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Real-time PCR-based microfluidic array chip for simultaneous detection of multiple waterborne pathogens

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ABSTRACT

We report disposable microfluidic devices harboring an array of unsealed reactors, which are pre-loaded with different primer pairs for simultaneous (parallel) real-time PCR-based detection of multiple waterborne pathogens. The PCR mixture loading among an array of reactors and subsequent isolation of the reactors was solely realized by a single step capillary-based flow scheme, which eliminates the use of pumps, valves and liquid handling instruments. We incorporated a localized thermal cycling scheme to minimize evaporative loss of PCR mixture in unsealed microreactors, which greatly reduces the complexity of the microfabrication and fluidic operation process, in cases, where valving or sealing of the reactors for PCR thermal cycling is required. Experiments were performed to determine the optimal microreactor design parameters, so as to perform bubble-free PCR with minimal evaporative loss in unsealed reactors. The potential of the microfluidic device was successfully demonstrated by specifically detecting genomic DNA of waterborne pathogens from a pool of genomic DNA template.

1. Introduction

Water is undoubtedly one of the major resources necessary for well-being of human and animal existence. According to the last report by World Health Organization (WHO) in 2004, about 2.9 million people die due to waterborne pathogens, in a year [1]. As per World Health Organization (WHO) guidelines for drinking water quality, it is unrealistic to examine drinking water for every probable microbial pathogen. Thus water quality monitoring for presence of waterborne pathogens, relies on detection of indicator bacteria such as coliforms (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*), and other potential waterborne pathogens like *Aeromonas hydrophila*, *Staphylococcus aureus*. *E. coli* is a member of faeceal coliform group and is a specific indicator of feceal pollution than any other coliforms. It is therefore highly recommended by WHO, US-Environmental Protection Agency (US-EPA), and other environmental health agencies that public water systems must conduct analysis for faecal coliforms or *E. coli* for routine water quality checks. The control of microbiological hazards to reduce the incidence of infectious disease requires rapid and simultaneous detection of multiple microbial pathogens including bacteria, viruses, and protozoa. Traditional cell-culture methods are time consuming, and some pathogens are not cultivable. With recent advances in PCR methods and microarray assays, rapid methods for waterborne pathogen community composition and quantitation of pathogen load are being realized.

In the past, multiplex PCR and microarrays were reported as a high-throughput platform for simultaneous analyses of multiple gene targets. Simultaneous detection of different types of waterborne bacterial pathogens including *Aeromonas* spp., *Salmonellae* spp., *Shigella* spp., *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Y. enterocolitica* was demonstrated using multiplex PCR system [2]. In another work, simultaneous detection of multiple microbial pathogens including a *V. cholerae* (bacterium), *Calicivirus* (virus) and *Aureococcus amophagefferens* (protist) in ships' ballast water was reported using multiplex PCR [3]. However, multiplex PCR involves an arduous design process for multiple primer pairs that should perform equally well in a single reaction tube. The probability of primer dimer formation is very high, and this might lead to poor sensitivity and preferential amplification of certain targets. Alternatively, microarrays have been successfully used to analyze the pathogen content in wastewater [4]. However, the sensitivity of microarray technology is low and the multiple steps involved in this technology introduce variability in the results when compared to real-time PCR [5]. In addition to the microarray technology for parallel analyses of multiple pathogens, a number of platforms have been developed to integrate PCR with microarray techniques [6,7]. Most of these platforms combine solid-phase PCR with microarray platform. However, studies show that solid-phase PCR is 20–30% less efficient than solution-phase PCR [8]. Lee et al.

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compared genomic DNA microarray and amplified DNA microarray (PCR followed by microarray) with real-time PCR assay for detection of waterborne pathogen, and found that real-time PCR assay is ~10^8 times more sensitive than genomic DNA microarray and ~70 times more sensitive than amplified DNA microarray (PCR product tested on microarray) [4]. Currently, real-time PCR is used as the "gold standard" to validate the data generated by microarray platforms, and it has been successfully used for enumeration of waterborne pathogens from sewage samples in microliter reaction volume scale using a conventional real-time PCR instrument [9–13]. Unfortunately, real-time PCR in conventional systems is limited by its capability to perform parallel analyses of multitude of samples and assays (<384 wells plates). Recently, Roche Applied Science (Indianapolis, USA) released LightCycler® 1536 Real-Time PCR System, which uses a 1536 multiwell plate. Again, this system requires a robotic liquid handling instrument to the load samples. The performance of this system is yet to be evaluated by researchers. In order to simultaneously analyze multiple gene targets (>1536 samples/assays), research groups are making immense effort to develop PCR-based microchip devices as a high-throughput platform.

