


Advances in multiplex nucleic acid diagnostics for blood-borne pathogens: promises and pitfalls

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The large number of blood-borne viruses, bacteria and parasites currently of concern, as well as many newly emerging pathogens, presents a daunting challenge to protection of the safety of blood for transfusion and diagnosing infectious diseases. Focusing on nucleic acid diagnostic tests, multiplex devices are coming into use with many more in various developmental stages that promise to offer solutions to the clinical need. The characteristics, advantages and disadvantages of platforms in clinical use and at the research and development stage are examined here. The presence of multiple assays and associated reagents operating simultaneously on one platform, implementation in traditional clinical laboratories and regulatory review will present special challenges. Fortunately, clinical laboratories have made dramatic technical progress in the last two decades and regulatory agencies have publicly expressed support for development of multiplex devices.

KEYWORDS: multiplex diagnostics • nucleic acid tests • blood-borne • pathogen

The problem

Safety of blood and blood products is considered the first priority for transfusion and tissue transplantation. The risk of transfusion-transmitted viral infections was significantly reduced in the US and in many other countries after the implementation of nucleic acid tests (NATs) in parallel with the conventional methods for blood donor screening.[1,2] Every unit of donated blood undergoes a series of the tests for well-recognized blood-borne pathogens, HIV, HBV, HCV and West Nile virus (WNV) using molecular assays approved by the US FDA. Currently, NATs are the routine approach for testing blood components and tissue transplants in the US using the mini-pool strategy.[1,3] Viral RNA or DNA appears very early in infection, within 1–2 weeks, but antibody production will follow after

10–12 weeks. It is important to have highly sensitive tests for detection of potential infection before the appearance of antibodies (window period) to identify blood units containing pathogens. Although these tests have successfully increased the sensitivity and reduced the diagnostic window periods, the number of pathogens tested and newly emerging agents threatening the blood supply make individual pathogen testing burdensome and costly.

New exotic and emerging arboviruses have migrated from their historic endemic areas and expanded in new geographic locations where susceptible vectors and hosts provide permissive conditions for them to spread. The example of Chikungunya virus illustrates the threats imposed by this emerging infectious disease; prior to 2006, the virus caused outbreaks in countries in Africa, Asia, Europe, Indian and

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Pacific Oceans.[4] In late 2013, the first local transmission of Chikungunya virus was identified in the Caribbean region,[5] since then other local transmissions had been identified all around the Americas, increasing the risks to transfusion safety in those regions. Dengue virus is another emerging arbovirus that burdens public health worldwide.[6] It infects as many as 100 million people yearly and kills 25,000 people worldwide.[7] Approximately 50–80% of human infections with Dengue virus do not produce flu-like symptoms while virus can be present in the blood of asymptomatic donors. Thus, to avoid missing the asymptomatic donors during screening, testing of blood units for dengue virus is recommended in endemic areas for this infection such as Puerto Rico or Brazil. Furthermore, exotic viral, bacterial and protozoan parasite pathogens could be considered for detection in different parts of the world, which will increase the testing burden for blood banks. The challenge of additional pathogens of concern is revealed in a list published by the AABB's Transfusion Transmitted Diseases Committee and the Blood Safety Council established by the US Public Health Service.[8,9] The list includes viruses like Chikungunya and Dengue as well as bacteria and parasites (*Plasmodium falciparum* and *Babesia microti*), mutants of already known viruses (HIV, HCV and HBV) that would escape detection using the existing blood donor screening tests and many other nonroutine blood-transmissible agents. *Babesia*, as an example of a protozoan parasitic infection endemic to the Northeastern United States, is linked to at least 10 deaths related to transfusion since 2006. WHO policy for blood screening recommends mandatory testing for HIV, HBV, HCV and *Treponema pallidum* (syphilis), while screening for other infections, such as those causing malaria, Chagas disease or human T-lymphotropic virus should be based on regional epidemiological evidence. New technology that can detect all the pathogens of concern with a single multiplex test without sacrificing sensitivity and specificity is needed to solve this growing problem.

An alternate approach to assuring transfusion blood is free of infectious agents that would not involve pathogen detection is chemical and light treatment of blood products called pathogen reduction. It is in use in Europe and the US for platelets; yet in its infancy for use with whole blood or red blood cells.[10] Wide application of pathogen reduction would alter the need for pathogen detection and deserves comprehensive treatment in a separate review article.

The challenge of the multitude of blood-borne infectious agents also plagues clinical diagnosis. Vast strides have been made with molecular diagnostic assays and devices for enteric [11] and respiratory [12] infections; yet the burden of multiple tests and the uncertainty of diagnosis to guide therapeutic choices for blood-borne pathogens remain. Bloodstream infection is a leading cause of morbidity and mortality in intensive care units, impacting more than one million Americans annually. The mortality rate associated with bloodstream infection ranges from 10 to 40% and has aggregate healthcare costs of approximately \$16 billion a year.[13] A rapid (within 24 h) and accurate identification of a broad range of microbial or

