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Review Article

Reducing Uncertainty for Acute Febrile Illness in Resource-Limited Settings: The Current Diagnostic Landscape

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Abstract. Diagnosing the cause of acute febrile illness in resource-limited settings is important—to give the correct antimicrobials to patients who need them, to prevent unnecessary antimicrobial use, to detect emerging infectious diseases early, and to guide vaccine deployment. A variety of approaches are yielding more rapid and accurate tests that can detect more pathogens in a wider variety of settings. After decades of slow progress in diagnostics for acute febrile illness in resource-limited settings, a wave of converging advancements will enable clinicians in resource-limited settings to reduce uncertainty for the diagnosis of acute febrile illness.

FINDING THE CORRECT DIAGNOSIS FOR ACUTE FEBRILE ILLNESS MATTERS

Fever is the most common symptom reported by persons seeking medical care in resource-limited settings.[1,2] There is no widely accepted, universal definition for acute febrile illness.[3] Abrupt-onset illnesses, lasting less than 2 weeks, with symptoms that include fever encompass a wide range of diseases including respiratory tract infections, diarrheal disease, malaria, and other vector-borne diseases which combined cause one-third of all morbidity and one-fourth of all mortality in low-income countries.[4,5] Rigorous acute febrile surveillance studies in resource-limited settings identify the etiology of acute febrile illness in 37–97% of cases, but rely on testing performed in capitals or foreign countries far removed from the majority of patients.[6–13] Without access to diagnostic tools, providers in resource-limited settings turn to syndrome-based treatment protocols. World Health Organization (WHO) syndrome-based guidelines often advise antimicrobials that prove ineffective for patients with acute febrile illness, even when effective ones are widely available in resource-limited settings if the correct diagnosis can be determined.[14] Reliance on such guidelines also results in over-prescription of antimicrobials to patients who do not need them.[15]

Despite concerns of worsening antimicrobial resistance, more patients perish worldwide from lack of access to antimicrobials than die of antimicrobial resistance.[16] As rickettsial illnesses do not respond to typical empiric antibiotic regimens, patients with rickettsial infections suffer higher mortality and complications when they are not treated with widely available tetracyclines.[17,18] Zoonotic bacterial infections that do not respond to standard antibiotics are common. *Rickettsia* or *Coxiella burnetii* were found in 13% of acute febrile illness cases in Tanzania. Rickettsial infections outnumbered conventional bacterial infections in central nervous system infections in Laos.[19,20] In Uganda, one quarter of human immunodeficiency virus (HIV)-infected patients with severe sepsis had mycobacteremia, a grave condition treatable with available targeted therapy, but frequently fatal with delayed diagnosis.[21]

Health-care providers in resource-limited settings prescribe antibiotics for 32–74% of patients presenting with fever amounting to antibiotic prescription for 22–73% of all health-care encounters.[22–26] Patients in resource-limited settings often receive unnecessary combinations of antimicrobials, which are continued for longer than necessary.[27] Lack of access to diagnostics drives inappropriate antimicrobial use.[28,29] Rollout of rapid diagnostic tests with accompanied training can reduce antimalarial use in patients who test negative, and, by proving an alternative diagnosis, has reduced antibacterial use in patients who test positive.[24,25,30–32] However, testing for malaria alone is insufficient. In some cases of malaria rapid diagnostic test deployment, overall antibacterial use rises as fewer patients are assigned a diagnosis of malaria.[30] Despite changing WHO guidelines to give antimalarials only to patients with confirmed malaria, 35–58% of smear or rapid diagnostic test negative patients receive antimalarials in certain settings in an environment of diagnostic uncertainty.[33–36] In resource-limited settings, up to 90% of patient encounters are with informal health-care providers[37]; patients and their families can purchase antibiotics without consulting a medical professional.[38] Out-of-pocket purchase of antibiotics is associated with more antimicrobial resistance.[39] Antimicrobial overuse is a driving factor behind the global surge in antimicrobial resistance.[40,41] Rising antimicrobial resistance in resource-limited settings affects antimicrobial effectiveness everywhere. Antimicrobial resistance genes that have emerged in India, the world's largest consumer of antimicrobials, are now the most common cause of resistance in Enterobacteriaceae infections in the United States.[42,43]

Emerging and reemerging infections are a constant threat to human health.[44] Public health measures may combat outbreaks of emerging infectious diseases, but only if they are detected. As a nonspecific acute febrile illness, Zika virus may have been circulating in Brazil for more than 1 year before its detection.[45] In rural Guinea, faced with poor diagnostic and laboratory infrastructure, 79 people died over 3 months before samples were sent to reference laboratories in France and Germany, which identified Ebola as the cause of a mysterious acute febrile illness characterized by diarrhea.[46,47] Once an outbreak such as Ebola is recognized, models show that earlier case detection using rapid

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diagnostics would reduce disease transmission and outbreak severity.[48,49] The capability to diagnose a broader array of infectious diseases in resource-limited settings where outbreaks emerge may allow for earlier recognition of public health threats and mobilization of a public health response before outbreaks become emergencies.

