Microchim Acta (2011) 173:503–512
DOI 10.1007/s00604-011-0584-5

ORIGINAL PAPER

Simultaneous detection of *Salmonella enterica, Escherichia coli* O157:H7, and *Listeria monocytogenes* using oscillatory-flow multiplex PCR

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Received: 23 November 2010 / Accepted: 4 March 2011 / Published online: 23 March 2011

Abstract An oscillatory-flow multiplex PCR method in a capillary microfluidic channel has been developed for the simultaneous determination of pre-purified DNA of multiple foodborne bacterial pathogens. The PCR solution passes three temperature zones in an oscillatory manner. The thermal stability and sample evaporation of the microfluidic device were investigated. Under controlled conditions, a highly efficient multiplex PCR was accomplished as demonstrated for the simultaneous amplifications of 278 bp, 168 bp, and 106 bp DNA fragments within 35 min after 35 cycles for simultaneous detection of *Salmonella enterica*, *Escherichia coli* O157:H7, and *Listeria monocytogenes*. This is much shorter than that of a conventional PCR machine. The detection limits of bacterial genome DNA for the three species are about 399, 314, and 626 copies per μL, respectively. This is comparable to those obtained with the conventional multiplex PCR. Consequently, the oscillatory-flow multiplex PCR technology holds good potential for rapid amplification and detection of nucleic acids of microbial foodborne pathogens.

Keywords Microfluidics · Oscillatory-flow · Multiplex polymerase chain reaction · Foodborne bacterial pathogens

Electronic supplementary material The online version of this article (doi:10.1007/s00604-011-0584-5) contains supplementary material, which is available to authorized users.

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Introduction

Polymerase chain reaction (PCR) is an in vitro nucleic acid amplification technique which was first reported by Kary Mullis [1]. It has the advantages of high sensitivity, fast speed, and relative simplicity and robustness, and therefore has been successfully applied in many fields such as health and clinical diagnosis, environmental monitoring, and food safety. Multiplex PCR, which is a variant of PCR, has been well used to reduce the cost and also to increase the diagnostic capacity of PCR [2, 3]. Because more than one target sequence can be amplified by including more than one pair of primers in the reaction solution, multiplex PCR has the potential to result in considerable savings of time and effort within the laboratory without compromising test utility [2]. Since its introduction [4], multiplex PCR has been successfully used in many areas of nucleic acid diagnostics, such as identification of foodborne bacterial pathogens [5, 6]. However, most of multiplex PCR amplifications are performed in a conventional thermal cycler. As a result, 2–3 h is often required for 30–40 cycles, and most time is spent on temperature ramping during the reaction process. In addition, the conventional multiplex PCR usually needs a relatively large reaction volume (ranging from 25 μL to 100 μL). Therefore, the multiplex PCR approach is still time-consuming and expensive when standard thermocyclers are used.

Recently, great attention has focused on developing microfluidics-based PCR devices, since they can offer rapid thermal cycling, reduced analysis times, low reagent/energy consumption, portability, and potential for high automation and integration of various analytical procedures. Nowadays, there are in general two types of microfluidic PCR: stationary microchamber PCR and continuous-flow PCR [7–9]. The micro-chamber PCR is miniaturization of

conventional PCR in nature, where PCR solution is kept stationary inside a confined chamber and temperature cycling of both solution and chamber is performed using different ways [10–17]. Within these PCR microdevices, the temperature transition rate and power consumption are still greatly affected by the thermal mass surrounding the chambers. Therefore, in order to reduce the reaction time and power consumption, the system's thermal mass has to be optimized considerably. For continuous-flow PCR, DNA amplification cycling occurs as the PCR solution is continuously pumped through a microchannel incorporating two or three thermally isolated reaction zones [18–23]. Using this approach, PCR can obtain a rapid temperature transition rate, and as a result the reaction time is greatly decreased. Up to now, the fastest PCR was obtained on the continuous-flow PCR microfluidic chip reported by Soper's group—amplification of a 500-bp DNA fragment in 1.7 min and a 997-bp fragment in 3.2 min, respectively [24]. An alternative method is oscillating-flow PCR, where PCR solution flows through two or three temperature zones in an oscillating manner. The oscillatory-flow PCR approach not only combines the cycling flexibility of the stationary microchamber PCR with fast temperature transitions associated with the continuous-flow PCR, but also offers the possibility of performing high-throughput PCR reactions in parallel. During the past years, several research groups have developed the oscillatory-flow PCR microfluidic device to perform fast DNA amplification [25–32]. For example, Wang et al. have reported a droplet-based micro oscillatory-flow PCR chip, where the amplification of a human papilloma virus (HPV) DNA fragment was accomplished within about 15 min, which was about one ninth of that for the conventional PCR machine [25]. Recently, Sista et al. have developed an electrowetting-based digital microfluidic platform for point of care testing [31]. In this microfluidic cartridge, the oscillatory-flow real-time PCR was performed within 12 min. In addition, the magnetic bead-based immunoassays, as well as sample preparation for bacterial pathogen (methicillin-resistant *Staphylococcus aureus*, MRSA) and for human genomic DNA, have been successfully demonstrated on the same microfluidic cartridge.