fungal pathogens is the key for successful management of patients with bloodstream infection. Blood culture is still considered the gold standard in the detection of bloodstream infection; the culture of patient blood, even utilizing an automated continuously monitored system, typically requires 1–5 days.[14] Identification will require subsequent Gram staining and subculturing. The positive predictive value of cultures may exceed 95%, but requires time for full bacterial identification and antimicrobial susceptibility profile determination. This procedure is frequently exceeding 72 h for bacteria and even longer for fungi.[15] In septic shock, each hour of delay over the 6 h after onset of hypotension decreases patient survival by 8%.[16] Thus, early and accurate detection of blood pathogens will greatly benefit patient care, but direct detection of pathogens from blood is more challenging than from blood culture. For sepsis, diagnostic tests with higher multiplex capabilities are preferable because approximately 20–25 pathogens are responsible for more than 90% of bloodstream infections.[17] Molecular diagnostics to detect pathogens directly in blood are still at the research stage.[18,19] One of the unfortunate outcomes of slow or incomplete diagnosis is “empiric therapy.” The definition of empiric therapy is treatment based on a clinically educated guess in the absence of complete information.[20] In the area of infectious diseases, where clinical presentation in different patient populations may be caused by a variety of different ethological agents, initial therapy for infection is often empiric and guided by clinical presentation alone.[20] The common approach to empirical therapy is to use a broad spectrum antibiotic (or a combination) to cover a large spectrum of pathogens. The superior, evidence-based diagnostics require isolation and identification of the specific pathogen, which may take from 24 to 72 h or even longer depending on the organism. Prolonged empiric antimicrobial treatment without clear evidence is one of the reasons for increased emergence of drug resistance in hospital and community settings.[21,22] New diagnostic technologies aimed at faster and more accurate pathogen identification are increasingly integrated into clinical microbiological laboratory practice. These technologies, including multiplex PCR tests, have already shown an effect on reduction of healthcare costs and improved antimicrobial treatment.[21] The Infectious Disease Society of America (IDSA) recently reviewed the diagnostic landscape and presented recommendations to address challenges for emerging diagnostics in clinical practice.[14] The recommendations advise that while new technologies enable the detection and quantification of pathogens with increased sensitivity and speed, there are barriers preventing implementation of NATs in clinical practice, which include lengthy and costly regulatory approval, integration of new tests into routine patient care and an appropriate level of reimbursement for clinical laboratories. With a clear shift from detection of single analytes to complex testing, the amount of information generated in multiplex tests presents its own challenges for data interpretation and reporting formats with clinically relevant information understood by clinicians. The engagement and coordination of a number of stakeholders, including

government, regulatory, public health, clinical practitioners and the diagnostics industry, are necessary to overcome obstacles for implementation of new diagnostic tests for patient care.

How do multiplex nucleic acid diagnostics promise to solve the problem?

The exquisite sensitivity and high discriminating power of molecular detection techniques has held the promise in recent years for rapid, multiplex detection. Diverse platforms that utilize enzymatic amplification, miniaturization, unique particle-based solid supports, advanced labels and detectors and automation among all of the platforms are advancing rapidly

in the research arena. The challenge, which the authors discuss below, is bringing them through the approval process and into the clinic. The number of commercially available multiplex assays is increasing rapidly, as is the number of laboratory-developed multiplex assays, and these use a variety of technologies and instrument platforms (see Table 1).

Multiplex platforms with demonstrated effectiveness

Multiplex real-time PCR

Multiplex PCR is the simultaneous co-amplification of multiple targets using multiple primer pairs in a common reaction. In the field of molecular diagnostics, the capacity to amplify more than

Table 1. Molecular diagnostic platforms for detection of blood-borne pathogens.

Technology	Devices	Methodology	References
Multiplex real-time PCR	LightCycler Multiplex DNA or RNA Master products (Roche Applied Science)	MPX-RT PCR in glass capillaries that could measure up to four DNA targets per capillary	[18,23]
	Cobas Taqscreen MPX (Roche Molecular Diagnostics)	Multiple fluorophores detected in a single PCR reaction	[24,25]
	Dual-Labeled Probes (Life Technologies, Biorad, Biosearch Technologies, IDT)	Multiple fluorophores with distinct excitation/emission wavelength	[26,27]
Bead-based probes, end point PCR	Multi-Analyte Profiling (xMAP, Luminex, Inc.)	Biotinylated PCR products hybridized to target-specific probes attached on beads. Flow cytometer is used for signal detection and bead count	[28–30]
	Barcoded, magnetic beads (Applied Biocodes, Inc.)	PCR products hybridized to target specific probes attached to beads. Fluorescent reader is used for signal detection and bright field microscopy for identification of barcoded beads	[31]
DNA microarrays	Pathogen detection arrays, ViroChip (UCSF)	Target-specific oligonucleotide probes attached to solid matrix. Detection is based on hybridization of PCR products to specific probes	[23,32–35]
	Resequencing microarrays (TessArae, Inc.)	High-density overlapping probes that reveal the nucleotide sequence of a PCR product	[36]
Multiplex PCR with mass spectrometry identification	Plex-ID (Abbott Ibis Biosciences)	Measure the mass of PCR amplicons with sufficient accuracy to enable the calculation of the base composition	[37–40]
Multiplex PCR with nanoparticle T2 magnetic resonance for detection	T2 Magnetic resonance (T2 Biosystems, Inc.)	Target-specific oligonucleotide attached to derivatized superparamagnetic nanoparticles that produce the magnetic resonance signal	[41–43]
Isothermal multiplex amplification	Loop-mediated isothermal amplification	Multiple oligonucleotides designed to amplify the target at a single temperature. Detection by DNA binding dye or fluorescent probes	[44–47]
	Recombinase polymerase amplification	Amplification at a constant temperature with a combination of enzymes and a fluorescent probe	[48–50]
Next-generation sequencing	Ion Torrent PGM (Life Technologies, Inc.), Miseq (Illumina),454-Titanium (Roche)	Massively parallel determination of nucleotide sequence of short fragments with sophisticated bioinformatics tools to perform target identification	[34,51–53]
Spatial multiplexing of real-time PCR	TaqMan OpenArray (Life Technologies, Inc.)	Wafer patterned with 3072 open reaction wells (through-holes) allowing to perform separate PCR reactions. Target-specific assays are printed in each through-hole	[54]

