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Advances in Microfluidic PCR for Point-of-Care Infectious Disease Diagnostics

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Abstract

Global burdens from existing or emerging infectious diseases emphasize the need for point-of-care (POC) diagnostics to enhance timely recognition and intervention. Molecular approaches based on PCR methods have made significant inroads by improving detection time and accuracy but are still largely hampered by resource-intensive processing in centralized laboratories, thereby precluding their routine bedside- or field-use. Microfluidic technologies have enabled miniaturization of PCR processes onto a chip device with potential benefits including speed, cost, portability, throughput, and automation. In this review, we provide an overview of recent advances in microfluidic PCR technologies and discuss practical issues and perspectives related to implementing them into infectious disease diagnostics.

Keywords

PCR; microfluidic; diagnostic methods; infectious disease; point-of-care

1. Introduction

POC testing is defined as analytical testing performed outside the central laboratory using devices that can be easily transported to the vicinity of the patient (College-of-American-Pathologists, 2001). The value of near-patient testing for routine infectious disease diagnosis is well recognized given that real-time test results can direct timely therapeutic interventions and improve patients' clinical outcomes. With the increasing threat of accelerated epidemic-to-pandemic transitions of new or reemerging infectious disease outbreaks owing to globalization, decentralizing diagnostic testing closer to frontline clinical settings could facilitate earlier implementations of public health responses to contain and mitigate such events (Nichols, 2007, Rajan and Glorikian, 2009). In the developing countries where high infectious disease burden is compounded by diagnostic challenges due to poor clinical laboratory infrastructure and cost constraints, the potential utility for POC testing is even

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greater (Urdea et al. , 2006, Yager et al. , 2008, Yager et al. , 2006). In fact, among the 'Grand Challenges for Global Health' identified by the Bill and Melinda Gates Foundation and the NIH, one of the major priorities involves developing POC technologies for diagnosing infectious diseases (Mabey et al. , 2004).

Culture remains the mainstay of microbiological diagnosis, and enables antimicrobial susceptibilities to be determined; however, it is time-consuming, expensive, and poorly sensitive in cases of fastidious organisms or prior antibiotics exposure. Moreover, culture requires well-maintained laboratory-based equipment, a constant supply of reagents and electricity, and adequately trained and supervised technologists. Meanwhile, molecular approaches to amplify microbial nucleic acids have clear theoretical advantages over culture methods in terms of detection accuracy and turnaround time (Cuchacovich, 2006, Yang and Rothman, 2004). Among the various nucleic acid amplification methods, polymerase chain reaction (PCR) has been the most mature and popular due to its simplicity (Fredricks and Relman, 1999, Yang and Rothman, 2004). Variations of the method have been modified to expand its utility and versatility. Multiplex PCR enables simultaneous detection of several target sequences by incorporation of multiple sets of primers. Nested PCR, a double amplification step with appropriately designed primers, can increase sensitivity and specificity (Fredricks and Relman, 1999). Reverse transcript PCR (RT-PCR) enables the detection of RNA, which is converted into a complementary DNA copy and amplified (Erlich et al. , 1991). Real-time PCR allows simultaneous amplification and product detection without additional post-PCR processing. Digital PCR enables direct quantification of nucleic acids through parallel single-molecule amplification (Vogelstein and Kinzler, 1999). With the increasing number of genomes of infectious pathogens being sequenced, catalogues of genes can be exploited to serve as amplification targets fundamental to the design of clinically useful diagnostic tests. As a result, the number of PCR assays developed to identify either specific pathogen or classes of pathogens by amplifying unique or conserved sequences, respectively, continues to expand. Widespread clinical applications, particularly in detecting difficult-to-culture, limb- or life-threatening, biothreat or emerging infectious pathogens, have demonstrated significant performance and cost advantages over traditional methods (Rothman et al. , 2010, Yang et al. , 2008a, Yang and Rothman, 2004, Yang et al. , 2008b). While commercial development of PCR-based diagnostics has progressed significantly in recent years, all FDA-cleared PCR test kits to-date are still categorized as high or moderate complexity under Clinical Laboratory Improvement Amendments (CLIA) (Holland and Kiechle, 2005). Inefficient nucleic acid preparation from complex sample types (e.g. whole blood, stool, etc.) requires highly skilled personnel to manually perform multiple processing steps in a dedicated laboratory space, batched testing with next-day reporting of results, and costly reagents and instrumentation. These features are some of the major hurdles which still preclude PCR-based assays from being classified as a "simple test" based on FDA recommendations for POC use (Table 1) (Holland and Kiechle, 2005).

The emerging field of microfluidics, in combination with micro-electro-mechanical systems (MEMS) technology, to create so called lab-on-a-chip (LOC) or micro total analysis system (μTAS), promises exciting solutions to realize PCR-based, POC diagnostics to the POC. Microfluidics involves the behavior, precise control and manipulation of fluids in the micro-scale environment. Bioanalytical systems based on microfluidics allow miniaturization and integration of processes that were previously done at larger scale in separate operations with enhanced speed and efficiency (Whitesides, 2006). By exploiting the strengths of microfluidics, miniaturization of PCR devices can be achieved with significant advantages, including shorter assay time, lower reagent consumption, rapid heating/cooling, portability, as well as the possibility of integrating multiple pre- and post-PCR processing modules in a self-contained system with full automation. This unique set of capabilities has the potential

to bring about decentralization of PCR-based diagnostic testing for use in resource-limited settings.