To analyze different DNA samples in diagnostic laboratories, several groups have developed the multiplex PCR chips, where amplification of different DNAs was performed in different microchambers on a single chip [33–35]. For example, Ramalingam et al. reported a real-time PCR array chip that was preloaded with different primer pairs for simultaneous detection of multiple waterborne pathogens [33]. The development of this type of multiplex PCR microfluidics provided a potential means for high-throughput nucleic acid analysis for different target samples. However, precise and accurate transfer of multiple reaction solutions from the macroscale systems to the microchamber array still faces challenges. In 2008, Christensen et al. developed the multiplex PCR in a SU-8 chip for detection of *Campylobacter* at species level [36]. Similar to the conventional multiplex PCR, three DNA target fragments of different lengths were simultaneously amplified in a single reaction chamber. Compared with the aforementioned multiplex PCR on an array chip, this type of multiplex PCR has several obvious advantages, such as decreased reagent consumption, increased simplicity in sample handling and structure designing, and easier integration with various functions. However, these two kinds of microfluidic multiplex PCR still have the inherent shortcomings of the stationary microchamber PCR amplification.

To overcome these shortcomings, amplification of multiple DNA fragments on the continuous-flow microfluidics has been developed. This kind of microfluidics is usually performed by using a continuous segmented-flow of different PCR solutions containing different DNA samples [19, 20]. This format of amplification can save much time and labor. However, such systems easily suffer from cross-contamination between samples and sample dispersion. The alternative method is performing parallel PCR in different channels. For example, Sun et al. reported a multichannel closed-loop magnetically actuated microchip for high-throughput PCR and four gene fragments were simultaneously amplified in four different channels of the PCR microchip [22]. In 2007, Frey et al. demonstrated a multi-channel oscillatory-flow chip for real-time PCR integrated with liquid handling, and proposed the possibility of realizing high-throughput PCR in different channels by synchronous actuation of the pumping membranes [30]. However, this work does not provide the high-throughput PCR results. The aforementioned serial or parallel formats for continuous-flow amplification of multiple target fragments can perform very well in many aspects. However, they usually involve complicated fluid handling and control so as to perform amplification of different DNA fragments. Moreover, the chip-based multi-channel design potentially increases the total fabrication cost and complicates the structure assembly.

In this work, we present a simple capillary-based microfluidic device for the novel oscillatory-flow multiplex PCR, where three target fragments of different lengths are amplified in a single reaction solution for simultaneous detection of three foodborne bacterial pathogens—*Salmonella enterica* (*S. enterica*), *Escherichia coli* O157:H7 (*E. coli* O157:H7) and *Listeria monocytogenes* (*L. monocytogenes*). The device mainly consists of three heated copper blocks that provide temperatures for oscillatory-flow multiplex PCR, and a single polytetrafluoroethylene (PTFE) capillary tube that is embedded on the grooves of copper blocks as the reaction

channel. To perform multiplex PCR, the PCR solution is moved back and forth over different temperature zones by a precision syringe pump.

Materials and methods

Design of the experimental arrangement

The experiments were performed on the computer-controlled, LabView-based temperature control and measurement platform developed in our laboratory [37]. The setup mainly consists of the following parts: three grooved copper heating blocks (40 mm×25 mm×15 mm) which were machined by Automation Engineering R&M Centre, Guangdong Academy of Sciences (Guangzhou, China, www.autocenter.gd.cn), three resistance cartridge heaters (100 W, 8 mm×40 mm (o.d. × length), Guangzhou Haoyi Thermal Electronics Factory, China), three K-type thermocouples (0.005 in. diameter, Omega Engineering Inc., Stamford, CT, USA, www.omega.com), the PCI control module (PCI-4351, National Instruments Corp., Austin, TX, USA, www.ni.com), the terminal block (TBX-68 T), and the relay module. Each of the copper blocks includes one larger central hole (8 mm diameter) for the resistance cartridge heater and two small holes for the K-type thermocouples. To keep three temperatures for denaturation at 95°C, annealing at 56°C, and extension at 72°C, a computer received the temperature signal through a PCI-4351 interface and determined the power input to the heater using a home-made fuzzy proportional/integral/derivative (PID) control algorithm that was programmed with LabView 8.0 (Version 8.0, National Instrument Corp., Austin, TX, USA, www.ni.com). A single-use polytetrafluoroethylene (PTFE) tubing (~15 cm) (inner diameter 500 μm, Wuxi Xiangjian Tetrafluoroethylene Product Co. Ltd., Wuxi, China, www.wxxj.com) was used as a reaction channel, where the oscillatory-flow multiplex PCR was carried out when the solution was driven to shuttle between three heat zones by the precision syringe pump (CZ-74901-15, Cole Parmer, Illinois, USA, www.coleparmer.com). The temperatures in the PTFE channel were measured by the K-type thermocouple. During these measurements, the channel was filled with the 1 × Taq DNA polymerase buffer, and no bubble formation was observed. Figure 1 is a schematic diagram of the oscillatory-flow multiplex PCR thermocycler set-up.