one target in a single-reaction tube is known as multiplexing. Multiplex assays are defined as assays where at least two targets are simultaneously detected and/or identified through a common process of sample preparation, target or signal amplification, allele discrimination, detection and collective interpretation.[55] Nucleic acid-based devices employ technologies such as PCR, real-time PCR (RT-PCR) or transcription-mediated amplification for amplification. The detection of the amplified product can be TaqMan-style real-time fluorescence, bead-based hybridization probes, microarray-based probes or resequencing microarrays. While multiplex PCR devices for detection of 10 or more pathogens are increasingly accepted in the area of respiratory [12] and gastrointestinal [11] disease diagnostics, there is a limited number of multiplex PCR-based devices for testing blood-borne pathogens, focusing primarily on viral detection and the multiplicity is limited. For example, the *cobas Taqscreen MPX Test* from Roche Molecular Systems, Inc. uses RT-PCR for detection of several viruses such as HIV-1 (groups M and O), HIV-2, HCV and HBV in plasma as an FDA-licensed assay for blood, organ and tissue donors. The Gen-Probe, Inc. Procleix Ultrio Plus Assay is an FDA-approved blood donor screening assay that uses transcription-mediated amplification for multiplex detection of HIV-1, HCV and HBV.

The advancements on the signal detection and their use in molecular biology laboratories made the establishment of new multiplex assays easier with high performance, reproducibility and sensitivity equal to the individual PCR testing approaches. RT-PCR uses one set of specific primers and probe to enable continuous monitoring of fluorophore signal during the generation of PCR products in a closed tube format. The basic principle of multiplex RT-PCR (mRT-PCR) is similar to conventional RT-PCR except in mRT-PCR assays more than one primer and probe set are included in the reaction pool, allowing two or more different targets to be amplified and quantified in a single-reaction tube. The closed-tube aspect of this methodology contributes greatly to preventing amplified products from contaminating other subsequent reactions.

The most crucial step for a successful mRT-PCR assay is the correct design of primers and probes for each target to minimize possible interactions between oligonucleotides and fluorescence and to obtain maximum performance for each reaction.[56] Other important factors are the selection of suitable fluorescent reporter and quencher dyes that can work together with minimal spectral overlap. Also, it is very important to minimize competition and allow amplification of each target by optimizing the master mix conditions. Currently, most commercial mRT-PCR mixes are formulated to amplify up to four targets simultaneously in a single reaction with performance that compares to singleplex reactions.[57]

Several fluorescent systems have been developed for mRT-PCR reactions, and they are adapted to be used for detection and quantification of multiple blood-borne pathogens. Common ones are those using the TaqMan probes, TaqMan probes combined with fluorescence resonance energy transfer

(FRET) systems, as well as the melting point analysis of probe hybridizations and the beacon multiplex system.

The TaqMan probe is a single-stranded oligonucleotide containing a fluorophore dye in the 5'-end and quencher dye in the 3'-end. The TaqMan probe-based PCR system depends on the 5'-3' exonuclease activity of Taq DNA polymerase that hydrolyzes the oligonucleotide probe bound to the template DNA during the amplification in order to generate the fluorophore signal.[58] The TaqMan probe-based multiplex PCR methods use the same fluorophores that are widely available for individual RT-PCR, but the detection of generated signals needs to be performed in instruments equipped with multiple optical channels, such as the ABI7500, ViiA 7 and QuantStudio 6 Flex Real-Time PCR Systems (Applied Biosystems, Life Technologies, Grand Island, NY, USA). Each channel requires its corresponding excitation light source, filter set tuned to the emission wavelength and detector. On the other hand, the multiplexing capability using multiple fluorescent dyes is limited when the instruments are equipped with only one light source, such as the LightCycler 1.2 and LightCycler 2 (Roche Applied Science, Indianapolis, IN, USA), the Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.) instrument (Idaho Technology, Salt Lake City, UT, USA).