In this review, we concisely discuss the latest advancement in microfluidic PCR technologies for infectious disease diagnostics. We first review the current status of on-chip PCR technologies. Key aspects of the recent advancements toward a fully integrated POC platform based on microfluidic technologies are discussed, including pre-PCR sample preparation, post-PCR amplicon analysis, system integration/automation, adaptation for use in developing countries, as well as current challenges and future perspectives in this field. It is beyond our scope to provide an exhaustive review of all the related technologies in this domain. Instead, our goal is to present a snapshot of the promising technical advances with a focus on practical concerns appropriate for infectious disease applications.

2. On-chip PCR

PCR is an enzyme-driven process for amplifying short regions of DNA *in vitro*. It can create millions of DNA copies by cycling between different temperatures to allow repeating steps (denaturation, annealing and elongation) of DNA replication to take place. Despite the simplicity and amplification power of PCR chemistry, limitations in its supporting hardware still hinder PCR from reaching its full potential. In particular, improvements in thermal cycling speed, instrument size, and reaction volume are still much needed. The bulky instrumentation and large reaction volume required in conventional bench-top thermal cyclers lead to large thermal mass which reduces the temperature transition speed and reaction efficiency. These shortcomings can all be directly addressed through miniaturization of the PCR device. Rapid thermal cycling can be achieved through rapid heat transfer in microfluidic-based PCR due to the small reaction mass and the high surface to volume ratio of the small reactor. The small length scale can also lead to a more uniform temperature distribution and enhance the yield and integrity of PCR.

2.1 Device materials and fabrication

Miniaturizing the PCR device onto a single-use, self-contained, disposable microchip, which can reduce cross-contamination and biohazard risks, would be most practical for infectious disease testing. To that end, cost and performance are important considerations when selecting substrate materials to create the chip device. Silicon and glass substrates are popular with well-established microfabrication methods commonly used in the microelectronics fields. However, expensive material and fabrication costs associated with these substrates preclude them from disposable use. Recently, polymeric materials such as polydimethylsiloxane (PDMS) (Thorsen et al. , 2002, Unger et al. , 2000), polymethylmethacrylate (PMMA) (Hataoka et al. , 2004, Lee et al. , 2004), and polycarbonate (PC) (Hashimoto et al. , 2004, Liu et al. , 2002) have become more popular owing primarily to their lower material and fabrication costs. In particular, PDMS has emerged as the most promising substrate given its inert and non-toxic properties, improved optical transparency, high thermal stability, and PCR compatibility (Melin and Quake, 2007, Thorsen, Maerkl, 2002, Whitesides, 2006). Soft lithography, which refers to a replica molding process on micro-fabricated master templates, provides a simple, low cost, and rapid prototyping of micron scale fluidic circuits on elastomeric polymers such as PDMS, and thus has become one of the most popular approaches to fabricate microfluidic PCR system (Xia and Whitesides, 1998). Other thermoplastics including PMMA and PC can be fabricated either in a batch process by using hot embossing and injection molding techniques, or in a sequential process by using laser ablation. Many substrates bonding technologies such as adhesive, thermal, and plasma treatment bonding are available for making enclosed fluidic environments (Dittrich et al. , 2006, Sun and Kwok, 2006).

2.2 Chip designs

Two distinct design approaches, namely the stationary reaction chamber system and the continuous flow system, have been adopted for on-chip PCR devices to reduce the reaction volume. The stationary reaction chamber system works in the same manner as conventional PCR devices in which the PCR mixture is kept stationary inside the reaction chambers while the thermal cycling is done by alternating the temperatures of the heating units (Figure 1a). Since the first PCR chip based on this format was introduced by Northrup *et al* (1993) (Northrup, 1993), substantial improvements have been made, including implementing a multi-chamber design to increase throughput. Stationary systems generally allow a small reaction volume and simple system configuration. Detection of micro-organisms in pL scale chamber volumes with chip configurations of up to 1176 parallel reaction chambers have been reported (Marcus et al. , 2006b, Ottesen et al. , 2006, Pal et al. , 2005). Precise sample handling and processing, in addition to ensuring temperature uniformity between chambers, in the setting of increasing numbers of them still pose challenges. Further miniaturization of the reaction volume may also lead to nonspecific absorption of PCR samples on the walls of the chamber due to increased surface-to-volume ratio.

A continuous flow system transports the PCR mixture through different pre-fixed temperature zones for PCR thermal reactions (Figure 1b). Compared to a stationary chamber based format, the continuous system approach provides faster thermal cycling in general because thermal inertia depends only on the thermal mass of the sample, rather than the chip. In this system, the required heating and cooling sequence and the residence time are controlled by channel routing and flow speed (Kopp et al. , 1998). Multiple closed-loop microfluidic channels through different temperature zones can be effectively used for continuous cycling of small volumes of reaction mixtures while reducing cross-contamination risks (Jian et al. , 2002). Higher fabrication cost and fixed cycle number dictated by the channel layout are some of the drawbacks as compared to the stationary chamber system. Nonetheless, the dynamic nature of fluidic transport in this format may facilitate integration with other functional components towards μTAS.