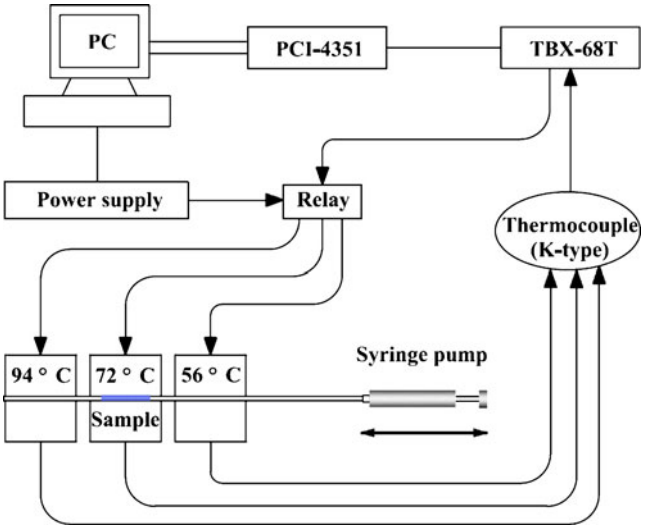

Fig. 1 Schematic diagram of the oscillatory-flow multiplex PCR microfluidics

Bacterial strains, growth conditions, and DNA isolation and quantification

Three bacterial strains including *S. enterica* CMCC50040, *E. coli* O157:H7 GW1.0202 and *L. monocytogenes* CMCC54007 were used in this study to evaluate the applicability of the presented method. They were obtained from Guangzhou Institute of Microbiology (Guangzhou, China, www.gdim.cn). All the bacterial strains were grown in Nutrient broth (0.3% beef extract, 1% soy peptone, and 0.5% NaCl) at 37°C overnight with rotary shaking at 200 rpm to obtain the desired concentration. The genomic DNA was extracted from 1 mL of overnight bacterial culture using the TIANamp Bacteria Genomic DNA Extraction Kit (Tiangen biotech (Beijing) Co., LTD., Beijing, China, www.tiangen.com) following the manufacturer's instructions, and re-suspended in a 100-μL TE buffer. The quantity and quality of the extracted DNA was determined by measuring A260 and the ratio of A260/A280 on the Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany, www.eppendorf.com).

Reagents and samples

The reaction reagents, 10 × Taq DNA polymerase buffer (500 mM KCl, 100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$), thermostable Taq polymerase (5 U μL^{-1}) and deoxynucleotide triphosphate (dNTPs) (2.5 mM each of dATP, dGTP, dCTP, and dTTP) were all purchased from TaKaRa Biotechnology Co., Lid (Dalian, China, www.takara.com.cn). The oligonucleotide primers (Table S1, Electronic Supplementary Material, ESM) were synthesized by Invitrogen Biotechnology Co., Ltd (Shanghai, China, www.invitrogen.com). The gene targets chosen for this study were the *invA* (invasion protein A) gene for *S. enterica*, the *rfbE* (O157-antigen) gene for *E. coli* O157:H7, and the *hlyA* (listeriolysin O) gene for *L. monocytogenes*, since they are described in the literature as being

among the most specific and reliable genetic targets for the considered microorganisms [38–40].

Bovine serum albumin (BSA) (Fraction V, Purity ≥ 98%, Biotechnology Grade, No. 735094), which was used to dynamically passivate the inner surface to decrease the surface adsorption, was bought from Roche Diagnostics GmbH (Mannheim, Germany, www.roche.com). Concentrated sulfuric acid, which was utilized to remove the possible PCR inhibitors from the microchannel before each run, was obtained from Tianjin Hongyan Chemical Reagent Factory (Tianjin, China, www.tjhongyan.b2b.cn). GoldView™ was purchased from SBS Genetech Co. Ltd. (Beijing, China, www.sbsbio.com). The DNA markers, which contain 500, 400, 300, 250, 200, 150, 100 and 50 bp DNA fragments, were from Dongsheng Biotech Co., Ltd. (Guangzhou, China, www.dongshengbio.com).

Modification of the inner surface of capillary

To decrease reaction inhibition and reagent absorption, the PTFE capillary was cleaned and passivated. First, the PTFE capillary was rinsed with concentrated sulfuric acid to eliminate the possible PCR inhibitors, and then washed with deionized water. Here, the use of concentrated sulfuric acid for PCR inhibitor removal is considered for the following two possible reasons. Strong acid can cause the degradation of DNA and protein that adsorb on the inner surface of the reaction channel. In addition, strong acid can destroy the intermolecular interactions which cause the surface adsorption. To further protect the reaction from surface inhibitory effects, the dynamic passivation method was used for the sacrificial surface adsorption of chemical additives comprising 0.5 μg μL^{-1} BSA. Moreover, in order to avoid contamination, the reaction capillary tube was discarded after each run in this study.