Most of the reported PCR-based microchip devices required tedious, manual PCR mixture loading into individual reaction wells in a PCR array chip, similar to that used in a well-plate operation, required an expensive liquid dispensing robot/pneumatic pressure source, or involved immobilization of primers in a matrix [14–17]. In an effort to achieve high-throughput PCR on-chip, Nagai et al. developed a microchamber array on silicon substrate for picoliter PCR using manual sample loading step, and the amplification product was characterized by comparing the fluorescence intensity at the beginning and at the end of the PCR process [14]; while Matsubara et al. developed a microchamber array in which the DNA sample was loaded by using a nanoliter dispensing robotic system and the PCR data was analyzed using a DNA microarray scanner [15]. Brenan et al. reported a rectilinear array of 3072 through-holes in a stainless steel platen, in which the primer pairs were immobilized in a matrix and the array of through-holes were sealed inside a cassette with UV curable epoxy [16]. This chip is commercially sold by BioTrove (OpenArray® DLP Real-Time qPCR System; BioTrove, Woburn, USA; www.biotrove.com). Using this system, Stedtfeld et al. reported detection and identification of multiple pathogens from environmental waters by targeting a large number of virulence and marker genes [12]. Although, this system provides solutions to high-throughput real-time PCR, the loading is based on differential surface treatment of the steel platen, this requirement makes the chip fabrication process laborious and expensive.

One of the major challenges associated with the development of cost-effective, high-throughput PCR-based biochips is to develop a non-robotic/micropump-less based manual liquid loading method to facilitate development of point-of-care diagnostic devices. The manual liquid loading method includes nucleic acid sample loading into a large number of reaction wells in micro and nanoliter volume ranges and subsequent isolation of all these wells to avoid cross-contamination. In the literature, few researchers have employed micropumps in conjunction with an array of valves for performing PCR on-chip, among them Liu et al. [17] demonstrated the microfluidic distribution of 2 μl of PCR mixture among 400 independent reactors, using 2860 integrated hydraulic valves and pneumatic pumps, and Mathies and co-workers [18] demonstrated multiple PCR (four) on-chip with a microfluidic PCR mixture distribution, using a mechanical valve array for sample loading and well sealing. Implementation of microvalve array, adds complexity to the chip fabrication and operations processes, and also reduces the space on the microchip. Another challenge is to seal an array of reactors. Although mineral oils and pressure-sensitive adhesive tapes have been successfully utilized for sealing microreactors [19,20], most of the reported nucleic acid amplification systems utilized microvalves to seal the reactors [17,18,21,22]. In order to reduce the complexity of the chip fabrication and operation process, we developed a microPCR array chip harboring open or unsealed reactors so as to overcome the requirement of microvalves for sealing the reactors, while capillary microfluidics was implemented to overcome the requirement of robotic/micropump based sample loading methods.

In this paper we report a microPCR chip comprising an array of reactors, pre-loaded with primer pairs for simultaneous (parallel) PCR analyses of multiple waterborne pathogens. The microfluidic operation is simple, involves a single manual pipeting step, and can be performed in a point-of-care setup, without the need of sophisticated liquid handling systems for sample loading, and isolation of microreactors. The capability of the PCR array chip was demonstrated by simultaneously detecting four waterborne pathogens: *P. aeruginosa*, *A. hydrophila*, *K. pneumoniae* and *S. aureus*. However, the number of reactors on our chip can be expanded to analyze a panel of waterborne pathogens.

2. Experimental

2.1. Chip design

The aim of our PCR array chip is to simultaneously (parallel) detect multiple waterborne pathogens. The PCR array chip, incorporating microreactors, sample loading and waste channels, inlet and outlet bridge channels was fabricated on poly(dimethylsiloxane) (PDMS) substrate by a laser cutting technique, and then bonded to a 0.1 mm thick glass substrate. Such a hybrid design circumvents the expensive glass microfabrication processes, but still utilizes the superior properties of glass such as rigidity, flatness, and higher thermal conductivity compared to PDMS. The schematic layout of the PCR microreactor and channel networks on the chip is shown in Fig. 1(A). During the chip fabrication process, different microreactors are deposited with different primer pair (dry form) for detection of multiple waterborne pathogens. The loading channel is used for distributing PCR mixture (without primer pair) containing a pool of DNA templates, into an array of microreactors. During PCR mixture loading step, the liquid flows into the microreactor and re-suspends the dried primer pair, while the air inside the microreactors is purged out through the air venting port. After complete filling of the microreactors, the liquid samples inside the microreactors are isolated from each other by autonomous removal of the excess PCR mixture in the loading channel by an absorbent pad. The microreactors on our array chip are not sealed during the PCR thermal cycling, and the sample evaporative loss in these open/unsealed reactors was well controlled by reactor design, reactor surface and heating region of the PCR array chip. Reduction in sample evaporative loss in our open/unsealed reactors was achieved by (1). Localized heating of the microreactors with a peltier device, while the long narrow bridge channels connected to the microreactor is automatically maintained at the room temperature Fig. 1(C). (2) Smooth internal surface of the microreators, so as to prevent generation of air bubbles inside the reactors during PCR thermal cycling. Fabrication of a smooth internal surface of the reactor is critical for success of PCR in our array chip, since presence of air bubbles inside the microreactors will purge the liquid out of the reactors due to bubble expansion.

A photograph of the prototype PDMS-glass chip, comprising an array of nine microreactors for PCR is shown in Fig. 1(D). The chip contains four microreactors connected to a common loading channel for target sample (positive control reaction), four microreactors connected to another common loading channel for no-template