As vaccines for dengue, Ebola, and other infectious diseases become available, effective diagnostic tools will be essential to demonstrate vaccine efficacy and to provide surveillance for outbreaks to most efficiently guide vaccine deployment. Clever use of cluster-randomized ring vaccination, inspired by the success of a similar strategy used for smallpox eradication, generated preliminary data showing Ebola vaccine efficacy, a study design that critically required accurate case ascertainment.[50] In regions where malaria incidence is declining, more sensitive diagnostics are required to identify cases of submicroscopic malaria, a key barrier to elimination.[51]

TRADITIONAL MICROBIOLOGY TECHNIQUES REINVENT THEMSELVES

Culture and serologic detection modalities have undergone relatively little advancement over the decades since their discoveries. Bacterial culture, identification, and drug susceptibility testing require little capital, but complex procedures and incubators requiring reliable electricity limit the use of culture outside large referral hospitals in resource-limited settings. A low-tech approach using a phase-change material has been developed, which provides warmth for culture without a machine or electricity.[52] Multiple approaches to miniaturize, automate, and integrate bacterial culture and antimicrobial susceptibility testing may promise rapid results in a format that does not require a traditional bacteriology laboratory (Table 1). The Sensititre MYCOTB MIC Plate (Thermo Fisher Scientific, Waltham, MA) is preconfigured with lyophilized antibiotics; it can perform drug susceptibility testing for *Mycobacterium tuberculosis*, diagnosing drug-resistant tuberculosis faster than conventional methods.[63] QuantaMatrix (Seoul, Korea) has commercialized an automated single-cell analysis platform to yield antimicrobial susceptibility test results within 4 hours.[62] In early development, the 1 cm × 1 cm ePetri on-chip microscopy platform incorporates culture media directly over an image sensor to accurately count microcolony growth under 6 hours.[59] Other bacterial culture techniques in early stages of development use hundreds of nanoliter-scale droplets that may dramatically reduce the size of a microbiology laboratory.[60,61]

Instead of relying on variable visual inspection or a cumbersome microplate reader for enzyme-linked immunosorbent assay, technology has been developed to use smartphones to accurately read the results of serologic tests.[64,65] As with nucleic acid amplification test (NAAT), the ability to diagnose multiple infections allows for more cost-effective and streamlined diagnosis of acute febrile illness. Multiplex point-of-care immunoassays for HIV and syphilis as well as mosquito-borne diseases have been tested, and are commercially available for HIV and syphilis.[66–69] Using bacteriophage display libraries, epitopes for thousands of viral strains have been generated to detect exposure to almost every virus.[58] Diagnosis of neglected tropical diseases such as human African trypanosomiasis

TABLE 1
New approaches to traditional microbiology laboratory practices for diagnosing acute febrile illness

Traditional technique	New approach	Benefit over traditional technique					Performance against ref test*			Evaluated in RLS	Ref
		Less hands-on time	Smaller/portable	Faster result	Other	Reference test	Sensitivity (%)	Specificity (%)		
Parasite blood smear	Mobile phone microscopy	✓	✓			Filaria blood smear	100	94	Yes	53,54
	Transdermal hemozoin detection	✓			Needle-free	Malaria smear	100†	100†	No	55,56
ELISA and agglutination tests	Lateral flow assays	✓	✓	✓	Instrument-free, multiplex possible	Trypanosomiasis card agglutination test	89	95	Yes	57
	Viral phage display library				Highly multiplex	HIV ELISA	95	100	No	58
Bacterial culture and identification	Culture with on-chip microscopy	✓	✓	✓		Not reported in clinical samples			No	59
	Nanoscale droplet culture	✓				Not reported in clinical samples			No	60,61
Antimicrobial susceptibility testing	Single cell analysis	✓		✓		Standard bacterial culture	92% agreement		No	62
	Microtiter plates	✓	✓	✓		TB drug susceptibility on Middlebrook agar	≥ 92% agreement		Yes	63

ELISA = enzyme-linked immunosorbent assay; Ref = reference; RLS = resource-limited setting; TB = tuberculosis.
*For tests that can identify more than one pathogen, one reference pathogen was selected.
†Test described in only one patient with malaria and four without.