Oscillatory-flow multiplex PCR protocol

In the oscillatory-flow multiplex PCR, the assay was performed in 5 μL of PCR solution containing 2×PCR buffer, 0.3 mM of each dNTP, three pairs of primers (0.4 μM), 0.5 μg μL^{-1} BSA, 0–0.5 U μL^{-1} Taq DNA polymerase. In the reaction solution, unless stated otherwise, the concentration of each bacterial genomic DNA was 1.0 ng μL^{-1}. After being introduced into the capillary tube, 5-μL PCR solution was driven by the syringe pump. The PCR solution was flanked by 2 μL mineral oil to avoid evaporation. And, PCR-biocompatible bromophenol blue was also added to the PCR solution to easily observe the movement of the PCR solution. To verify the performance of the oscillatory-flow multiplex PCR, the positive-control multiplex PCR was performed on a conventional PCR instrument (Eppendorf Mastercycler Gradient, Hamburg, Germany, www.eppendorf.com). The thermocycling parameters included an initial incubation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s, with a final incubation at 72°C for 2 min. The products were analyzed with a 2% agarose gel electrophoresis prestained with GoldView™ dye.

Oscillatory-flow multiplex PCR at various flow rates

To demonstrate the rapid detection ability of the presented microfluidic device, oscillatory-flow multiplex PCR was performed at different flow rates of the corresponding multipex PCR solution through the thermal-cycling capillary. The flow velocity controlled by the syringe pump ranged from 1.70 to 12.73 mm s^{-1}. For comparison, the positive-control multiplex PCR was performed in the conventional machine using the same PCR solution. In addition, the negative-control multiplex PCR, without DNA sample in the reaction solution, was also run on the microfluidic device, where the flow rate of 2.55 mm s^{-1} was used.

Oscillatory-flow multiplex PCR using various concentrations of Taq polymerase or input DNA targets

Oscillatory-flow multiplex PCR was performed using a series of Taq polymerase concentrations ranging from 0.025 to 0.5 U μL^{-1}. In addition, the multiplex PCR solution without Taq polymerase was also prepared. Taq polymerase of various concentrations was included in 10 μL of the multiplex PCR solution. Then, 5 μL of the solution was introduced to the capillary for the oscillatory-flow multiplex PCR, where the flow rate was selected to be 2.55 mm s^{-1}, giving a cycle time of about 1 min. All of the remaining solution was subjected to a standard cycling procedure on the conventional PCR machine.

The oscillatory-flow multiplex PCR was also performed using ten-fold serial dilutions of DNA targets to evaluate the detection limit of the present microfluidic device, where the concentration of each bacterial genome DNA ranged from 1.0×10^{0} to 1.0×10^{-6} ng μL^{-1}, and the solution moved in the reaction channel at the flow rate of 2.55 mm s^{-1}. The positive-control multiplex PCR from the conventional machine was carried out, with 1.0 ng μL^{-1} of each bacterial genome DNA in the reaction solution. And, the negative-control PCR without DNA sample in the reaction solution was run at the flow rate of 2.55 mm s^{-1}.

Oscillatory-flow multiplex PCR at various cycle numbers

When the multiplex PCR of different number of cycles (40, 35, 30, 25, and 20 cycles) were done, the reaction solution

contained the same components as those mentioned above. However, 1.0 ng μL^{-1} of each bacterial genomic DNA and 0.2 U μL^{-1} of Taq DNA polymerase were used. The setting of cycle number could be accomplished on the precision syringe pump, and the flow rate of 2.55 mm s^{-1} was utilized.

Analysis of amplification products

The collected products were analyzed using a 2% agarose gel stained with GoldView™. Loading buffer contained 30 mM ethylenediaminetetraacetic acid (EDTA), 36% (v/v) glycerol, 0.05% (w/v) xylene Cyernol FF, and 0.05% (w/v) bromophenol blue (BPB). The gel was prepared with 2% (w/v) agarose in 20 mL of 1× TAE buffer (40 mM Tris-acetate 2 mM EDTA, pH 8.5) containing 1 μL of GoldView™ dye. The gel was run for 30 min at a constant voltage of 100 V, and then the amplified fragments were visualized under UV light. The DNA markers were used as the evaluation standards for the amplified DNA fragments.

