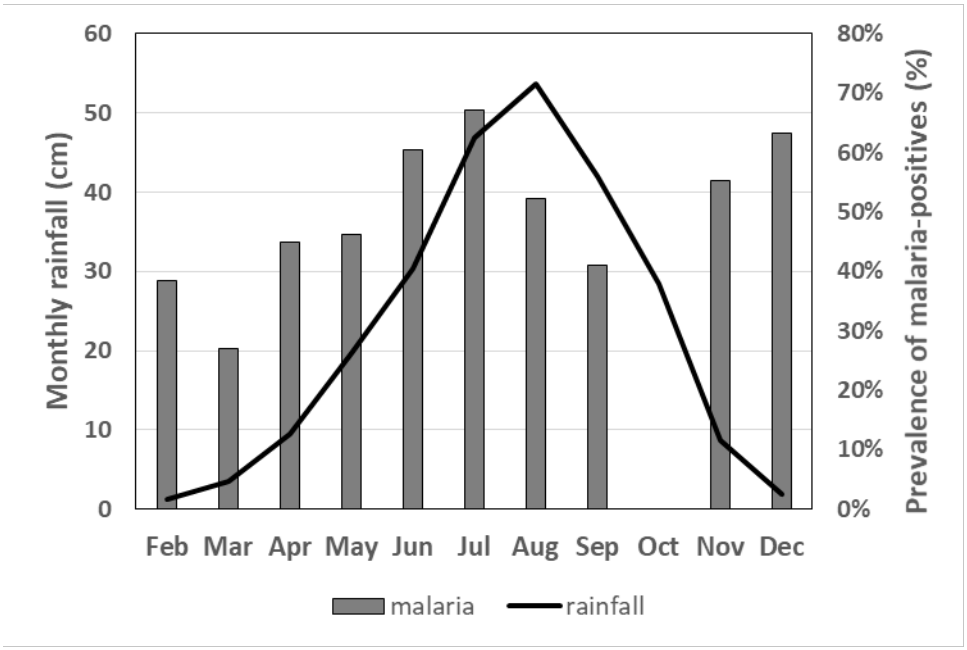


Fig. 7—Prevalence of malaria-positives and monthly rainfall

As expected, and as previously observed by other groups (31, 32), PCR detected a significantly greater number of samples with malaria-causing parasites than by smears or by either of the RDTs ($p<0.001$, Table 8); the presence of *any* malaria-specific amplicon or marker was counted as a positive. In spite of the higher sensitivity of PCR, a small subset of samples (n = 14) was deemed positive by one of the two RDTs, but not by PCR (Fig. 8). These samples may represent false-positives by the RDTs or false-negatives by PCR. In the former situation, these samples may harbor cross-reactive proteins from underlying health conditions (33–35) or other infectious diseases (35), or may contain circulating malarial antigens that persist even after elimination of parasites (36). The latter situation may arise (PCR false negative) if malaria parasites harbor mutations in the primer binding sites, resulting in poor amplification; three of these samples amplified the *P. falciparum* marker but were not identified as positive due to a high Ct value (>35). Table 8 also shows concordances between COTS LFIs (where available), MEDx tests, and PCRs for BURK, anti-CHIKV IgM, and DENV.

Table 8—Concordance between PCR, MEDx RDT, and COTS RDT for DENV, BURK, and Malaria

		PCR (Overall Result)			Overall Concordance	More Sensitive Method
		Positive	Negative	Total		
MEDx RDT (DENV NS1)	Positive	0	1	1	99.8%	nd*
	Negative	0	526	526		
	Total	0	527	527		
Dengue Duo (NS1)	Positive	0	1	1	99.8%	nd
	Negative	0	517	517		
	total	0	518	518		
MEDx RDT (BURK - urine)	Positive	0	0	0	99.6%	PCR (p>0.25)
	Negative	2	462	464		
	total	2	462	464		
SD Bioline Pf/Pan malaria RDT	Positive	118	12	130	70.3%	PCR (p<0.001)
	Negative	144	249	393		
	total	262	261	522		
MEDx RDT (malaria)	Positive	85	3	88	65.7%	PCR (p<0.001)
	Negative	177	258	434		
	total	262	261	522		