Recently, microfluidic droplet technology dramatically decreased the thermal inertia of the microfluidic PCR system. Droplet emulsion PCR system, which utilizes discrete aqueous droplets dispersed in a continuous oil phase as illustrated in Figure 2a, enables high throughput testing at the single copy level with automated generation and control of multiple droplets, providing a digital PCR platform. Many microfluidic technologies are available for integrating the required microfluidic functions on a single chip, such as rapid generation of water-in-oil droplets by using electric field, flow focusing, and T-junction methods (Huebner et al. , 2008, Rane et al. , 2010, Teh et al. , 2008), and fusion or sorting of multiple droplets by applying localized electric field, droplet surface modification, and variation of channel geometries (Agresti et al. , 2010, Chabert and Viovy, 2008, Mazutis et al. , 2009b). An integrated platform that can detect single copy RNA and virion through RT-PCR in a pL droplet system has been reported (Beer et al. , 2007, Beer et al. , 2008). High throughput amplification and quantification in millions of droplets have been demonstrated for large scale genomic sequencing (Kiss et al. , 2008, Mazutis et al. , 2009a, Tewhey et al. , 2009) and digital detection of pathogenic *E. coli* O157 cells in a high background of normal K12 cells (Zeng et al. , 2010). Despite high throughput testing capability of emulsion PCR system with reduced risk of cross-contamination by compartmentalizing samples and reagents into pL or nL size droplets, the development of a compact and portable system for POC testing is still limited because external instruments and tubings are required for continuous actuation of fluid and droplet manipulation.

Another major form of droplet microfluidic device manipulates droplets in open space (Figure 2b). Free aqueous droplets containing PCR mixtures on an open surface platform

provide simple stationary reaction vessels (Juergen et al. , 2008, Pipper et al. , 2007, Zhang et al. , 2009b, Zhang et al. , 2011, Zhang et al. , 2010). The reaction droplet encapsulated by immiscible oil is insensitive to vapor generation during thermal cycling, thus avoiding the problem of micro bubble formation and expansion often found in conventional microfluidic PCR chambers of fixed volume. Moreover, given that each discrete droplet can function both as a reaction chamber and a fluid transportation unit (Guttenberg, Muller, 2005, Hsieh, Zhang, 2006, Juergen, Yi, 2008, Pipper, Inoue, 2007), no additional microfluidic components are required and the fluid handling is relatively simple. As a result, the open surface droplet platform is particularly advantageous to POC applications because the valve- and pump-less droplet manipulation enable easy implementation of upstream sample preparation into a fully functional μTAS without added complexity such as bulky external fluid couplings.

2.3 Thermal management

As the temperature ramping rate of the PCR system limits the speed of the reaction, a compact and efficient heater design with reduced thermal mass is essential to achieve fast target amplification. Various on-chip heaters have demonstrated improved speed of temperature cycling as compared to that of conventional bench top systems. In general, on-chip heaters can be categorized into contact and non-contact types. Many contact type heaters such as thin films, metal heating blocks, and Peltier units are commercially available at low cost and are thus widely adopted in on-chip PCR systems, while the temperature cycling speed can be limited by the thermal loss due to external coupling (Zhang and Xing, 2007). Despite the complexity and cost of fabrication, micro-fabricated thin film heaters are also widely used as a contact type method to achieve high speed reactions as fast as 6 minutes for 40 thermal cycles at the heating speed of 175°C/sec and cooling speed of −125°C/sec (Neuzil et al. , 2006). On the other hand, non-contact methods are more favorable for simple chip designs as they provide more flexibility for system integration. Several researchers have successfully integrated IR-mediated (Huhmer and Landers, 2000, Legendre et al. , 2006) or laser assisted heater units (Slyadnev et al. , 2001) with amplicon detection components. Recently, investigators used microwaves to achieve local heating with a 15 msec response time in conjunction with a droplet based microfluidic system (Issadore et al. , 2009). Although its reported heating speed is lower than that of common contact methods, the benefit from the simplified device configuration and the capability of selective heating of aqueous phase of droplets while maintaining surrounding oil streams at room temperature more than offset this deficiency.

3. Post PCR amplicon analysis

As part of the effort to miniaturize PCR-based bioassays for POC testing, developing chip-scale amplicon detection units capable of automated operations with the reduced risk of cross-contamination have become one of the major challenges towards realizing a fully integrated system. In conventional bench top PCR assays, amplicons are either collected or analyzed off-line in a separate analysis device (e.g., gel-electrophoresis, capillary electrophoresis), or monitored directly in the same reaction chambers using an on-line method (e.g. fluorescent probe or intercalating dye for real-time detection). Although on-chip PCR products can also be detected off-line using the same methods which require transferring of samples, on-line detection would be more ideal in improving throughput and reducing risk of contamination. To this end, much effort has been made to miniaturize and integrate conventional techniques as well as explore alternative approaches capable of highly sensitive detection within the appropriate volume range of on-chip PCR (down to subnanoliter level) and without the need for any off-chip apparatus such as external illumination sources or imaging devices. In this section, we discuss several relatively mature on-chip detection techniques for microfluidic PCR amplicon analysis.

3.1 Electrophoresis

With the aid of microfabrication technology, existing detection assays have been miniaturized into chip-scale devices. Among them, capillary electrophoresis (CE) and capillary gel electrophoresis (CGE) in microfluidic channels are the most common on-chip detection methods (Burns et al. , 1998, Govind et al. , 2006, Lagally et al. , 2001, Lagally et al. , 2004). In these electrophoretic separation methods, PCR amplicons are first labeled with intercalating dyes before they are loaded into a microfluidic channel. Upon the application of an electric field, amplicons migrate along the microfluidic channel filled with gel matrix. DNA molecules of different sizes migrate at different speeds and are subsequently separated. Advantages of electrophoretic-based detection include simple, fast analysis and high amplicon separation efficiency. Easley *et al*. reported an integrated on-chip PCR coupled CE which successfully detected *Bacillus anthracis*, *Bordetella pertussis, and Salmonella typhimurium* in as early as 12 minutes (Easley et al. , 2006b). A similar approach has been applied for detection of BK virus in unprocessed urine samples from renal transplant patients with limit of detection of 1-2 viral copies (Govind, Ryan, 2006). Given its size-based approach for amplicon analysis, one of the major limitations of electrophoretic separation is the inability to differentiate amplicons of similar size but different sequence.