TABLE 2

Emerging nucleic acid amplification and sequencing platforms for diagnosing infectious causes of acute febrile illness

								Utility in clinical specimens												
Device	Producer	Amplification technique	Run time (minutes)	Isothermal	Portable *	Integ prep	Multiplex	Malaria	Arboviruses	Zoonotic bact	Meningitis	Resp viruses	Tuberculosis	GI pathogens	Ebola	Reference example test	Sens (%)	Spec (%)	Lowest device cost ($)	Lowest per sample cost ($)	Ref
Alere i	Alere	Nicking endonuclease amp	15	✓		✓						✓				FilmArray influenza	79	100	8,628	94	78,79
FilmArray	BioFire	Nested multiplex RT-PCR	65			✓	✓				✓	✓		✓	✓	Ebola PCR	83	97	40,000	109	80–84
Genedrive	Epistem	Multiplex RT-PCR	45–75		✓								✓			TB culture	45	98	NCA	NCA	85,86
GeneXpert	Cepheid	Heminested RT-PCR	90–120			✓	✓				✓	✓	✓	✓	✓	Ebola PCR	100	96	17,500	10	71,87–92
Multiple†	Multiple†	RT loop mediated isothermal amp	15–75	✓	✓			✓	✓	✓	✓	✓	✓	✓	✓	Malaria PCR	95	98	2,561	5	71,93–100
MinION	Oxford Nanopore	Nanopore sequencing	60‡		✓		✓		✓						✓	Data not available	–	–	1,000	600	101–104
T-8	TwistDx	Recombinase polymerase amp	15–30	✓	✓				✓	✓		✓	✓	✓	✓	Ebola PCR	97	97	5,495	7	105–110
TaqMan Array Card	Applied Biosystems	Multiple parallel singleplex RT-PCR	40–90				✓	✓	✓	✓		✓		✓	✓	PCR for AFI pathogens	88	99	–§	34	88,89,111–113
two3	Biomeme	Multiplex RT-PCR	60		✓		✓									Data not available	NA	NA	4,000¶	30¶	114

AFI = acute febrile illness; GI = gastrointestinal; Integ prep = integrated sample preparation; Amp = amplification; NA = not applicable; NCA = not commercially available; Ref = references; RT-PCR = real-time polymerase chain reaction; Sens = sensitivity; Spec = specificity; TB = tuberculosis; Tech = Technologies.

*A platform was considered portable if it has the ability to run on an integrated battery.

†There are multiple RT loop mediated isothermal amplification protocols that make use of instruments providing isothermal heating systems with RT fluorometric or turbidimetric detection.

‡Sequencing time to target identification only. The entire process including sample and library preparation may take 24 hours.

§TaqMan array cards can be run on a variety of real-time thermocyclers.

¶J. Zhang, personal communication.

and visceral leishmaniasis has traditionally required complex or unreliable serologic methods, but lateral flow tests to replace them have now been developed and commercialized.[57,70–72] Imaging technologies can replace microscopes with smartphone-based designs for the identification of malaria and filarial parasites.[53,54]

NUCLEIC ACID AMPLIFICATION TESTS ARE BECOMING EASIER TO USE

NAAT using real-time polymerase chain reaction (RT-PCR) are becoming the standard for diagnosing many infectious diseases in resource-rich settings.[73] Previously limited to reference laboratories by advanced training needs of laboratory staff, high capital costs, and high power usage, recent advances promise to push NAAT to the bedside in resource-limited settings. Isothermal nucleic acid amplification techniques such as recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP), and nucleic acid sequence-based amplification (NASBA) use alternative cocktails of enzymes to accomplish the same goal of replicating pathogen DNA or RNA for the purpose of detection without the need to quickly vary the temperature as required for PCR.[74–76] RPA can even be performed using body heat as the isothermal heat source.[77] Obviating the need for expensive thermocyclers, isothermal NAAT remove the high capital cost barrier to uptake of NAAT in resource-limited settings. Systems using LAMP, RPA, and NASBA have shown promising test characteristics in laboratory settings detecting a full range of infectious causes of acute febrile illness including Ebola, Zika, influenza, and scrub typhus (Table 2).[93,105,106,115] Reagents and an instrument to perform RPA are commercially available from twistDx (Cambridge, United Kingdom), which have worked effectively for the field diagnosis of dengue.[107] The most mature applications of LAMP use an instrument with an isothermal heat source and real-time fluorometric or turbidimetric detection, available from multiple manufacturers.[94,116] Field testing with RealAmp, a RT-LAMP protocol developed by the Centers for Disease Control and Prevention, has proved effective for detecting malaria.[94]

