Talanta 122 (2014) 246–250

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Microfluidic chip integrating high throughput continuous-flow PCR and DNA hybridization for bacteria analysis

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ARTICLE INFO

Article history:
Received 4 December 2013
Received in revised form
21 January 2014
Accepted 24 January 2014
Available online 31 January 2014

Keywords:
Continuous-flow PCR
16S rDNA
Hybridization

ABSTRACT

Rapid identification of clinical pathogens is the initial and essential step for antimicrobial therapy. Herein, we successfully developed a microfluidic device which combines high-throughput continuous-flow PCR and DNA hybridization for the detection of various bacterial pathogens. Universal primers were designed based on the conserved regions of bacterial 16S ribosomal DNA (16S rDNA), and specific probes were designed from a variable region of 16S rDNA within the amplicon sequences. In the chip operation, after the continuous flow PCR was achieved in the first microfluidic chip, the product was directly introduced into a hybridization chip integrated with microarray containing the immobilized DNA probes. The target-probe hybridization was completed within 1 h at 55 °C, and fluorescence signals were obtained as the readout. The presented device is simple, versatile and with less sample consumption compared with traditional instruments. It can perform high-throughput bacteria detections continuously in a single assay, which makes it a promising platform for clinical bacteria identifications.

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1. Introduction

Rapid detection of pathogenic microorganisms related to infectious disease is essential for clinical diagnostics and public health. Among the various techniques currently used, methods based on nucleic acid detection are more accurate and sensitive than traditional assays, such that culturing is not required for microbial identification [1]. Bacterial 16S rDNA has been widely utilized as a target nucleotide for bacterial identifications, because it is highly conserved in most prokaryotic pathogens [2,3]. Therefore, the universal PCR primer set (27F and 341R) targeting conserved regions of the 16S rDNA, is often chosen to amplify the fragments from a wide range of bacterial species. The species-specific hybridization probes were designed from the variable regions using published bacterial 16S rDNA sequences.

Microfluidic technology for molecular identifications is developing rapidly, providing advantages like high-throughput, precise fluid control and low reagent and power energy consumptions [4]. Thus, chip-based PCR amplification has become a promising technique for many biological and clinical applications [5]. The first continuous-flow PCR chip was developed in 1998 by Kopp group [6]. Comparing to conventional PCR devices, the thermal-cycling time can be significantly reduced using a continuous-flow PCR chip, due to the very short time of the PCR mixture passing through the temperature zones (denaturation and annealing) in micro-channels [7]. The miniaturized PCR and detection device is portable, perfect for field application. Furthermore, comparing with chamber stationary PCR, the temperature controlling system is much simpler, since only different temperature zones are needed, instead of complicated accurate heating and cooling cycle systems in conventional chamber PCR systems. Rapid thermal cycling of 17 s/cycle was achieved in the continuous-flow PCR chip, because of the easy temperature zone control and fast thermal transmission [8]. Moreover, the amount of the PCR product from the continuous-flow-based PCR chip can be unlimited theoretically.

Detection methods for a PCR chip are mostly gel electrophoresis or capillary electrophoresis, which are unable to facilitate specific identifications [9,10]. Compared with these traditional electrophoresis methods, microfluidic microarray has advantages including (i) enabling detection at low DNA concentration, (ii) the hybridization time can be considerably decreased because of the enhanced reaction rate inside a small chamber, and (iii) high-throughput analysis can be achieved by printing all the concerned probes on the microarray. Researches based on PCR and DNA hybridization to microarray probes have been reported [1–8,11,12], but rarely refer to PDMS chips for PCR amplification and hybridization.

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0039-9140/$ - see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2014.01.053

In this report, a PDMS-based device harboring two-step PCR and on-chip hybridization for detections of five frequently encountered bacteria was successfully fabricated. After on-chip amplification, the PCR amplicons were directly transferred into a hybridization chip and hybridized to the DNA probes immobilized on the aldehyde-activated slide. The device is simple, has low-cost and is portable, which suggests its potential applications in clinical and environmental bacteria detections.