Results and discussions

Evaluation of the oscillatory-flow multiplex PCR microreactor

To perform a successful multiplex amplification, good temperature control is necessary since it is one of the most crucial factors affecting the PCR efficiency and even specificity. In this study, the multiplex PCR was performed in a capillary microchannel, and therefore the temperature distribution of the microfluidic channel was first investigated. Figure 2a displays the schematic drawing of temperature data points along the capillary microchannel. In order to obtain a relatively accurate temperature measurement of the fluid in the channel, the thermocouple was inserted into the capillary channel filled with the 1 × Taq DNA polymerase buffer. No bubble formation was observed in any of the experiments. Points 1–4 were located in denaturation zone, points 6–9 in extension zone, points 11–14 in annealing zone, point 5 in adjacent zones between annealing zone and extension zone and point 10 in adjacent zones between extension zone and annealing zone. Figure 2b shows the temperature distributions in the capillary tube incorporated into the annealing, extension and denaturation zones with predefined temperatures of 56, 72, and 94°C, respectively. As seen from Fig. 2b, three desired temperature zones were created, and the temperature kept stable in each zone. In addition, the temperatures changed sharply between the two adjacent zones. This can be explained by the fact that the air gaps have resulted in the proper thermal isolation for oscillatory-flow multiplex PCR. Therefore, a complete three-temperature PCR cycling can be obtained when PCR solution flows back and forth through the three temperature zones. In the oscillatory-flow cycling process, when the reaction solution in the capillary flows into a temperature zone, several seconds are usually required to obtain the desirable reaction solution temperature. This time is influenced by the transport velocity of the reaction fluid in the capillary. The related works have been reported in the literature [25, 29].

For microfluidic PCR, sample evaporation is often a problematic issue because PCR volumes are usually small. In addition, the open reaction channel is also often used within the oscillatory-flow PCR devices, which helps the sample evaporation. The evaporation loss of reaction solution will lead to the changes in the concentration of PCR components and thus an ineffective DNA amplification. In this study, non-miscible mineral oil was used as a liquid cover to prevent evaporation. During run, a 5 μL PCR volume plug was flanked by 2 μL of mineral oil. By this method, the evaporation loss is only about 5% after an amplification process of 35 cycles (Fig. S1, ESM). In addition, it should be noted that the used oil cap can also effectively avoid the breakup of the reaction plug during thermocycling [29].

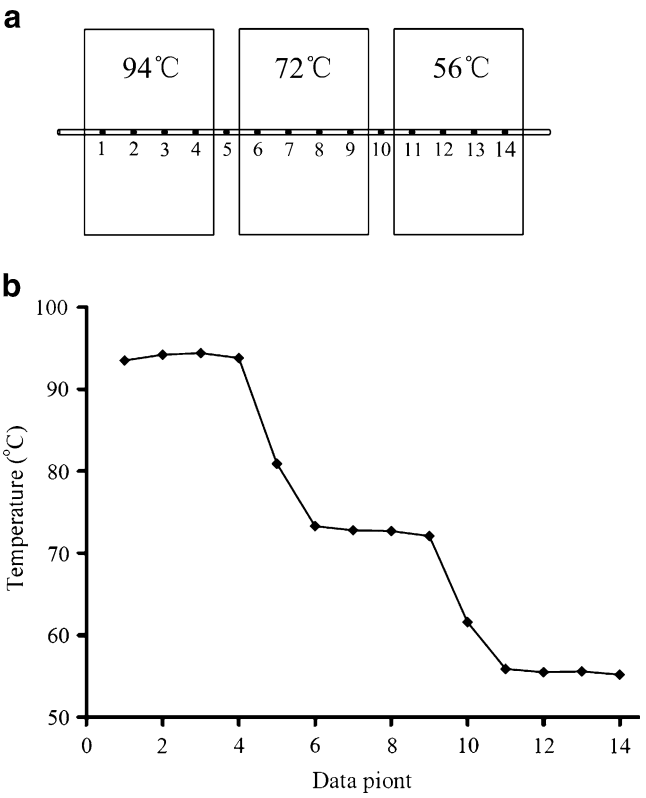

Fig. 2 **a** Schematic drawing of temperature data points along the microchannel. **b** Temperature distribution in the microchannel. Data at each point was measured five times

Specificity and efficiency of the oscillatory-flow multiplex PCR

After a preliminary setting of suitable primer concentrations, master mixture composition and temperature control, the multiplex PCR was performed for its specificity in detecting three target templates. Figures S2a, b (ESM) are the amplification results on the oscillatory-flow PCR microfluidics and the conventional PCR thermocycler, respectively. As seen from Fig. S2, when only single pathogen DNA template was added into the multiplex PCR solution including three primer pairs, only single corresponding product was observed, without any non-specific amplified fragments found (lanes 3–5 in Fig. S2, from left to right). However, three target fragments were amplified and detected when the mixed DNA solution was utilized (lane 6 in Fig. S2, from left to right). These results show that the specificity of the oscillatory-flow multiplex PCR is desirable. In addition, the comparison of the corresponding band intensities between Figs. S2a and b reveals that the efficiency of the oscillatory-flow multiplex PCR is comparable to that of the conventional multiplex PCR.