3.2 DNA hybridization microarray

The DNA hybridization microarray is a powerful amplicon sequence analysis method. Advances in microfabrication technology has made it possible to immobilize large numbers of oligonucleotide probes, each having complementary sequences against a specific target pathogen's DNA/RNA, all on a predefined, array-patterned microchip. Fluorescent patterns generated through hybridization with labeled PCR amplicons can be analyzed to extract sequence information. By taking advantage of microfluidic technology, PCR has been successfully coupled with microarrays on the same platform. For example, various combined PCR-microarray devices have been developed for genotyping HIV (Anderson et al. , 2000), Chinese medicinal plants (Trau et al. , 2002), and point mutations in human genomic DNA (Hashimoto et al. , 2006, Hashimoto et al. , 2005). While providing high throughput analysis of multiple amplicon sequences, microarray technology is limited by the prolonged assay time for hybridization reaction and the need for sensitive fluorescent pattern scanning and analysis methods.

3.3 Fluorescence based real-time detection

Real-time PCR combines amplification with detection in synchrony without post-PCR processing. Using fluorescently labeled probes or intercalating dyes, increasing fluorescent signals generated from PCR amplification can be measured during the assay run and greatly reduce overall assay time. The number of cycles required to reach a detectable threshold (Ct) of PCR products can be used to further assess PCR efficiency and determine the initial template concentration or pathogen load. To ensure accurate acquisition and analysis of the emitted fluorescent signal after each PCR cycle, the choice of excitation light source and optical detection apparatus requires much attention. Unfortunately, conventional bench-top reading instruments are limited to complex laser optics and imaging components, such as charge-coupled devices (CCD), with large footprints. To develop a POC device requires use of miniaturized and low cost external light sources and photo-detecting units. Light emitting diode (LED) and photo-diode (Cady et al. , 2005) are inexpensive alternatives although the illumination power, bandwidth, and the detection sensitivity are limited compared to the conventional laser optic system. Various microfluidic-based real-time PCRs using either SYBR green, ethidium bromide dye, or TaqMan probes for specific pathogen detection have been reported (Belgrader et al. , 1998, Lee et al. , 2006, Northrup et al. , 1998, Pipper, Inoue, 2007).

3.4 Electrochemical method

Despite the use of relatively miniaturized external fluorescence detectors such as LEDs and photodiodes for on-line amplicon detection, a fully integrated detection system microfabricated as part of the chip device would be most ideal. Electrochemical based detection offers a promising alternative with inherent features including high sensitivity, adjustable selectivity, low power, low cost, independence from external optical components, and compatibility with microfabrication technology. Electrochemical methods require sensor electrodes, which are often functionalized with probes or other chemicals, to generate electrical signals which can be correlated with the concentration of target amplicons. In most cases, the detected electrical signals such as voltage, current, and impedance need to be amplified or processed. Few researchers have reported electrochemical detection of PCR amplicons. An integrated chip device that can detect *E. coli* from whole blood samples based on DNA hybridization and electrochemical sensing has been developed with a reported limit of detection of 10^3 cells/mL (Liu et al. , 2004). Alternatively, Yeung *et al*. demonstrated an integrated system combining sample preparation, DNA amplification and subsequent multiplexed electrochemical detection of *E. coli* and *B. subtilis* in a single silicon-glass microchamber (Yeung et al. , 2006a). They further advanced the technology to develop the first electrochemical real-time PCR (ERT-PCR) (Yeung et al. , 2006b, 2007). Conductivity-based electrical DNA detection using functionalized gold nanoparticles has also reported significant improvement in detection limit down to as low as 500 femtomolar of target DNA, which is comparable to the sensitivity of an optical fluorescence detection system (Park et al. , 2002).

4. Pre-PCR sample preparation

Direct pre-processing of crude biological samples with efficient purification and extraction of target analytes is a key prerequisite of a POC device with sample-in answer-out capability. In general, sample preparation is a multi-stage process including sample collection, cell separation and concentration, cell lysis and nucleic acids extraction. Depending on the sample type, the complexity of the sample preparation process may differ significantly. In samples with ample quantities of target organisms in relatively inert sample matrices, one can lyse the target cells and directly analyze the nucleic acids from the crude samples (Govind, Ryan, 2006, Legendre, Bienvenue, 2006). In the majority of cases, however, tests are utilized to establish early diagnosis by detecting pathogens which may be present in extremely low quantities. Large sample volumes are often required to ensure sufficient capture of pathogens for detection. As a result, most samples need a concentration step to increase the pathogen load. Samples with a complex matrix, such as whole blood, which contain complex constituents, including large amount of blood cells and PCR inhibitory anticoagulant additives, may require more than centrifugation to concentrate target pathogens (Lim et al. , 2005, Toner and Irimia, 2005). In addition, the large human genomic content from the blood cells contributes to the PCR background and can significantly compromise the amplification efficiency of the target sequence (Al-Soud and Radstrom, 2001). Depending on the target organism, the specific blood fractions in which the microbe preferentially resides may also differ. For example, malaria diagnosis relies on the separation of parasite infected RBCs from uninfected cells. Thus, prior separation of the target pathogens from background cells is essential for the accurate and sensitive PCR detection downstream. Moreover, effective lysis of the target organisms, which may have variable protective outer layers, to release their genetic contents for analysis also plays an important role in sample preparation. In the following sections, we shall discuss in detail how microfluidic technology contributes to these key aspects of sample preparation.