2. Experimental

2.1. Bacteria samples preparation

Five bacterial strains including *Klebsiella pneumoniae*, *Staphyloccocus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* were isolated from clinical samples from Neweast Hospital (Shanghai, China). All bacteria were separately cultured in Luria-Bertani (LB) medium at 37 °C, and then mixed to give a variety of target combinations (*E. coli* and *S. aureus*, *K. pneumoniae* and *P. aeruginosa* and all five targets). Next, the bacteria solutions were centrifuged at 10,000g for 2 min to remove LB medium, followed by incubation with lysis buffer (DEAOU Biotechnology, China) at room temperature for 30 min, then centrifuged and washed with double distilled H_2O (ddH_2O), and 0.1 μl of the prepared bacteria suspension used as template.

2.2. Chip design and fabrication

A 5 cm × 4.5 cm PCR chip with a channel width and depth of 200 μm and 30 μm, respectively, was fabricated as shown in Fig. 1A. Access holes of 700 μm diameter were drilled for inlet and outlet. The thermal cycler for micro-PCR was built using polyimide heating membrane (40 mm × 60 mm × 0.2 mm, Jiari Electronics Co., China). A temperature sensor (SEMITE, Japan) was used as feedback to maintain stable temperature inside the chip channel. The PDMS was clamped to the heating membrane by PMMA plates (100 mm × 90 mm × 8 mm) to achieve close contact between the chip and the membrane, which helps to improve heat transmission efficiency. The inlet and outlet of the microchannels were separately connected to a syringe pump for fluid injection and collection, respectively (Fig. 1B). For the hybridization chip, a glass substrate containing aldehyde groups for DNA microarray was used. The arrangement of oligo probes immobilized on the microarray was designed for detections of the five bacteria, with it replicated 3 times for each bacteria (Fig. 1C). The printed slide was then served as the bottom slide of the fluid layer, and bonded to the PDMS layer to give a DNA hybridization chip (Fig. 1D).