To overcome this limitation, a new concept of multilabeled oligonucleotides is used to achieve multiplexing. It consists of TaqMan probes combined with the FRET system. The technology relies on a distance-dependent interaction between two dye molecules, where the excitation is transferred from a donor to an acceptor fluorophore without the emission of a photon. As a consequence, the donor fluorophore is quenched while the fluorescence intensity of the acceptor molecule increases.[59] A previous study [26] showed that a FRET-TaqMan triple-labeled probe can be used for multiplexing fluorophore-labeled oligonucleotides with instruments equipped with only one blue light emitting diode (LED) excitation source and three corresponding detection channels. The FRET-TaqMan probe comprises three labels: a black hole quencher at the 3'-end, an emitter fluorophore (Cy5.5) and a receptor fluorophore (FAM) joined together at the 5'-end. The FAM efficiently absorbs energy from blue LED as a light source. In the absence of targets, the probe is dark because the energy absorbed by the FAM is transferred to Cy5.5 and, in turn, the energy released from Cy5.5 is transferred to the quencher. In the presence of targets, the TaqMan probe is hydrolyzed by the exonuclease activity of the Taq polymerase and black hole quencher is released; the energy absorbed by the FAM is transferred to Cy5.5 through the FRET mechanism to emit fluorescence. This example illustrates the benefit of using the FRET-TaqMan probe system to design a duplex RT-PCR capable of quantifying a target sequence with an internal positive control. One of the probes was labeled with 5'-FAM/3'BHQ-1 and the other one with 5'-FAM-Cy5.5/3'BHQ-3. Both probes have a common acceptor molecule (FAM), which is excited using the same at 470 nm (blue LED). After subsequent cleavage of the probes by enzymatic

primer extension, the two probes were detected with separate wavelength emissions, respectively at 520 and 705 nm fluorescence wavelength channels.

Another approach for multiplex PCR was developed to examine more than 24 pathogens with the power of discriminating between fungi, Gram-positive and Gram-negative bacteria in single-tube multiplex PCR reaction.[60] In this example, a modified FRET system was used together with an accurate measuring of melting temperature (T_m) of both the probes and the amplicons. The system combined the usage of nonspecific SYBR green dye as a fluorescence donor molecule (instead of a specific anchor probe), the discrimination via the melting temperature of the overall PCR products and the melting point analysis of species-specific probes, measured separately in different optical channels of the instrument.

The Light Cycler SeptiFast (www.roche-diagnostic.us) is one of the very few diagnostic multiplex PCR tests capable of detection of 25 clinically relevant bacteria and fungi directly from whole blood in about 6 h.[61,62] The test requires 1.5 ml of whole blood used in the extraction process (manual or automated on the MagnaPure system) followed by three separate PCR reactions, one for Gram-positive bacteria, one for Gram-negative bacteria and a third for fungi. The SeptiFast assay is the most widely studied and validated assay using direct blood samples and has yielded sensitivities ranging from 60 to 95% and specificities from 74 to 99% depending on the target pathogen.[63] The comparison of the SeptiFast system to blood culture on blood samples collected simultaneously from critically ill patients showed that molecular multiplex PCR test detects more organisms that could be otherwise missed by blood cultures.[62,64]

Thus, the simplicity of a single-tube assay, the potential for reasonably high levels of multiplexing and the rapidity of the mRT-PCR platform show promise for the simultaneous identification of many frequent blood infectious agents in blood donor screening as well as diagnostics.

Multiplex PCR amplification with probe hybridization for detection

Bead-based systems. Luminex xMAP Technology uses colored beads to carry biological probes similar to nucleic acid hybridization assays. By color-coding microscopic beads into many spectrally distinct sets, each bead set can be coated with a nucleic acid capture probe specific to a particular PCR amplicon, allowing the simultaneous capture of multiple amplicons from a single reaction. Because of the microscopic size and low density of these beads, assay reactions exhibit virtually solution-phase kinetics. However, once an assay is complete the solid phase characteristics allow each bead to be analyzed discretely. By incorporating magnetic properties into xMAP Microspheres, assay washing is simplified while maintaining desirable solution-phase properties. Multiple light sources inside the Luminex analyzer excite the internal bead dyes that identify each microsphere particle and the fluorescent reporter molecules captured during the assay (with emission wavelengths distinct from the

beads). The instrument records dozens of readings for each bead set and produces a distinct result for each amplicon in the reaction. Using this process, xMAP Technology allows multiplexing of many probes in one assay. This technology has been applied to multiplex detection of enteric pathogens [65] and veterinary pathogens.[66,67] Applied to blood-borne pathogens, though promising, the only published results were with mold species spiked in blood [28] or detection of yeasts in blood cultures.[29]

Applied Biocode offers barcoded, magnetic beads with a functionalized surface for attachment of DNA molecules that can be used in hybridization assays. The company's technology employs a permanent digital barcode, bonded to the beads using a semiconductor photolithography process, which is used to identify the beads microscopically in the reaction. The surface of the barcoded magnetic beads can also be functionally modified with carboxyl, streptavidin or passive hydrophobic absorption for probe immobilization. Carboxyl beads permit attachment of probes or specific primers to bind the bead surface covalently via NH₂-modified 5' termini. Target-specific amplicons can be generated in multiplex PCR and then hybridized to the functional group on that set of beads. The detection of a positive signal can be done using a fluorescent dye attached to a captured target. The amount of fluorescence given off by each set of unique barcodes is used to identify a positive or negative reaction, or to quantify the amount of each target in the sample.[31] Diatherix developed target-enriched multiplex PCR, [68] which is compatible with the barcoded magnetic beads detection system. With this technology, multiple RNA and DNA pathogen targets can be amplified in one reaction. For each target in the multiplex PCR, nested gene-specific primers were designed and included in the reaction. These primers are used at extremely low concentrations and are only used to enrich the targets during the first few cycles of PCR. The two gene-specific primers have proprietary tag sequences that are recognized by the universal primers. The universal primers are included in the reaction at a concentration necessary for asymmetric PCR amplification and only the reverse universal primer is labeled with biotin. Biotin-labeled PCR products are hybridized with a complementary detection probe attached to barcoded beads and the captured target is detected by streptavidin phycoerythrin. This approach was used to assess the sensitivity and reproducibility of mock samples (low-prevalence pathogens spiked in plasma or blood) produced by standardized methods for the validation of diagnostic devices for detection of potential biothreat agents (Duncan, unpublished results). The compact nature of the bead-flow systems like Luminex or the 96-well plate system of Applied Biocode suggests they could be adapted to the throughput requirement of blood donor screening as long as good sensitivity and specificity is achieved.