4.1 Cell separation and concentration

As mentioned earlier, a typical microfluidic device handles a liquid volume in the range of nanoliter to microliter. In contrast, the sample volume required for infectious disease detection ranges from microliter to milliliter depending on the disease and type of sample under investigation. The volume mismatch may raise the issue of stochastic sampling resulting in false negative results (Mariella, 2008, Rådström et al. , 2004). Therefore sample pre-concentration as well as additional cell separation steps to exclude unwanted cell types and matrix constituents are often required to enhance the accuracy of detection. Disparities in the intrinsic properties of the different cell populations can be exploited to achieve cell separation. Physical and mechanical properties (e.g. size, density, shape, deformity) are popular parameters for differentiation. Conventional target cell concentration is done by pelleting the cells using centrifugation. Although some microfluidic platforms utilize centrifugal force, it is mainly used as means for fluidic transport instead (Gorkin et al. , 2010, Mark et al. , 2010). Filtration is one of the common concentration techniques implemented in microfluidic devices. Obstacle structures created inside microfluidic channels serve as selective filters based on cellular size and rigidity have been shown to separate white blood cells from other blood cells (Lee and Tai, 1999, Panaro et al. , 2005, Yuen et al. , 2001). In combination with hydrodynamic flow and variations in channel geometry, bacterial cells and human blood cells can be effectively separated (Wu et al. , 2009). Flow field fractionation is another common technique for continuous-flow sample concentration. In this method, cells are first confined within a thin flow stream with the application of a lateral force field, and the narrow cell stream is then directed to a split outlet, resulting in a reduction of fluid volume and sample concentration (Laurell et al. , 2007, Peter and Jody, 2002). Acoustic standing waves have been demonstrated as an efficient force field for cell separation and concentration based on size (Evander et al. , 2007, Petersson et al. , 2007). Additional force fields applied to enhance separation based on polarizability and magnetic characteristics of different cells are gaining popularity owing to their high sensitivity and efficiency. Dielectrophoresis (DEP) has been used to discriminate bacterial species and cell viability based on their distinctive dielectric properties (Bhattacharya et al. , 2008, Cheng et al. , 1998, Cho et al. , 2010, Lapizco-Encinas et al. , 2004, Park and Beskok, 2008, Park et al. , 2009a). External magnetic fields have also been applied to separate a heterogeneous population of cells through a process called magnetophoresis (Blakemore et al. , 1979, Pamme and Wilhelm, 2006). Alternatively, using antibodies conjugated on a solid substrate, such as magnetic particles, cell sorting and concentration can also be achieved through immunoaffinity mechanisms (Cho et al. , 2007, Juergen, Yi, 2008, Kang-Yi et al. , 2008, Kell et al. , 2008, Shih et al. , 2008).

4.2 Cell lysis

Effective cell lysis to liberate microbial genetic content for downstream PCR detection can improve the overall sensitivity of the assay. Many microfluidic devices have adopted various traditional methods of cell lysis, which may be mechanical, chemical/enzymatic, thermal or electrical (Belgrader et al. , 1999, Marentis et al. , 2005). Minisonication has been shown to induce chaotropic disruption of bacterial spores despite their rigid coats. Knife-like nanostructures fabricated inside the microfluidic channels can create shearing effects on cell membranes (Di Carlo et al. , 2003). A microfluidic counterpart of ball milling has been used to lyse cells by packing the cells and beads together into a microfluidic CD (Kim et al. , 2004). The addition of chemicals (e.g. chaotropic salt, detergent, alcohols) to induce osmotic pressure, or enzymes (e.g. lysozyme, lysostaphin, protease) for cell wall/membrane digestion, can be incorporated in the cell medium as further augmenting measures (Irimia et al. , 2004, Schilling et al. , 2002, Sethu et al. , 2004). Recently, a laser-irradiated magnetic bead heating system has been proposed as a convenient and efficient thermal cell lysis platform (Cho, Kim, 2010, Cho, Lee, 2007, Lee, Cheong, 2006). Electroporation of cell

membranes can be induced via a high-intensity pulsed electric field or high frequency AC current (Lee and Tai, 1999). To achieve the optimal result, various combinations of the aforementioned cell lysis methods may need to be adjusted based on the microbial pathogen of interest and the strength of its outer protective layer (Mahalanabis et al. , 2009, Stachowiak et al. , 2007).

4.3 Nucleic acids extraction

The last step before performing PCR is to extract and purify the nucleic acids from cell lysates. This process usually involves multiple manual operations with specific instruments, especially when the number of target copies is low, or the sample is in the form of complex mixtures. The cell lysates may contain PCR-inhibitory materials that limit the efficiency of the amplification process or even cause complete failure of the amplification. Some of the common PCR inhibitors in blood include heme, leukocyte DNA, immunoglobulin G or anticoagulant additives such as EDTA and heparin (Al-Soud and Radstrom, 2001). Although much of the PCR inhibitors are removed during the earlier cell separation and concentration stages, residual can remain without a proper nucleic acids purification step. Various on-chip nucleic acid purification methods have been developed, such as electrophoretic (Vulto et al. , 2009) and hybridization based purification in which poly(dT) conjugated beads are used to capture target mRNA (Hong et al. , 2004, Marcus et al. , 2006a). Nonetheless, silica based solid phase extraction (SPE) is the mainstream approach. The mechanism of the silica based SPE is believed to be the combined effects of dehydrating nucleic acids molecules, hydrogen bonding and electrostatic interactions (Melzak et al. , 1996). The purification process starts with binding of nucleic acids to the silica surface in high-ionic-strength chaotropic salt, followed by several washing steps to remove debris before eluting in low ionic strength buffers. When implementing SPE in microfluidic devices, the solid substrate is usually in the form of micropillars/microposts (Cady et al. , 2003, West et al. , 2007) or immobilized silica beads/particles (Breadmore et al. , 2003, Gijs, 2004, Juergen, Yi, 2008, Pipper, Inoue, 2007). Several microfluidic SPE devices have been reported to have high extraction efficiencies of up to 80% with small elution volumes in the microliter range, however, issues with sample volume capacity and flexibility, as well as process automation with minimal contamination, still require substantial improvement (Gijs et al. , 2010, Wen et al. , 2008).