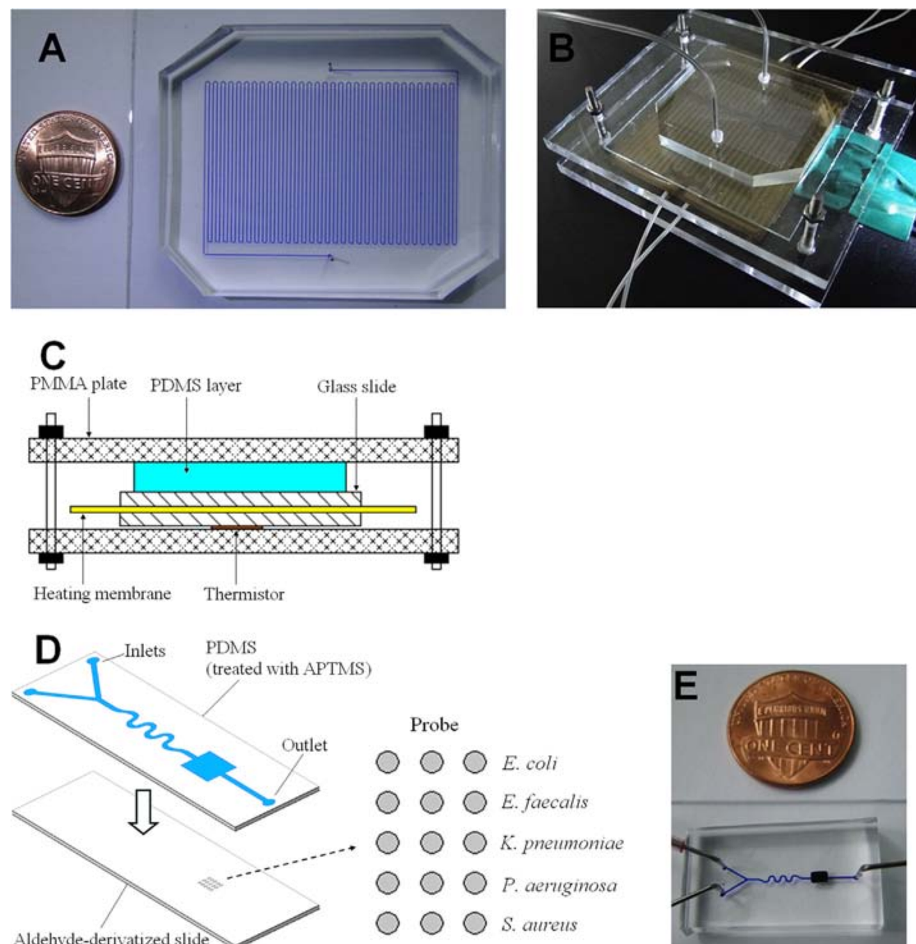

Fig. 1. Schematic illustration of PCR and DNA hybridization chips. (A) Photograph of the continuous-flow PCR chip. The PCR channel was loaded with blue, (B) photograph of the experimental set-up of the PCR chip. The chip was placed over two heating membranes (95 °C and 55 °C), and was clamped by PMMA plates, (C) schematic drawing of the assembled PCR chip viewed from the side, (D) schematic illustration of the assembly of the DNA hybridization chip. The upper PDMS layer was bonded onto an aldehyde-activated slide on which surface DNA probes were printed prior to the bonding, and (E) top view of the hybridization chip, showing a slanted direction of the two inlet flows, which is diagonal at an angle of 45°. The square represents the DNA hybridization chamber. The paths and chamber were colored blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The hybridization chip contains reagent channels (width, 200 μm; depth, 30 μm) and a DNA hybridization chamber (length, 2 mm; width 1.25 mm; depth, 0.5 mm) with a volume of 1.25 μl (Fig. 1E).

The PCR chip and hybridization chip were fabricated by soft lithography as described previously [4]. Briefly, a silicon mold was prepared by photolithographic processes to create the fluidic channels using negative photoresist SU-8 (Microchem Corp.). Next, poly-dimethylsiloxane (PDMS) (GE, RTV 615 A and B in a 5:1 ratio) was mixed and poured on the mold, followed by degassing for 40 min to build the fluid layer containing the microchannels. Afterwards, the PDMS was cured at 80 °C for 1 h, and then peeled off from the mold. Then inlet and outlet holes at the end of the PCR channels were introduced for loading reagents. Subsequently, the fluid layer was pressed on a PDMS (A and B in a ratio of 20:1) coated glass slide and incubated overnight at 80 °C to achieve maximum bonding strength.

2.3. Bonding process

For bonding of PDMS and aldehyde-derivatized glass slide (25 mm × 75 mm × 1 mm, CapitalBio, China) the lower surface of the PDMS layer was immersed in 1% H_2O_2 and HCl solution for 10 min, followed by 1 h treatment with 30% APTMS so as to attach amino groups onto the PDMS surface. Subsequently, the PDMS layer was thoroughly washed using ethanol two times, and then dried with N_2. The prepared amino-containing PDMS substrate was then attached to the aldehyde-derivatized glass slide to form a chip, and clamped lightly with two polymethylmethacrylate (PMMA) plates to facilitate close contact of the PDMS and the slide for 8 h to facilitate amino-aldehyde bonding.

2.4. Continuous-flow PCR amplification of 16S rDNA

The 1.9 μl PCR reagent consists of 0.2 U/μl ExTaq (3′–5′ exonuclease Taq DNA polymerase), 1 × Taq-polymerase buffer, 2.5 mM deoxynucleotide triphosphate (dNTP), 0.05 μg/ul Bovine Serum Albumin (BSA), 0.1 μM forward primer and 1 μM Cy3-labled reverse primer. Before PCR amplification, the inner surface of the chip channel was prepassivated under a continuous flow of 0.1 μg/μl BSA so as to reduce the adsorption of Taq-polymerase [13]. To prevent air bubble generation when PCR solution passes through high temperature zones, a 2 μl fluorinated oil (3 M) cap was introduced immediately before and after the introduction of the PCR solutions.