Microarrays. DNA microarrays have emerged as a valuable tool for detection of infectious agents in blood samples. The current pathogen detection arrays similarly amplify a nucleic acid target associated with the pathogen, then the amplified

products are hybridized to a single specific probe or a series of probes that accomplish DNA sequencing of the PCR product in a target. Each of these platforms has their advantages and disadvantages in terms of sensitivity, depth of information, cost and speed of assay completion. The biggest advantage of DNA microarray based detection is the highest multiplicity achieved by several thousand oligonucleotide probes printed on a chip representing the blood-borne pathogens and easily interpretable results can be obtained by data analysis using the sophisticated statistical algorithms that are becoming readily available. Several strategies for chip design and assay platforms have been employed by different groups for pathogen detection arrays [32] including ViroChip, Universal detection array, GreenChip and Lawrence Livermore microbial detection array. Accordingly, bioinformatics tools and data analysis algorithms have been developed to interpret the results that take into account the array design, sample material preparation and hybridization conditions, etc. However, multiplexing the amplification step for detection of several pathogens without compromising the sensitivity and specificity is one of the biggest challenges of DNA microarrays.

Resequencing microarrays. Unlike the printed oligonucleotide arrays used mostly for gene expression experiments,[69,70] the Affymetrix GenChips (Affymetrix, Santa Clara, CA, USA) have been adapted for resequencing target DNA fragments. The photochemical *in situ*-synthesized GenChip process is one of the keys that distinguish the GenChip from other microarray technologies. This feature enables extremely high-density resequencing microarrays of oligonucleotide probes. Short fluorophore-labeled DNA fragments (20–200 bp) derived from the sample hybridize to these probes.[71,72] The technology uses closely overlapping 25 base oligonucleotide probe sets to determine the nucleotide sequence of a selected genetic region of target DNA. Multiple probe sets are included per target organism to improve the sensitivity, specificity and accuracy of the assays providing statistical rigor to data interpretation.[72]

A resequencing microarray was designed previously for high-throughput SNP discovery and genotyping.[73] It has been shown to be effective for high-throughput detection of microorganisms in clinical, environmental, food and water samples.[74–76] Though not in use for routine clinical pathology practice, research has shown pre-amplification of the target sequence achieves higher sensitivity and better resolution, allowing the application of this technology to a broad range detection of respiratory tract infectious pathogens,[77,78] human platelet antigens genotyping,[79] bacterial identification and detection of resistance genes from positive blood culture,[80] biodefense application [81] and for testing multiple blood-borne pathogens in blood donors.[82] The sensitivity, specificity and complexity are acceptable for blood donor screening; however, the throughput rate may not be sufficient.

Multiplex PCR amplification with mass spectrometry for detection Recently developed by Ibis Biosciences, a method detects pathogen directly from blood using multiplex PCR followed by electrospray ionization mass spectrometry.[37] This detection platform can measure the mass of PCR amplicons with sufficient accuracy to enable the calculation of the base composition of the amplicon. The pathogen identification is conducted by comparing the base composition of the detected amplicons to the database of amplicons of known organisms. Accurate detections, obtained within 6–8 h, would allow for the initial antimicrobial therapy to be based on the organism(s) present, resulting in more optimal outcomes, reduced toxicity, lower costs and the preservation of existing antimicrobials from development of bacterial resistance.

Multiplex PCR amplification with nanoparticle T2 magnetic resonance for detection

T2 Biosystems (Lexington, MA, USA) developed a unique whole blood test for diagnosing of candidemia. The platform utilizes two pools of target-specific oligonucleotide-coupled supermagnetic nanoparticles that hybridize to each end of the single-stranded PCR-generated amplicons. This hybridization induces a cascade of events such as induction of nanoparticle clustering around the target, changes the molecular environment of water molecules in that sample, which results in alteration of the magnetic resonance signal, or the T2 relaxation signal that is measured, indicating the presence or absence of the target.[41] A recent study conducted on 1801 hospitalized patients with suspected sepsis demonstrated an overall specificity of 99.4% and sensitivity of 88–99.9% depending on the target.[83] The limit of detection was 1 colony-forming unit (CFU)/ml for *Candida tropicalis* and *Candida krusei*, 2 CFU/ml for *Candida albicans* and *Candida glabrata*, and 3 CFU/ml for *Candida parapsilosis*. [41] The negative predictive value was estimated to range from 99.5 to 99.0% in a study population with 5 and 10% prevalence of candidemia, respectively.