5. System integration and automation

A unique advantage of microfluidic PCR is the feasibility of integrating pre-PCR sample preparation with post-PCR analysis into a streamlined and automated system for POC applications. System integration and automation are critical for avoiding manual operation errors, minimizing cross-contamination risks, and reducing sample loss caused by multiple sample transfers between instruments (Mariella, 2008). However, to develop a fully integrated μTAS capable of completing an assay from sample-in to answer-out in clinical settings remains challenging mainly because operation of these platforms still requires complex and bulky peripherals. Large gas tanks and syringe pumps are often used as the pressure source for fluid actuation. Fluid control requires incorporation of micro-pumps, micro-valves, and micro-mixers with extensive external tubings. Bulky electrical instruments and microscopy are often required for signal detection and imaging. All these accessories are not portable. Therefore, current platforms are often considered as chip-in-lab rather than lab-on-chip. A truly POC platform should have compact actuating and controlling modules with universal interfaces for easy operation and high portability. In the past decade, great effort has been put into creating a μTAS by integrating a PCR module with the aforementioned pre- and post-PCR modules. PCR based whole blood sample preparation and amplification platforms that utilize micro-filter separation (Munchow et al. , 2005, Panaro, Lou, 2005, Wilding et al. , 1998, Yuen, Kricka, 2001), embedded SPE

(Legendre, Bienvenue, 2006), and immuno-magnetic beads (Easley et al. , 2006a, Juergen, Yi, 2008, Kang-Yi, Chien-Ju, 2008, Liu, Yang, 2004, Pipper, Inoue, 2007) have been reported. Other types of crude samples including oral fluid (Legendre, Bienvenue, 2006, Pipper, Inoue, 2007), nasal aspirate (Easley, Karlinsey, 2006a, Legendre, Bienvenue, 2006), urine (Govind, Ryan, 2006), semen (Legendre, Bienvenue, 2006), and serum (Anderson, Su, 2000, Lee et al. , 2009) have also been reported. Table 2 summarizes examples of such on-chip biological sample preparation devices integrated with downstream analysis modules. Many of these devices utilize magnetic beads based nucleic acid purification and transportation mainly because the nucleic acids can be precisely handled via manipulating the magnetic bead with syringe pumps and magnets.

Recently, open surface droplet based microfluidic devices have attracted increasing attention due to their benefits for system integration with pre- and post-PCR modules (Fan et al. , 2009, Guttenberg et al. , 2005, Hsieh et al. , 2006, Juergen, Yi, 2008, Lehmann et al. , 2006, Ohashi et al. , 2007, Pipper, Inoue, 2007, Shastry et al. , 2005, Teh, Lin, 2008, Velev et al. , 2003, Zhang et al. , 2009a, Zhang, Bailey, 2009b, Zhang, Park, 2011, Zhang, Park, 2010). The on-chip actuation methods of free droplets, either active actuation such as surface acoustic wave (Guttenberg, Muller, 2005), electrowetting (Fan, Hsieh, 2009), dielectrophoresis (Fan, Hsieh, 2009, Velev, Prevo, 2003), magnetic force (Hsieh, Zhang, 2006, Juergen, Yi, 2008, Lehmann, Vandevyver, 2006, Ohashi, Kuyama, 2007, Pipper, Inoue, 2007, Zhang, Park, 2011) or passive actuation (Shastry, Case, 2005, Zhang, Cheng, 2009a) are being extensively investigated. Recent optoelectronic approaches demonstrated functional flexibility for electrowetting or dielectrophoresis based droplet manipulation by employing a light-activated reconfigurable electrode (Chiou et al. , 2003, Park et al. , 2009b, Valley et al. , 2011). The magnet-actuated droplet is especially preferred as the entire operation can be performed through a single magnet, enabling valveless and pumpless POC sample preparation and analysis with reduced cost and complexity. In addition, the magnetic particles used for droplet actuation are often functionalized to serve as carriers for biomolecules, such as silica superparamagnetic particles (SSP) for nucleic acid binding and transfer. This approach has been applied for DNA preparation module for whole blood samples (Sista et al. , 2008) and a fully integrated droplet based system for detecting H5N1 avian flu virus from crude throat swab sample (Pipper, Inoue, 2007). Advances in an open surface droplet based system have enabled automated droplet control with relatively simple device configurations, but large-scale integration for high throughput applications with such devices is still limited (Mohamed and Aaron, 2009).

6. Design constraints in developing countries

Although use of POC devices is feasible in a number of settings (e.g. physician’s office, emergency first responder, home, etc.) with different design constraints, for maximum range of use, the ideal POC device should be designed for remote testing in developing countries. The outskirts of the medical care system typically lack laboratory infrastructure. Electrical power, clean water, and cold storage are intermittently present or absent. Given such physical limitations, devices should ideally be battery- or solar-powered, and not rely on external water if a high quality supply is needed. Also, reagents must be able to withstand large fluctuations in temperatures. The lack of trained personnel requires devices to be simple to use with easy-to-interpret results. Self-calibration with controls along with test samples should be incorporated into the design. Turnaround time ranging from a few minutes up to 1 h would be ideal to implement treatment during the same health-care encounter given lack of resources to ensure patient follow up. To ensure affordability of the end product, single-use, disposable microfluidic chips composed of inexpensive polymer materials through advanced fabrication methods would reduce the cost of production. The small quantities of sample and reagents consumed should be retained within the disposable