Effect of various concentrations of Taq polymerase on the oscillatory-flow and conventional multiplex PCR

For multiplex PCR, more DNA polymerase is usually needed because more than one target DNA fragments will be amplified [41]. In addition, the surface adsorption effect of the biomolecules, especially Taq DNA polymerase, will become serious as the surface-to-volume ratio (SVR) in a micro-scale environment is increased. Therefore, it is necessary to evaluate the effect of the Taq polymerase concentration on the oscillatory-flow multiplex PCR. Figure 3 shows the effect of Taq DNA polymerase concentration on oscillatory-flow multiplex PCR, where the PCR solution was driven to flow through the microchannel at 2.55 mm s^{-1}. The bands of the multiplex PCR product were detected by the gel electrophoresis when the Taq DNA polymerase concentrations ranging from 0.5 to 0.2 U μL^{-1} were used. If the Taq DNA polymerase concentration was further reduced, the uncomplete or unsuccessful multiplex amplification was observed. Therefore, in order to achieve an efficient multiplex amplification, an appropriate Taq DNA polymerase concentration is essential in the oscillatory-flow multiplex PCR. The similar experiments were also performed on the conventional PCR machine (Fig. S3, ESM). In these experiments, when the wider range of the Taq DNA polymerase concentration was used, which changed from 0.5 to 0.025 U μL^{-1}, the multiplex PCR products could all be observed, although the band intensities of the corresponding multiplex PCR product decreased gradually. It should be pointed out that the Taq polymerase concentration of 0.025 U μL^{-1} is the recommended concentration of monoplex PCR. The comparison of the corresponding band intensities in Fig. 3 and Fig. S3 reveals that within the present oscillatory-flow multiplex PCR system, there still is greater possibility of adsorption of Taq polymerase molecules onto the capillary inner surface, although BSA of 0.5 μg μL^{-1} has been included in the multiplex PCR solution to dynamically passivate the reaction channel. To resolve this problem, the present BSA passivation may be required to couple with other surface treatments [29].

Effect of various flow rates of the reaction solution on the oscillatory-flow multiplex PCR

Pursuing fast PCR is one of the major motivations in the development of PCR microfluidics. The thermocycling speed of continuous-flow PCR depends, to some extent, on the flow rate of the PCR solution, the substrate material and the size of the microchannel. Therefore, for an oscillatory-flow PCR device, changing the flow rates of the multiplex PCR solution through the microchannel can regulate the amplification rates of multiplex PCR. As shown in Fig. 4, when the flow rates increased from 1.70 to 12.73 mm s^{-1}, the multiplex PCR amplification speed were gradually enhanced, but the amount of PCR products was decreased gradually. When the flow rate was 1.70 mm s^{-1}, three target DNA fragments were successfully amplified in about 43 min after 35 PCR cycles (lane 2 in Fig. 4). The comparison of the band intensities reveals that the efficiency of the oscillatory-flow multiplex PCR (lane 2 in Fig. 4) is comparable to that of the conventional multiplex PCR (lane 1 in Fig. 4). When the flow rate of 4.24 mm s^{-1} was used, the time for the successful amplification of three target DNA fragments was about 24 min (lane 4 in Fig. 4), which is about one-third of the time required on the

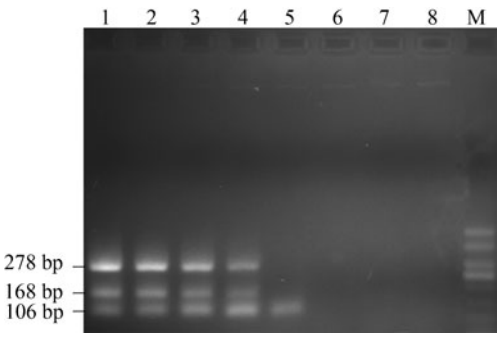

Fig. 3 Effect of various concentrations of Taq DNA polymerase on the oscillatory-flow multiplex PCR amplification. *Lanes 1–8*: 0.5, 0.4, 0.3, 0.2, 0.10, 0.05, 0.025, and 0 U μL^{-1} Taq DNA polymerase in 5 μL of multiplex PCR solution, *Lane M*: DNA marker

conventional machine (70 min). However, when the flow velocity is increased to reduce multiplex amplification time, the required cycle rate to generate product for multiplex PCR is primarily determined by the extension time of Taq DNA polymerase. The extension rate of Taq polymerase is usually 60–100 bases s^{-1} at 72°C in conventional PCR [42], and therefore an extension time of 5 s is considered sufficient for the amplification of the 278 bp and even shorter DNA fragments (lane 4 in Fig. 4). If the extension time was less than 5 s (i.e., the flow rates were more than 4.24 mm s^{-1}), the oscillatory-flow multiplex PCR amplification was ineffective and even completely unsuccessful (Lanes 5–8 in Fig. 4), where only shorter target fragments were amplified. For oscillatory-flow multiplex PCR, therefore, the following points should be considered when the length of the amplified target fragment is chosen. First, choosing the size of the amplified fragment facilitates resolution of each fragment by agarose gel electrophoresis following multiplex reactions. Second, for the amplified target fragments, the difference in length should be relatively small. As a result, the flow velocity can be as fast as possible to obtain the shortest cycling time for simultaneous amplification of multiple target fragments. Under the present testing conditions, the 106 bp target fragment was still successfully amplified when the flow rate was increased to 12.73 mm s^{-1} (the corresponding cycling time for 35 cycles was about 9 min). Therefore, it is possible for the present oscillatory-flow PCR system to complete multiplex amplification within several minutes if the lengths of three target fragments are properly chosen.