Spatial multiplexing on nanofluidic PCR platforms

There are several nanofluidic RT-PCR platforms with thousands of reaction wells that can be used with a panel of characterized PCR assays with SYBR or dual-probe chemistry. These reaction wells are spatially separated allowing the same sample to be used across multiple singleplex assays. The OpenArray technology is based on a metal plate the size of a microscope slide that has been photolithographically patterned and etched to form a rectilinear array of 3072 through-holes, organized in 48 subarrays with 64 through-holes each. Each through-hole is loaded with individual TaqMan assays and contains 33 nl of PCR mixture. Previous work has shown that the PCR assay performance in the nanoplates is equivalent to the same assay in microplates but with a >150-fold lower reaction volume (33 nl vs 5 μ l PCR reaction volumes) and with the ability to profile multiple targets using the same sample.[84] The combination of proven dual-probe chemistry with spatial assay separation on a

nanofluidic device, such as the OpenArray platform (Thermo Fisher Scientific, Waltham, MA, USA) was successfully used for simultaneous detection of four viral, two bacterial and three protozoan pathogens in blood.[54] A more recent study was conducted to evaluate the performance of highly multiplex blood-borne PCR panel built on the OpenArray platform for simultaneous detection of 17 viral strains in human plasma samples and 13 bacterial and protozoan pathogens in human plasma samples.[85] Although the current regulation requires that donor testing should be performed using assays approved by the FDA, this OpenArray system has a potential to screen large numbers of samples across different types of blood-borne pathogens.

Fluidigm and Wafergen have high-throughput PCR platforms allowing high level of PCR reaction partitioning (spatial multiplexing) through integrated fluidic circuits called the SmartChip. The loss of sensitivity inevitable for nanofluidic PCR reaction volume can be compensated by a target enrichment, pre-amplification step conducted off-site prior to qPCR. Recently introduced by Fluidigm, the FLEXsix has six 12-assay-by-12-sample partitions and it can be used on multiple days/PCR runs. Both systems have very limited application in the area of molecular microbiology. The Ariyoshi group [86] has utilized Fluidigm nanofluidic RT-PCR system for molecular serotyping of pneumococcus generating quantitative data of 50 pneumococcal serotypes in 29 groups directly from 45 test samples in a single run. One of the reported limitations of the utilization of Fluidigm nanofluidic PCR system was the initial cost of instrument. With the price of about 5- to 10-times higher than a conventional RT-PCR instrument, it presents a significant barrier for adoption in clinical microbiology. No reports were found in the published literature about utilization of Wafergen SmartChip platform for bacterial/viral identification or strain subtyping.

Isothermal multiplex amplification

Isothermal nucleic acid amplification is used for inexpensive, rapid and point-of-care nucleic acid detection of pathogens with sensitivity and specificity similar to PCR but with no thermocycling required. In isothermal techniques, the amplification reactions proceed at constant temperature without needing expensive thermal cycling instruments and analysis software. Isothermal nucleic acid amplification methods include loop-mediated isothermal amplification (LAMP)[87] and recombinase polymerase amplification (RPA).[88]

The LAMP method uses 4–6 different primers that form a stem loop structure and are specifically designed to recognize eight distinct regions on the target gene. Amplification and detection of a gene can be completed in a single step by incubating the mixture of samples, primers, DNA polymerase (usually *Bacillus stearothermophilus*) with strand displacement activity and substrates at a constant temperature (60–65°C) for 60 min. RNA template can also be amplified with reverse transcription and LAMP, known as RT-LAMP in one step. Target nucleic acid can be detected by RT measure

of turbidity as the reaction produces precipitate, which correlates with the amount of DNA synthesized. Disadvantages of LAMP are complex primer design and multiplex limitations.

Several pathogens can be detected with LAMP including HBV,[44] *Leishmania* species,[89,90] *Plasmodium*,[91] Chikungunya virus,[45] Dengue virus [46] and WNV.[47] The six major HBV genotypes, A–F, were detected with 92% diagnostic sensitivity in HBV-positive donor plasma specimens (n = 75) and 100% specificity in healthy donors (n = 107).[44] The assay detected 10–100 IU per reaction of HBV DNA in a 25 µl reaction volume incubated for 60 min at 60°C in a digital heat block. There was no cross-reactivity with *Leishmania major*, *Leishmania tropica* and *Trypanosoma cruzi*.

The multiplex potential of isothermal amplification was demonstrated by an RT-LAMP assay developed to detect and differentiate six flaviviruses including DENV serotypes 1–4, Japanese encephalitis virus (JEV) and WNV with no cross-reactivity with each other.[47] The RT-LAMP reaction detected 1–10⁶ copies per reaction viral genomic RNAs within 20 min. The RT-LAMP assay tested patient sera (n = 168) with 100% sensitivity and specificity for DENV1–4 and JEV with no cross-reactivity with 12 strains of influenza virus and 12 strains of Hantavirus. Lu *et al.* designed primers and used a loopamp RNA amplification kit (Eiken Chemical) for RT-LAMP to rapidly identify CHIK and DENV.[45] There was viral detection within 60 min without cross-reactions with other viruses (WNV, JEV, yellow fever, H5N1 influenza) with sensitivity for CHIK and DENV detection of 27 and 12 copies/reaction compared to 270 and 120 copies/reaction respectively by RT-PCR. The RT-LAMP sensitivity was 100% and the specificity was 95.25% in patient sera (n = 68) and healthy donor sera (n = 18).