*could not be determined

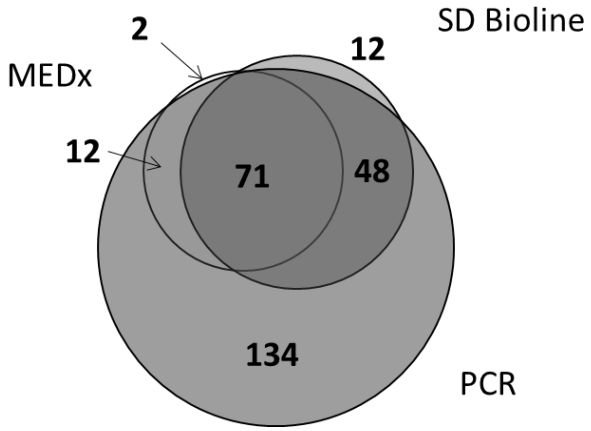


Fig. 8—Venn diagram—overlap of malaria-positives by MMSR, MEDx, and SD Bioline COTS RDT

3.3.2 Differentiation of Plasmodium species by MMSR

Although RDTs are effective, rapid, and user-friendly methods for diagnosis of malaria, some—such as both the COTS RDT and the MEDx platform used here—have been optimized for detection of *P. falciparum* and may often not detect other *Plasmodium* spp. parasites (37). This was apparent in the COTS RDT, as all samples positive for the "Pan" stripe (*Plasmodium* spp.) on the commercial RDT were also positive for *P. falciparum*. The ability to detect other *Plasmodium* spp. is a potential advantage of the MMSR test. This multiplex test includes a set of primers/probes specific for *P. vivax* and another set

targeting an 18S rRNA sequence highly conserved amongst *Plasmodium* spp. (38). No sensitivity or specificity information of the pan-*Plasmodium* primer/probe set in the MMSR assay has been published.

None of the 526 samples with valid MMSR results were positive for the presence of *P. vivax*. This result was unsurprising, as WHO has documented low prevalence of *P. vivax* in West Africa (23). Although many samples tested positive for both *Plasmodium* spp. and *P. falciparum*, eight samples were positive for *Plasmodium* spp. but negative for both *P. falciparum* and *P. vivax* (Fig. 9). Concordance between MMSR and the two RDTs for both the species-specific and pan-*Plasmodium* markers was poor for both RDTs (Table 9).

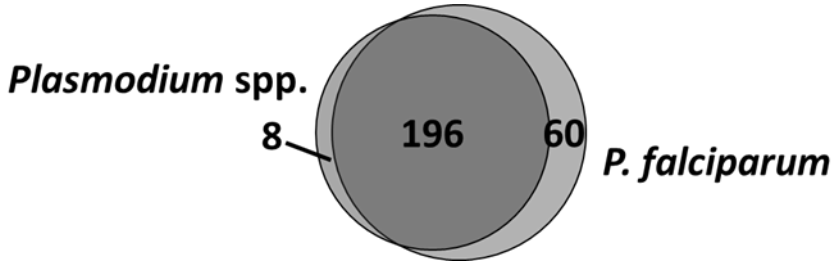


Fig. 9—Venn diagram—Overlap in samples positive for *P. falciparum* and *Plasmodium* spp. in MMSR assay

Table 9—Concordance between MMSR, MEDx, and COTS RDTs for Detection of Species-specific and Pan-*Plasmodium* Markers

		MMSR (*P. Falciparum*)			**Overall Concordance**
		Positive	**Negative**	**Total**	
SD Bioline Pf/Pan RDT—*P. falciparum* line	Positive	118	12	130	71.4%
	Negative	136	256	394	
	total	254	268	524	
MEDx RDT (*P. falciparum*)	Positive	84	4	88	66.8%
	Negative	170	264	436	
	total	254	268	524	
		MMSR (*Plasmodium* spp.)			**Overall Concordance**
		Positive	**Negative**	**Total**	
SD Bioline Pf/Pan RDT—*Plasmodium* spp. line	Positive	28	0	28	66.6%
	Negative	174	321	496	
	total	202	321	524	

3.3.3 Plasmodium speciation by a panel of single-plex PCRs

In addition to the eight *Plasmodium* spp.-positive / *P. falciparum*- and *P. vivax* negative samples, eight other samples were identified that did not meet the criteria for positive detection, but that demonstrated weak logarithmic amplification in the *Plasmodium* spp.-specific assay only. 15 of the 16 samples were subjected to a panel of real-time PCR amplifications to determine the species of parasite (39). This panel of PCRs targets 18S rRNA sequences common to all *Plasmodium* species, *dhfr* sequences specific to *P. vivax*, *P. malariae*, and *P. ovale*, and species-specific repetitive sequences specific to *P. falciparum* and *P. knowlesi*.