The PCR amplification was performed by pumping the injection plugs (fluorinated oil and PCR sample) continuously into the microchannels at a rate of 80 nl/s, passing through two temperature zones (95 °C for 20 s and 55 °C for 20 s) alternately for 33 cycles. The PCR products were collected and centrifuged to remove the fluorinated oil, then used directly for on-chip hybridization, without purification process. To evaluate the limit of detection (LOD), overnight cultured *E. coli* was used in serial dilution as PCR templates.

2.5. Preparation of DNA microarray

For each bacteria, 40 μM oligo probes were mixed with DMSO at a ratio of 1:1, then immobilized onto the aldehyde-derivatized glass slide through conventional spotting by a SmartArray™-48 Microarray Spotter (CapitalBio, China). The spot diameter was set to be approximately 100 μm–120 μm, with spacing between each spot of 120 μm. The slide containing the probes was then bonded with the amino-containing PDMS as described. Next, a continuous flow of 0.2% SDS was supplied into the hybridization chamber for 3 min, followed by a ddH_2O wash and injection of a freshly prepared 0.3% $NaBH_4$ solution for 5 min to block the unused surface area of the slide. The chamber was subsequently washed with ddH_2O, and dried carefully under an air stream.

2.6. Hybridization

The Cy3-labeled PCR product was heated for 5 min at 95 °C and placed on ice immediately for 2 min to give single stranded DNA (ssDNA). 2 μl hybridization reagent was prepared, containing 5 × Denhardt's (Sangon Biotech, China), 3 × SSC and 0.2% SDS. Hybridization was carried out by simultaneously pushing the PCR product and hybridization reagent toward the DNA hybridization chamber through two inlets separately. After completely filling the hybridization chamber, the chip was heated at 55 °C for 1 h. Then the hybridization chamber was washed by 0.2 × SSC/ 0.2% SDS and 0.2 × SSC and ddH_2O. After wash step, the PDMS layer was peeled off from the slide. Then the slide was washed by ddH_2O and dried with N_2. Fluorescence data were collected using a GenePix™ 4200 Scanner (Molecular Devices, US).

3. Result and discussion

3.1. Device quality

Accurate temperature and rapid heat transmission are important for PCR efficiency. The polyimide heating membrane used in this assay has a thickness of 0.2 mm, and exhibited fast heat transmission ability. But the temperature of the membrane surface can be affected by the influence of the air movement and environmental temperature due to the low power of the membrane. In traditional reports, PCR chips were generally placed over on the upper surface of the thermal source, and the chips were placed in close contact with the thermal source by gravitational effect [14]. However in the proposed assay, by placing a temperature sensor inside the PCR microchannel, we observed the temperature error within the channel can be as high as 5 °C, which was mainly due to the non-compact contact as a result of tiny interspaces between the undersurface of the chip and the upper surface of the polyimide membrane because the membrane's upper surface was with slight surface irregularity. To solve the issue, PMMA plates were utilized to clamp the PCR chip and the polyimide membrane to facilitate very close contact of the chip and the thermal source. The temperature inside the microchannels was monitored to achieve an accurate error < 0.2 °C.

Generation of air bubbles is a common and fatal problem in continuous flow PCR when the solutions are passing through high temperature zones (denaturation at 95 °C), which adversely affect PCR amplifications [15]. Liquid gasification happened mostly at the frontal part of the liquid flow direction because of the relatively higher pressure within the latter part compared with the frontal part due to the pump force. The problem was solved by introducing fluorinated oil intervals before and after the introduction of the PCR plug, to increase pressure within the channel. Fluorinated oil has been reported to reduce air bubble generation [7], and was very important for stable PCR process in the assay, without which air bubbles were immediately generated. Mineral oil and silicon oil (Sigma) were also tried in the experiment. The high viscosity of the two oils caused increase of the fluid resistance in the microchannels, which made the fluid flow very slow after passing several cycles. PMMA plate is also helpful for reducing air leakage from PDMS wall, which benefits in stabilizing pressure within microchannels and thereby inhibiting air bubble generation.