Most isothermal NAAT still require laborious sample preparation by skilled technicians and at times nuanced interpretation of results. Walk-away PCR platforms can perform PCR with only minimal user input and training. Performed directly by nurses or clinicians, simply by changing a self-contained cartridge in which extraction, amplification, and detection occurs, the robotic GeneXpert platform (Cepheid, Sunnyvale, CA) can detect a variety of pathogens such as tuberculosis and Ebola.[87,117,118] GeneXpert has been deployed successfully to the point-of-care at Ebola treatment centers during the recent outbreak in west Africa.[88,89] The GeneXpert has been shown to have a low tolerance for heat and humidity[119]; improvements to increase robustness and decrease maintenance requirements will allow more widespread adoption. A competing platform to GeneXpert, BioFire FilmArray (bioMérieux, Marcy l'Etoile, France), uses cartridges to simultaneously test for multiple pathogens within a syndrome, ranging from diarrhea to meningoencephalitis.[80,81] Using an isothermal technique named nicking endonuclease amplification reaction, Alere (Waltham, MA) has developed a NAAT for influenza that gives a result in 15 minutes.[78] Walk-away PCR platforms do offer simplicity, but using proprietary cartridges, only manufacturers can expand testing for additional diseases and control pricing, limiting dissemination of such systems to diagnose neglected tropical diseases in resource-limited settings. Volume discounts provide GeneXpert cartridges to resource-limited health-care systems for $10, but the instrument itself costs $17,500.[71] Costs for the BioFire FilmArray instrument and per-test consumables exceed $40,000 and $100, respectively.[82,83] The Alere isothermal instrument costs $8,628 and each kit $94.[79] Given high capital and consumable prices, inability to tailor to local needs, and high electricity demands, current walkaway PCR systems are still far out of reach for the routine diagnosis of acute febrile illness in resource-limited settings.

Several devices in development, however, can perform PCR in a miniaturized, low-cost device that can be battery powered. Cepheid is developing a portable version of its GeneXpert system, Omni.[120] BioFire has adapted its cartridge technology to develop a portable PCR unit for biodefense purposes.[121,122] Genedrive (Epistem, Manchester, United Kingdom) is a portable machine whose cartridges use a paper-based extraction system that has so far been evaluated for tuberculosis diagnosis with inconsistent results.[85,86] Biomeme (Philadelphia, PA) is developing a handheld PCR device that runs off of an iPhone, using its camera for fluorometric detection and screen for interface.[114]

In an era of global malaria decline, recognition of the increasingly vast possible etiologies for acute febrile illness in patients presenting for care in resource-limiting settings means that sequential testing for individual infectious diseases may be too slow to guide patient care and are not cost effective. Though potentially cumbersome in their current formats, increasingly multiplex PCR assays allow for the screening of a wide range of pathogens with one assay. Multiplex PCR for arboviruses include multiple offerings for a multiplex dengue, chikungunya, and Zika assay, although there is some controversy regarding the sensitivity of the Trioplex assay developed by the Centers for Disease Control and Prevention.[123–125] Taqman array cards, sold by Applied Biosystems (Foster City, CA), can be customized for any target, and can perform 26 singleplex PCR assays for infectious diseases in parallel.[111] Advances in molecular biology are allowing for increasingly multiplex detection strategies.[126] Although no single existing modality can perform sample extraction, amplification, and detection of numerous pathogens in a simple, affordable, customizable package without a heavy power requirement, advances in each individual component are paving the way for a future in which such a device will be surely available.

METAGENOMIC SEQUENCING CAN IDENTIFY ANY PATHOGEN

Despite increasingly multiplex molecular diagnostic strategies, such an approach would have been unlikely to identify Ebola in West Africa, Zika in Brazil, or Middle East respiratory syndrome coronavirus in Saudi Arabia as clinicians had no reason to suspect a disease that never in history had occurred in their respective settings of emergence or reemergence. Approaches to diagnose any infectious disease without a priori suspicion would enable surveillance

programs and clinicians to recognize emerging and reemerging infectious diseases. Metagenomic sequencing using next-generation technologies can perform unbiased sequencing on high-throughput systems at remarkably shrinking costs.[127] Use of metagenomic sequencing as a diagnostic tool has successfully identified expected and wholly unexpected existing pathogens.[128–131] Novel viruses have also been identified using metagenomic sequencing.[132,133] Although high-throughput, second-generation sequencing techniques require large machines with expensive capital and consumable costs, third-generation sequencing using nanopores by a device named MinION (Oxford Nanopore Technologies, Oxford, United Kingdom) can perform metagenomic sequencing in a package no larger than a thumb drive that can be powered by a laptop computer.[134] MinION has already identified bacteria and viruses.[134–136]