Effect of the number of cycles on the oscillatory-flow multiplex PCR

Compared with the serpentine or spiral channel continuous-flow PCR systems, the oscillatory-flow PCR system can provide one a flexible cycle number by only modifying the pumping program, with no need to fabricate a new device. In addition, when time-sensitive diagnostics (e.g., detection of infectious diseases and related biowarfare agents) is needed, the PCR may be performed with a moderate cycle number so as to shorten the total detection time. Therefore, the effect of cycle number on the oscillatory-flow multiplex PCR was studied here. This was achieved by varying the number of oscillations of the solution within the microchannel. The flow speed of the solution was selected to be 2.55 mm s^{-1}, giving a cycling time of about 1 min. Figure 5 shows the effects of cycle number on the efficiency of oscillatory-flow multiplex PCR. As seen from Fig. 5, with the decrease of the number of cycles used within multiplex PCR, the amount of amplification products was gradually reduced. However, little difference in amplification yield resulted when the cycle number ranged from 40 to 25. Moreover, the multiplex PCR amplification was incomplete or unsuccessful when the cycle numbers of 20 or ever smaller were utilized. These results indicate that the multiplex PCR product from the oscillatory-flow device possibly started the exponential increase at the cycle number of between 20 and 25 and showed a trend to reach saturation gradually. When the cycle number of 25 or 20 was used at the flow rate of 2.55 mm s^{-1}, the reaction time was only about 24 or 19 min. The similar experiments were also completed on the conventional machine (Fig. S4, ESM). The comparison between Fig. 5 and Fig. S4 demonstrates that the present multiplex PCR system is comparable to the conventional multiplex PCR system at each cycle number. It should be noted that in two systems, the longer target fragments of 278 bp were both amplified at the cycle number of 20, indicating that there is still much room to optimize the present multiplex PCR reaction so as to obtain a successful multiplex amplification result in the case of low cycle number.

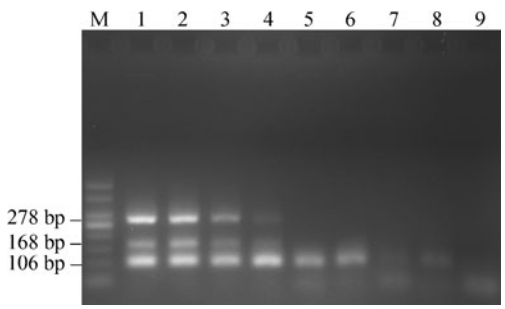

Fig. 4 Effect of various flow rates on the oscillatory-flow multiplex PCR amplification. *Lane M*: DNA markers, *Lane 1*: the positive-control PCR product from the conventional PCR machine, *Lanes 2–8*: the oscillatory-flow multiplex PCR products at various flow rates of 1.70, 2.55, 4.24, 5.94, 7.64, 10.19, and 12.73 mm s^{-1}, respectively, *Lane 9*: the negative-control multiplex PCR, the reaction solution with no DNA sample run at the flow rate of 2.55 mm s^{-1}

Sensitivity of the oscillatory-flow multiplex PCR

For some applications (for example detection of foodborne bacterial pathogens), it may be important to obtain a detectable amount of multiplex PCR product quickly from a low-abundant sample. Therefore, a study was performed to establish the minimum concentration of template that could be detected when amplified through 35 cycles using the oscillatory-flow multiplex PCR at a flow rate of 2.55 mm s^{-1}. As an analytical model, the concentration of each bacterial genomic DNA template within the reaction solution was approximately equal during each run. The results of these studies are demonstrated in Fig. 6. Seen from Fig. 6, the minimum template concentration that could be simultaneously detected at 2.55 mm s^{-1} was about 1.0×10^{-3} ng μL^{-1}, which corresponds to 399±53, 314±29, and