3.2. Bonding of the PDMS to the microarray slide

The conventional method of bonding PDMS with a glass slide (covalent bonding of siloxanegroups with hydroxygroups) such as plasma treatment, heating at 80 °C was not suitable for the proposed method. A novel strategy was established with APTMS,

and the bonding strength of the PDMS layer and the slide was strong enough for fluid injection, hybridization at 55 °C for 1 h and wash process. No leakage was observed during the hybridization assay. The bonding of PDMS and microarray slide that was applicable for proposed utilization, was still weaker than the conventional bonding method. Moreover, the immobilized amino groups on the inner surface of the PDMS channels might lead to adsorption of DNA oligonucleotide, which would theoretically cause loss of the amplified DNA. In our experiment, no obvious effect was observed in our assay. We also tried to place the PDMS layer directly onto the microarray slide surface, without any surface treatment, and clamp them slightly with PMMA plates. With this method, almost no leakage was observed, after liquid injections and incubation at 55 °C for 1 h.

3.3. On-chip PCR and hybridization

The five species were used as PCR templates because they were frequently associated with infectious diseases. The lysed bacteria were amplified for 33 cycles in the PCR chip using universal primers: 5′-AGAGTTTGATCCTGGCTCAG-3′ (forward) and 5′-Cy3-CTGCTGCCTCCCGTAGGAGT-3′ (reverse) to generate approximately 310-base pair (bp) amplicons for each bacteria. Based on the amplicon length, the double stranded DNA can be synthesized within 20 s according to the manual of the Taq-polymerase. Thus, the retention time for annealing and extension in the chip was both set to be 20 s. With one cycle representing approximately 45 s, 33 cycles take 25 min.

Table 1
Probe sequences.

Oligonucleotide probe sequence (5′–3′)	Organism
$(dT)_{15}$-tcttgccatcggatgtgccca	*Escherichia coli*
$(dT)_{15}$-cttctttcctcccgagtgct	*Enterococcus faecalis*
$(dT)_{15}$-agcacagagagcttgctctc	*Klebsiella pneumoniae*
$(dT)_{15}$-atcttcggacctcacgctatca	*Pseudomonas aeruginosa*
$(dT)_{15}$-tattttgaaccgcatggttcaa	*Staphyloccocus aureus*

The collected PCR product was directly introduced into the hybridization chip, on the surface of which all of the five specific probes were printed. Table 1 shows the designed DNA probes targeting on variable regions of 16S rDNA. A poly (T) chain in the 5′ end of the probes could help to increase the hybridization efficiency as a spacer [16].

As shown in Fig. 2A, specific hybridization results were achieved for each of the five bacteria, showing the specificity of the device, except that a weak non-specificity signal was observed for *E. faecalis* PCR products with *P. aeruginosa* detection probe. Furthermore, combinations of *E. coli* and *S. aureus*, as well as *K. pneumoniae* and *P. aeruginosa* were served as templates for the on-chip PCR and hybridization. The expected hybridization patterns were obtained for the two combinations (Fig. 2B). These results provide further confirmation for the specificity of the probes. Finally, the five bacteria were mixed and used as the PCR template, and positive hybridization result for all the five bacteria was observed (Fig. 2B). Considering good fluorescent signal was obtained for the PCR product of *S. aureus* in the case when *S. aureus* was individually served as PCR template for hybridization (Fig. 2A), it is suggested that the relatively low fluorescent signal for *S. aureus* when the PCR product of the five bacteria mixture was used for hybridization, is possibly due to the fact that the relatively less amount of *S. aureus* 16S rDNA was produced in the mixture. Since *S. aureus* is a gram-positive bacteria and is hard to lyse, less genomic DNA would be released in the lysis process of the bacterial mixture. When the lysed bacterial mixture was served as PCR template simultaneously using a single primer pair, the primers were easier to bond with other bacterial DNA in the suspension in the competition with the *S. aureus* DNA, which would cause less PCR products of *S. aureus* compared to the other four species.