RPA is another nucleic acid isothermal amplification method for pathogen detection. RPA's advantages over LAMP are less reaction time, lower temperature and greater multiplexing. Amplification depends upon a combination of two enzymes and proteins (recombinase, single-strand binding protein and strand displacing DNA polymerase), two primers and probes used at constant temperature (30–42°C) in a reaction time of 20 min. RT detection of RPA amplicons is done by specific probes with fluorescence signal measured by a simple point-of-care scanner. RPA has been used to measure early detection of DENV.[48]

The multiplex potential of RPA was demonstrated in a study by Teoh *et al.* [48] designed for simultaneous detection of the four DENV serotypes in a single-tube reaction incubated at constant 40°C for 20 min. The assay detected DENV RNA in <20 min for 12 genotypes of DENV including all four serotypes without cross-reacting with other arboviruses (CHIK, JEV). The RT-RPA assay had a detection limit of 50 copies of DENV RNA. The study compared diagnostic performance of the RT-RPA, RT-LAMP and qRT-PCR methods with patient sera. The RT-RPA assay had a good concordance with RT-LAMP and qRT-PCR in detecting Dengue virus. The qRT-PCR was the

most sensitive detection method (<50 copies), followed by RT-RPA and RT-LAMP.

Next-generation sequencing

Next-generation sequencing (NGS) has revolutionized genomic research by significantly shortening the run time and cost of nucleotide sequencing in a high-throughput manner. The entire genome sequence of bacterial or viral isolates or the much larger genome of eukaryotic parasitic protozoans such as *Plasmodium* species (causative agents of malaria) can be completed in a few days. Similarly, the cost of the Human Genome Project was US \$3.8 billion; now the cost of sequencing a human genome is approaching \$1000.[92] As a consequence, NGS is rapidly enhancing the nucleotide database for the pathogenic bacteria, viruses and parasites of global public health importance. NGS, in combination with microarrays, has allowed the prospect of rapid identification of pathogens in epidemics, discovery of novel pathogens in illness of unknown origin, and monitoring and surveillance of infectious diseases at the global level. In addition, NGS has opened new avenues to identify the novel antigenic forms and drug-resistant forms of existing pathogens through whole-genome sequencing or targeted genome resequencing.[51] To be applicable in the donor screening and blood-borne disease diagnostic settings, NGS technologies should be flexible in use and have a high throughput with a short run time. Several recently launched NGS systems appear to possess these characteristics.[32,51,93] However, many challenges remain including standardization of bioinformatics-based algorithms for data analysis and interpretation of results. Extensive preclinical and clinical studies accompanied by the manufacturing adaptations will be required for successful application of NGS in blood donor screening. An example of the role NGS can play is illustrated by the analysis of the 2014 Ebola outbreak that was published while the epidemic was still raging.[94]

What are the challenges for performance and regulatory approval of multiplex nucleic acid diagnostics? (the “pitfalls”)

Multiplex NAT provides significant challenges to the laboratory with regard to appropriate verification and validation testing, and especially the acquisition of appropriate control and reference materials to conduct the testing. The complexity of data analysis and reporting of results is increased relative to single-result assays. These challenges include

- *Multiplex assay target content.* The clinical utility of multiplex NAT PCR device is typically limited as it has a predefined subset of the targets relevant to transfusion or vector-borne diseases. Inclusion of emerging pathogens and therefore changes in the target content require revalidation of device performance. NGS for pathogen identification in blood has more clinical utility than testing of other nonsterile samples, such as rectal swabs or nasopharyngeal swabs, where the biggest challenge is to differentiate between colonization and infection.
- *Reliability of a positive or negative result.* The performance validation of multiplex devices leads to a large amount of analytical and clinical testing because each target analyte or pathogen has to be individually characterized for specificity and sensitivity as well as in combination with all other analytes. These performance studies are necessary for estimation of expected percentage of false positive and false negative test results for each analyte and, ultimately, the entire multiplexed device.
- *Specimen type.* Blood as a sample type presents a unique challenge for molecular multiplex devices. While whole blood versus conventional blood culture is more preferable for rapid pathogen identification, low pathogen bacteremia (1–10 CFU/ml) may have a significant impact on patient status. Extraction methods should be tested for isolation of a low number of pathogens and optimized to produce high-quality nucleic acids with no inhibitors present for downstream testing. Although the detection of a low amount of pathogen nucleic acid in a high background of human DNA may not necessarily affect PCR-based devices, it presents a significant challenge for NGS. Target enrichment procedures or removal of human DNA could be considered and included in the validation procedure. Further, if multiplex devices are intended for use in blood donor screening, not diagnostic detection of blood-borne pathogens, the challenges are even greater. The blood donor is expected to be uninfected; therefore, the specificity must be very high to avoid loss of healthy blood unit due to false positives. If the blood donor is infected, there must be no symptom to be acceptable for donation. This asymptomatic, infected state often occurs when the pathogen level in the blood is low; hence, the assay must be quite sensitive. The first highly multiplexed device will face additional challenges because the regulatory pathway for multiplex devices with a level of multiplicity higher than the five targets in the *cobas Taqscreen MPX Test* has not been established.
- *Result interpretation.* Simultaneous detection of multiple organisms may not present a problem for blood testing due to the nature of the tissue because it is not common for blood to be contaminated with multiple pathogens. Coinfection does occur so multiple pathogens may need to be distinguished simultaneously. A more challenging problem will be the clinical interpretation of the result. Increasingly broad ability to identify and increasingly sensitive platforms have revealed the presence of microbes of uncertain clinical significance. Some time and clinical investigation will be needed to understand the full significance of the microbes we will be able to detect.
- *Determination of “clinical truth” and sample availability.* “Clinical truth” for each organism tested is defined by the established Comparator Method aka “gold standard,” highly variable depending on pathogens. Multiplex molecular diagnostic devices may have a higher sensitivity and specificity for pathogen detection than comparator culture-based or histological methods, thus determination of clinical truth