Of these 15 samples tested with the PCR species identification panel, amplicons with the correct melt temperature for the *Plasmodium* spp. target were detected in six samples, for *P. falciparum* in five samples, for *P. malariae* in two samples, for *P. ovale* in two samples (Table 10). Sequencing of the pan-*Plasmodium* amplicons from two samples (MEDx0041 and MEDx0209) confirmed the identity predicted by the individual species-specific assays; the remaining samples did not generate sufficient amplicon to allow sequencing.

The value of the MMSR assay was demonstrated in the ability to identify non-*falciparum* malaria. However, five of the samples tested in the speciation assays generated amplicons indicating the presence of *P. falciparum*. As these same samples were previously declared negative for *P. falciparum* by MMSR, it is clear that the species identification assays are more sensitive. It is unclear how many more of the 263 samples deemed negative for all three *Plasmodium* targets in the MMSR assay were actually *P. falciparum* false-negatives.

Table 10—Speciation of *Plasmodium* spp.-Positive/*P. falciparum*- and *P. vivax*-Negative Samples

Sample no.	MMSR Results (*Plasmodium* spp. assay)	Real-time PCR Results						
		Plasmodium spp.	Species ID (*Plasmodium* spp. amplicon)	*P. falciparum*	*P. vivax*	*P. malariae*	*P. ovale*	*P. knowlesi*
MEDx0039	- (weak)	-	nd*		-	-		-
MEDx0040	+	+	nd	-	-	+	-	-
MEDx0041	+	+	*P. malariae*	-	-	+	-	-
MEDx0052	+	+	nd	-	-	-	+	-
MEDx0248	- (weak)	-	nd	-		-	-	-
MEDx0260	- (weak)	-	nd	-	-	-	-	-
MEDx0284	- (weak)	-	nd	-	-	-	-	-
MEDx0288	- (weak)	-	nd	+	-	-	-	-
MEDx0290	+	+	*P. ovale*	-	-	-	+	-
MEDx0360	- (weak)	+	nd	-	-	-	-	-
MEDx0378	+	-	nd	+	-	-	-	-
MEDx0379	- (weak)	-	nd	+	-	-	-	-
MEDx0404	- (weak)	-	nd	-	-	-	-	-
MEDx0409	+	-	nd	+	-	-	-	-
MEDx0411	+	+	nd	+	-	-	-	-

*not done

4. CONCLUSIONS

Here we determined operating parameters of the MEDx platform, its optical reader, and other diagnostic assays for use in resource-limited environments. Although the antibody-based lateral flow assays were less sensitive than PCR-based assays, the presence of antibodies directed against CHIKV and DENV—detectable in antibody-, but not nucleic acid-based platforms—suggests previous infections with these pathogens. These results also illustrate a possible limitation of many nucleic acid-based methods—namely, the inability to diagnose disease when the patient is not highly viremic or bacteremic.

The MEDx platform had significant issues with both sensitivity and specificity. A large number of samples had multiple cross-reactive stripes in Lane C, which unfortunately, included approximately half of the samples deemed positive for anti-CHIKV antibodies. This artifact confounds our ability to make accurate conclusions about the numbers of samples positive for anti-CHIKV antibodies. Furthermore, sensitivity of the MEDx test for malaria was significantly lower than that of the commercial malaria test.

Transmission and automated analysis of MEDx results were also troublesome. Deki readers were unable to make a positive/negative determination in almost a third of the blood samples tested, although automated calls were made on most urine samples. Furthermore, of the >500 urine samples for which automated calls were made, none of the 55 samples called positive for BURK by the Deki reader were also deemed positive by the on-site user. While images uploaded to the Fio website did prove valuable in detecting errors, the availability of these images was spotty at best, and in many cases, neither images nor result data were successfully transmitted and available on the website.

In general, results from complementary tests were in agreement with our previous observations (15, 17). Malaria was detected in almost 50% of the participant pool by PCR and in approximately 25% by COTS RDT. PCRs indicated a low prevalence of active CHIKV, DENV, and BURK infections; dengue results were confirmed by RDT. Interestingly, prevalence of anti-CHIKV IgM was much lower than observed in 2013 (12), but the prevalence of anti-CHIKV IgG determined by the MEDx test—if valid—is consistent with values expected several years after the suspected 2013 outbreak.

Overall, the MEDx validation data obtained here demonstrate that additional improvements are required to attain similar sensitivities and specificities of commercially available platforms. This information will drive further development and deployment of diagnostic tests and instruments capable of operation remote, austere locations. The current study also provided timely epidemiological data from febrile individuals, improving situational awareness and mitigating the potential for unexpected outbreaks of infectious diseases.

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