to avoid contamination with the external instrument and minimize spread of biohazards. Based upon the mathematical modeling analysis of disease impact as reported by Global Health Diagnostic Forum, the sensitivity and specificity requirements for diagnostic tests should range from 85%-95% and 76%-97% respectively depending on various clinical diagnostic considerations (Urdea, Penny, 2006). The WHO has developed a list of general characteristics that make a diagnostic test appropriate for resource-limited sites, which are abbreviated using the acronym ASSURED (Table 3) (Mabey, Peeling, 2004). Striving for the most accurate test, however, should not prevent the development of the most useful test. A test that is less sensitive but rapid may result in more infected people receiving treatment, as not all patients return for the results of tests. So, the best test is not always the most useful test; the context of the end-user's setting needs to be considered.

7. Current challenges and future perspectives

7.1 Multiplex detection

From the clinical perspective, the same clinical symptom can be caused by infections from many etiologic agents; therefore, a POC test which can simultaneously screen/detect multiple pathogens from a single specimen would be highly desirable. Test design strategies include comprehensive identification of all clinically-relevant pathogens, or a flexible platform that detects a panel of suspected pathogens which can be readily changed based on symptomatology and local epidemiology. Assay approaches that split the initial specimen for parallel simplex PCRs, each with a single primer set specific for a pathogen, are often not possible due to limited quantity of target DNA. Alternatively, multiplex PCR using multiple primer sets in the same reaction can decrease specimen and reagent consumption but is limited in detection sensitivity due to uneven amplification efficiencies of the different primer sets (Elnifro et al. , 2000). PCR using a single primer set targeting phylogenetically conserved sequences (e.g. *16S rRNA*) can allow broad-range pathogen detection without compromising sensitivity (Yang et al. , 2002, Yang and Rothman, 2004). Moreover, broad-range PCR can also serve as a "molecular petri dish" to identify unsuspected, mutated, or emerging infectious causes of diseases. It is also ideal for use to determine the presence of any infection in an otherwise sterile specimen. RT-PCR amplification of 16S rRNA has also been found to positively correlate with microbial viability, which may provide indications of active infection and prove useful in assessing efficacy of antimicrobial treatment (Keer and Birch, 2003). Sequence analysis of the amplified product from broad-range PCR using differentially labeled fluorogenic probes has been incorporated into real-time PCR assays to identify a small panel of pathogens. Oligonucleotide arrays can greatly expand the number of pathogens identifiable and the integration of PCR with microarray on a microfluidic platform has been developed (Liu, Yang, 2004). Nonetheless, the principle shortcoming of all probe-based approaches to amplicon analysis is still limited to a pre-defined set of pathogens identifiable. Direct sequencing of amplicons would allow identification of emerging or unsuspected pathogens, and a microfluidic array device integrated with an on-chip sequencing assay has recently been developed to identify mutant strains of influenza virus (Liu et al. , 2006). Alternatively, high-resolution melt analysis (HRMA), which detects sequence variations within the amplicons based on their melting curve profiles, offers a simple, low-cost, PCR-compatible solution to broad-scale pathogen identification (Yang et al. , 2009).

7.2 Multiple analytes analysis

Assisting clinicians in the differential diagnosis of diseases that produce common symptoms, identifying multiple classes of microbes (e.g. virus, bacteria, fungi, etc.) which may be involved, determining disease severity and predicting clinical outcomes based on host response, a having the capacity to analyze multiple samples and biomarker types are all

attributes of an ideal assay. For example, acute lower respiratory infection with complex etiologies may require testing multiple sample types, including sputum, nasopharyngeal aspirate, and blood, to ensure adequate detection of all relevant respiratory pathogens while determining if infection has progressed into the bloodstream. Detection of inflammatory or pathogen biomarkers derived from various types of analytes (e.g. DNA, RNA, proteins, toxin, etc.) would require integration of different assay formats, including PCR, RT-PCR, and immunoassays, onto the same platform. While conserved or specific DNA targets can be detected in conventional PCR, RT-PCR allows detection of RNA viruses as well as microbial rRNA/mRNA targets to assess microbial viability; however, given the highly labile nature of RNA molecules with short half-life, additional measures must be taken in sample preparation and reverse transcription of RNA to avoid degradation (Keer and Birch, 2003). Many microfludic-based devices have been developed for testing various analyte types individually (Chen et al. , 2007, Roper et al. , 2005), but combined testing on a single platform has yet to be achieved. On the other hand, a droplet-based microfluidic device has been reported to simultaneously analyze multiple sample types using the electrowetting effect for fluid actuation (Srinivasan et al. , 2004). Future research in this direction can significantly increase the utility of new tests.

7.3 Antibiotic susceptibility

A POC diagnostic for infectious pathogens will not supplant traditional culture-based methods if the former cannot rapidly characterize the antimicrobial susceptibility profile of the detected pathogen to help direct early therapy. Traditional laboratory approaches for susceptibility testing, which rely mostly on phenotypic assays utilizing in vitro growth inhibition of a microorganism in the presence of antibiotics, are slow and resource demanding. Although genotypic-based (e.g. PCR) detection of specific antimicrobial resistance genes may offer a more rapid alternative, the genetic mechanisms for resistance are diverse and the presence of genetic marker may not always confer resistance. Therefore, antimicrobial susceptibility will continue to be more accurately determined by phenotypic methods.