and determination of performance (especially specificity, negative agreement) for each targeted organism usually leads to more complex study designs. Bidirectional sequencing of target nucleic acids is increasingly used as a confirmation tool for positive or negative results.[95] The US FDA has recommended in a Guidance [96] approaches to reduce the amount of comparator testing by randomized, partial comparison. If a study is required to perform a comparator assay for each assay on a highly multiplexed device, the volume of sample consumed could be prohibitive, the availability of clinical samples, for example, CSF containing viral pathogens, could be too limited to perform a comparative study. In many cases, the commercial access to such samples could be cost-prohibitive or have very strict Institutional Review Board policy restrictions. The development of specimen depository providing positive and negative samples for development of new diagnostics had been discussed in IDSA guidelines,[14] but it requires support from public agencies and companies with interest in obtaining regulatory clearance.

- *Reliability of sequence databases.* The design of molecular test devices as well as test results is often based on sequence information queried against public databases that contain widely accepted information obtained from many sources and may not be appropriately validated or correctly annotated. Similarly, many devices may use proprietary databases to evaluate test results, which will also need validation to demonstrate reliability. Sequence information used for device design should be validated at least every year against new sequences submitted to databases to ensure assay specificity on existing multiplex devices. The sequence database problem, especially if molecular devices are to advance to clinical use, has been widely discussed, [97] and some solutions are being applied such as careful filtering of existing databases or building new ones from scratch with appropriate documentation.
- *Device modification.* When a new target is added or a modification is made to a previously cleared or approved multiplex device, not only the performance of the newly added assay must be demonstrated, but also the effect of the new analyte on the performance of the previously validated, cleared or approved analytes. As the number of targets of the device increases, the amount of validation necessary to modify the existing device also increases. This is an important consideration since some viruses have the potential to mutate with a higher frequency, thus necessitating the ability to modify devices rapidly to detect variable strains. An ideal

multiplex device should have a flexibility to expand to detect emerging agents and at the same time have relatively short validation time frame. Thus, expansion of a multiplex device needs to be built in at the design phase and regulatory authorities must become aware of the unique characteristics of multiplex devices.

Expert commentary and five-year view

The prospects for application of multiplex NATs to blood donor screening and diagnostics were enhanced when one of the branches of the US FDA that regulates devices issued a guidance document on highly multiplexed diagnostic devices, [96] and the branch that regulates blood donor screening devices held a workshop bringing together major stakeholders to discuss the ways to move these devices into clinical use.[98]

All of the diagnostic and blood donor screening platforms that have been discussed above depend on a nucleic acid sequence-specific interaction. Direct determination of the sequence of the infecting or contaminating pathogen is the future universal method. NGS is already fast enough to be clinically relevant and allows very specific identification and additional clinically important information where phenotypes can be associated with genotypes. Antibiotic resistance genes [99,100] and pathogenicity islands [101] are obvious examples. NGS is also the ultimate multiplex platform because no prior assumptions of which pathogens are present need be made. Whatever nucleic acid is present in the sample can be determined.

The biochemistry and mechanics of NGS are nearly advanced to routine use. The bioinformatics analysis remains as a complex and time-consuming hurdle. This hurdle is recognized in all the domains where NGS is being applied, resulting in advanced approaches to reduce the bioinformatics burden.[102]

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Key issues

- Although nucleic acid tests (NATs) have successfully increased the sensitivity and reduced the diagnostic window periods, the number of pathogens tested and newly emerging agents threatening the blood supply make individual pathogen testing burdensome and costly.
- The challenge of a daunting multitude of blood-borne infectious agents also plagues clinical diagnosis.
- The exquisite sensitivity and high discriminating power of molecular detection techniques has held the promise in recent years for rapid, multiplex detection.
- The challenge is bringing new multiplex detection devices through the approval process and into the clinic.
- Multiplex platforms with demonstrated effectiveness include multiplex real-time PCR, multiplex PCR amplification with probe hybridization for detection (probes on fluorescent beads, barcode beads, microarrays, resequencing arrays) multiplex PCR amplification with mass spectrometry for detection, multiplex PCR amplification with nanoparticle T2 magnetic resonance for detection, multiplex isothermal amplification and next-generation sequencing (NGS).
- Multiplex NAT provides significant challenges to the laboratory with regard to appropriate verification and validation testing, and especially the acquisition of appropriate control and reference materials to conduct the testing.
- A validation study could be required to include a comparator assay for each assay on a highly multiplexed device causing the volume of sample consumed to be prohibitive. The US FDA has recommended in a Guidance Document approaches to reduce the amount of comparator testing by randomized, partial comparison.
- NGS is the ultimate multiplex platform because no prior assumptions of which pathogens are present need be made. The biochemistry and mechanics of NGS are nearly advanced to routine use. The bioinformatics analysis remains as a complex and time-consuming hurdle.

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**** This article highlights an important common issue challenging the development, and implementation of multiplex molecular assays, the establishment of clinical quality genome reference databases.**



Advances in multiplex nucleic acid diagnostics for blood-borne pathogens: promises and pitfalls

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