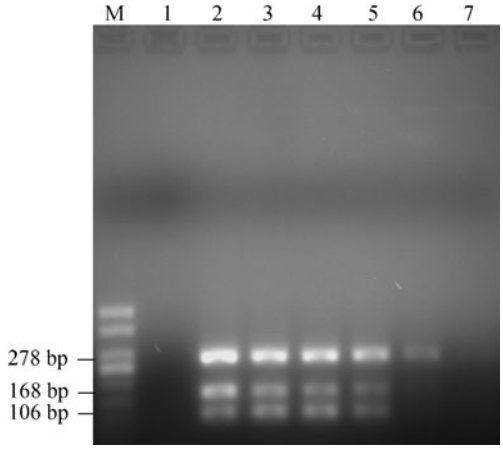

Fig. 5 Effect of the cycle number on the oscillatory-flow multiplex PCR amplification. *Lane M*: DNA marker, *Lane 1*: the negative-control multiplex PCR with no DNA sample in the reaction solution, *Lanes 2–7*: the numbers of cycles used in multiplex PCR were 40, 35, 30, 25, 20, and 15, respectively. When the oscillatory-flow multiplex PCR was performed, the solution ran at the flow rate of 2.55 mm s^{-1}

626±62 copies μL^{-1} for *S. enterica*, *E. coli* O157:H7, and *L. monocytogenes*, respectively (lane 5 in Fig. 6). Although no visible multiplex product band was apparent for 1.0×10^{-4} ng μL^{-1} template concentration (lane 6 in Fig. 6), this does not necessarily mean that no multiplex product was produced at those concentrations, but most likely the amount of product generated under these multiplex PCR conditions was lower than the detection limit of the presented agarose gel electrophoresis. In addition, it should be pointed out that when the template concentration was reduced to 1.0×10^{-6} ng μL^{-1}, the 106 bp target fragment was still successfully amplified and detected (here, only 0.62 copies μL^{-1} of *L. monocytogenes* DNA template was used), but other two longer target fragments were not successfully amplified.

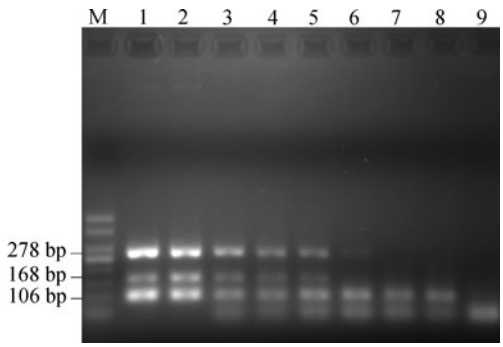

Fig. 6 Gel electrophoresis analysis of the oscillatory-flow multiplex PCR product from various concentrations of input DNA template. *Lane M*: DNA marker, *Lane 1*: the positive-control PCR product from the conventional PCR machine, *Lanes 2–8*: the concentration of each bacterial genomic DNA template in the oscillatory-flow multiplex PCR was 1.0×10^{0}, 1.0×10^{-1}, 1.0×10^{-2}, 1.0×10^{-3}, 1.0×10^{-4}, 1.0×10^{-5}, and 1.0×10^{-6} ng μL^{-1}, respectively, *Lane 9*: the negative-control PCR, PCR solution with on DNA sample run at the flow rate of 2.55 mm s^{-1}

Conclusion

\In this paper, we have successfully developed an oscillatory-flow multiplex PCR method for simultaneous detection of multiple foodborne bacterial pathogens in a single reaction. To perform an efficient multiplex amplification, the temperature distribution and sample evaporation were first investigated, and the results demonstrated that the temperature kept very stable in three zones, and that the evaporation loss of less than 5% was achieved. The present multiplex amplification system exhibited a relatively high amplification speed and the multiplex amplification of three target DNA fragments could be successfully completed in approximate 24 min after 35 cycles, which was about one-third of the time required on the conventional machine. Moreover, it is possible for the developed system to complete multiplex amplification within several minutes if the sizes of three target fragments are properly designed or an appropriate cycle number is chosen.

The specificity and efficiency of the oscillatory-flow multiplex PCR system presented here are comparable to those on the conventional benchtop PCR instrument. And, the minimum copy number of each bacteria DNA template that could be simultaneously detected at 2.55 mm s^{-1} was about 399, 314, and 626 copies μL^{-1} for *S. enterica*, *E. coli* O157:H7, and *L. monocytogenes*, respectively. This detection limit is close to that from the conventional PCR machine. However, compared with the conventional multiplex PCR, the presented multiplex PCR system needs more Taq DNA polymerases. But, this issue is common to almost all microfluidic PCR systems. Moreover, coupling BSA passivation with other surface treatments can resolve this problem. Therefore, the oscillatory-flow multiplex PCR presented here can provide a powerful tool for rapid diagnosis of multiple pathogens and facilitate the diagnosis and management of food- or waterborne poisoning outbreaks. Within future oscillatory-flow multiplex PCR system, the sample preparation, real-time fluorescence detection, or other analytical micro/nano-fluidic technology [43, 44] is likely integrated to further increase the detection speed of multiple foodborne bacterial pathogens.

Acknowledgments This research is supported by the National Natural Science Foundation of China (61072030, 30700155), the Key Program of NSFC-Guangdong Joint Funds of China (U0931005), the National Basic Research Program of China (2010CB732602; 2011CB910402), and the Program for Changjiang Scholars and Innovative Research Team in University (IRT0829).

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