NEW MOLECULAR TECHNIQUES ARE NOT PERFECT

Even with improvements in sensitivity, many infectious diseases do not release pathogen genetic material in the bloodstream in sufficient quantities to be diagnosed by NAAT using blood or serum samples. Blood culture theoretically can detect as little as one bacterium in a large volume culture bottle. Each bacteria, however, only has one genomic copy, making it difficult for PCR to equal the sensitivity of culture. One approach to overcome a paucity of genomic bacterial DNA has been to detect transcribed RNA, which for some transcribed targets may be present many fold more than genomic DNA.[137] Other approaches increase the volume of blood collected or make use of the white blood cell fraction of blood specimens, which for intracellular bacteria may be more sensitive.[138] As metagenomic methods require the nonspecific amplification of all genomic material by PCR during the library preparation phase, organisms not detected by RT-PCR using specific primers against the target of interest are unlikely to be detected by next-generation sequencing.[139]

In resource-limited settings, one-half of patients may wait until 4 days of illness and one quarter of patients 1 week to present to health care for evaluation of acute febrile illness.[19,33,140] A broad array of diseases that cause acute febrile illness such as Zika, hantavirus, and *Rickettsia* have only a brief window of detection in which circulating genetic material is detectable in blood samples, though symptoms may continue past this window.[141–143] New approaches to use NAAT to detect a host RNA expression signature in lieu of pathogen nucleic acids may circumvent these limitations.[144]

PUT IT ON PAPER, OR ON A CHIP, AND THROW AWAY THE NEEDLE

Advancements to produce smaller, simpler, and cheaper diagnostic instruments for acute febrile illness still leave users in resource-limited settings reliant on equipment that may break, disappear, or remain locked in a closet. Introduced more than a decade ago, the WHO ASSURED criteria challenges developers to produce ideal point-of-care diagnostic tests for resource-limited settings that are Affordable, Sensitive, Specific, User friendly, Rapid and robust, Equipment-free, and Delivered.[145–147] As has been shown with HIV, cryptococcal meningitis, and malaria, disposable paper-based lateral flow diagnostics have no infrastructure requirements, require minimal training, and do not break.[148–150] Iterative improvements can be disseminated quickly and do not require capital equipment upgrades. Addition of microfluidic channels to paper-based assays allows for the production of more complicated, multiplex systems.[151,152] Separate channels enable multiplexing of serologic tests for multiple pathogens.[69] Techniques have been developed to extract, amplify, and detect nucleic acids on paper.[153–155]

Performing PCR on a chip allows for rapid sample heating and cooling enabling performance of 30 PCR cycles in 2 minutes.[156] Further miniaturization of PCR allows for fast thermocycling at lower power, paving the way for small, disposable, low-power PCR platforms. One such example in development can perform 30 cycles in under 5 minutes with a power requirement that could be met by several AA batteries.[157] Noninvasive approaches are being tested to detect nucleic acids and antigens that cause acute febrile illness in saliva and urine.[158–160] An intriguing technique may detect hemozoin, a product of malaria parasite hemoglobin digestion, using a transdermal probe that would not require any sample (Table 1).[55,56]

In an age of constantly emerging pathogens with threat of global spread, declining efficacy of overused antibiotics, and large-scale efforts to control specific infectious diseases, we can no longer accept diagnostic uncertainty for acute febrile illness in resource-limited settings. Knowing the etiology of acute febrile illness at both the individual and population level will allow for targeted treatment, judicious use of antibiotics and, ultimately, rational vaccine deployment. Accelerating technologic innovation has led to rapid breakthroughs in NAAT, improvements in traditional serologic and culture techniques, imaging devices, and next-generation sequencing as tools for infectious disease diagnosis. Diagnostic testing for acute febrile illness in resource-limited settings has the chance to leapfrog over complicated, expensive tests to simple, low-cost detection assays. Many of these novel designs are driven by resource limitations, which demand the elimination of costly equipment to move diagnostics to the bedside. This frugal innovation has the capacity to improve patient-centered care and outcomes in all settings.

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