Microfluidics is an attractive approach for susceptibility testing by providing a miniaturized fluidic environment suitable for monitoring cell growth in the presence of antibiotics (Balaban et al. , 2004, Orit and Nathalie, 2009). High surface-to-volume ratio in microfluidic channels has been demonstrated to enhance bacterial oxygenation and reproduction, reducing susceptibility testing time to 2 hours as compared to days using traditional methods (Chen et al. , 2010). Droplet-based microfluidic platforms can potentially allow single cell testing with multiple antibiotics (Boedicker et al. , 2008). Monitoring physiologic stress responses, instead of cell count, through measurable changes in specific cellular properties (e.g. morphology, membrane potential, dielectric properties, metabolites, etc.) may offer more rapid alternative approaches for assessing drug susceptibility (Kohanski et al. , 2010, Mann and Mikkelsen, 2008).

8. Conclusions

With the pressing need for developing new technologies toward near-patient testing for infectious diseases, microfluidic PCR is well-positioned to contribute to this challenge by leveraging its inherent advantages and recent advances in many aspects of the field, including device material, microfabrication, thermal management, amplicon detection and sample preparation. A paradigm switch from component and device based research to a system and product oriented approach is currently underway in order to face the next challenge: translating technologies into reliable and cost-effective clinical diagnostics with flexible capabilities adaptable to the context of end users. Advanced design considerations based on clinical context may incorporateetiologic pathogens suspected, patient

symptomatologies, resource limitations, and treatment decisions. The new challenge will necessitate new ways of thinking in order to realize the full potential of microfluidic PCR technologies.

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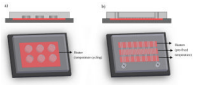

Figure 1.
Schematic illustrations of types of microfluidic PCR chip designs. a) stationary chamber system b) continuous flow system

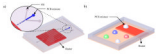

Figure 2.
Schematic illustrations of droplet based microfluidic PCR chips. a) Droplet emulsion system b) Open surface droplet based system

Table 1

FDA guidance for simple test

Is a fully automated instrument or a unitized or self-contained test.
Uses direct unprocessed specimens, such as capillary blood (fingerstick), venous whole blood, nasal swabs, throat swabs, or urine.
Needs only basic, non-technique-dependent specimen manipulation, including any for decontamination.
Needs only basic, non-technique-dependent reagent manipulation, such as “mix reagent A and reagent B.”
Needs no operator intervention during the analysis steps.
Needs no technical or specialized training with respect to troubleshooting or interpretation of multiple or complex error codes.
Needs no electronic or mechanical maintenance beyond simple tasks, e.g., changing a battery or power cord.
Produces results that require no operator calibration, interpretation, or calculation.
Produces results that are easy to determine, such as ‘positive’ or ‘negative,’ a direct readout of numerical values, the clear presence or absence of a line, or obvious color gradations.
Provides instructions in the package insert for obtaining and shipping specimens for confirmation testing in cases where such testing is clinically advisable.
Has test performance comparable to a traceable reference method as demonstrated by studies in which intended operators perform the test. If a reference method is not available for a test you are proposing for waiver, please contact OIVD to discuss your proposed plan prior to submitting your application.
Contains a quick reference instruction sheet that is written at no higher than a 7th grade reading level.
Sample manipulation should NOT be required to perform the assay. (For example, tests that use plasma or serum are not considered simple.) Sample manipulation includes processes such as centrifugation, complex mixing steps, or evaluation of the sample by the operator for conditions such as hemolysis or lipemia.
Measurement of an analyte should NOT be affected by conditions such as sample turbidity or cell lysis.

Recommendations: Clinical Laboratory Improvement Amendments of 1988 (CLIA) Waiver Applications for Manufacturers of In Vitro Diagnostic Devices (http://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm070890.pdf)

Table 2

Microfluidic PCR devices with on-chip crude sample processing capability

Ref.	Sample Type	Sample Volume	Detection Target	Sample Pre-processing	On-chip Detection
(Panaro, Lou, 2005, Wilding, Kricka, 1998, Yuen, Kricka, 2001)	Whole blood	< 3.5 μl	WBC	Filtration (micropillar)	N/A
(Liu, Yang, 2004)	Whole blood	1 ml	E. coli	SPE (bead)	Electrochemical
(Juergen, Yi, 2008, Pipper, Inoue, 2007)	- Throat swab - Whole blood	25 μl	- influenza virus H5N1 - THP-1 cells	SPE (bead)	Real-time (Taqman)
(Easley, Karlinsey, 2006a)	- Whole blood - Nasal aspirate	1 μl	- Bacillus anthracis spores - Bordetella pertussis	SPE (bead)	Electrophoresis
(Munchow, Dadic, 2005)	Whole blood	8 μl	WBC	Filtration (fiber matrix)	N/A
(Legendre, Bienvenue, 2006)	- Whole blood -Nasal swab - Semen	4~200 μl	- Human gene - Anthrax spores	SPE (packed bed)	N/A
(Kang-Yi, Chien-Ju, 2008, Lee, Lien, 2009)	- Whole blood - Serum	100~ 200 μl	- leucocytes - Dengue virus	SPE (bead)	N/A
(Govind, Ryan, 2006)	Urine	0.24 μl	BK virus	N/A	Electrophoresis
(Anderson, Su, 2000)	Serum	100 μl	HIV RNA	SPE (Cellulose)	Hybridization microarray

Table 3

Characteristics of the ideal diagnostic test – ASSURED (Mabey, Peeling, 2004)

Affordable by those at risk of infection
Sensitive (few false-negatives)
Specific (few false-positives)
User-friendly (simple to perform and requiring minimal training)
Rapid (to enable treatment at first visit) and Robust (does not require refrigerated storage)
Equipment-free
Delivered to those who need it