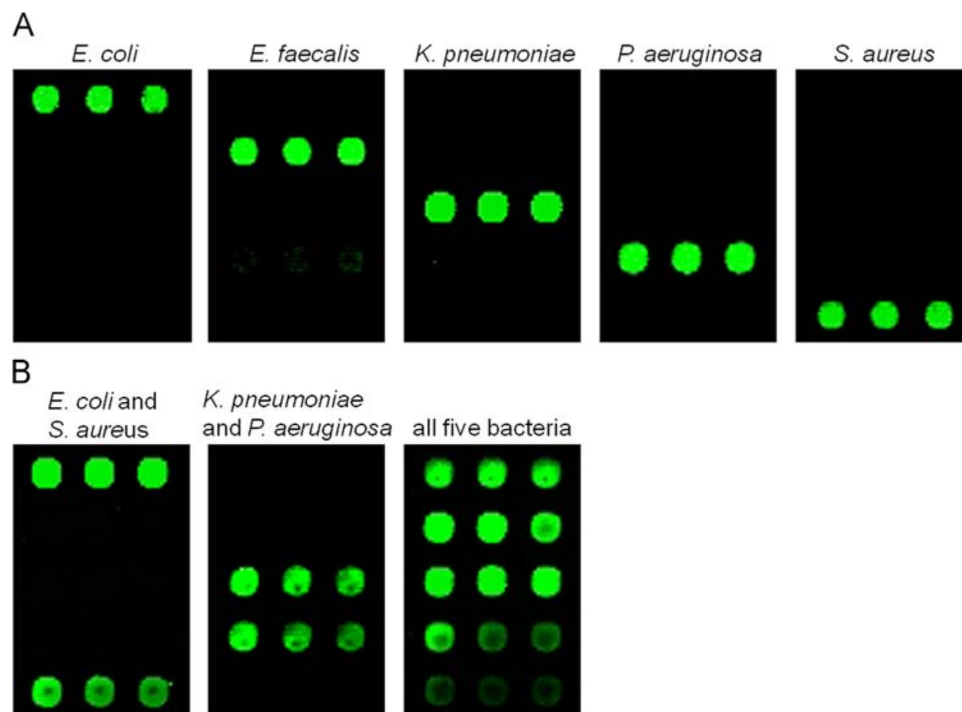

Fig. 2. Hybridization images of Cy3-labeled target nucleotides. (A) Determination of the probes specificity by amplifying each target individually with the same primer pair, then hybridize with all five probes printed on the slide. Following the direction of left to right and then top to bottom, the analyzed single targets were *E. coli*, *E. faecalis*, *K. pneumoniae*, *P. aeruginosa* and *S. aureus* and (B) a test with a variety of target combinations. From left to right and top to bottom, the combinations evaluated were *E. coli* and *S. aureus*, *K. pneumoniae* and *P. aeruginosa*, and all fives targets.

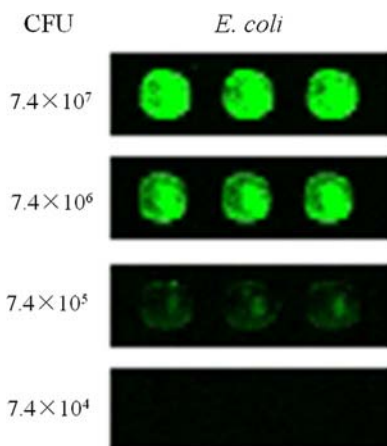

Fig. 3. Evaluation of the sensitivity of the microfluidic system using serially diluted *E. coli* ranging from 7.4×10^7 to 7.4×10^4 CFU/ml.

Since the diffusional mobility of DNA molecule is low, DNA hybridization times under static conditions were shown to be 2–5 h [16,17]. However, a good fluorescent signal was obtained within 1 h for the static hybridization in our assay. It can possibly be attributed to the small dimension of the microfluidic chamber, which decreased the diffusion length to improve the DNA hybridization efficiency. Considering the 33 cycle PCR which took about 25 min, lysis about 30 min, hybridization for 60 min, plus washing and fluorescence analysis for about 30 min, the total analysis took about 2 h 30 min.

3.4. Sensitivity of the device

For sensitivity determination, serially diluted *E. coli* bacterial solutions ranging from 7.4×10^7 to 7.4×10^4 CFU/ml were utilized as templates for the on-chip PCR. As shown in Fig. 3, visualized results were obtained when the bacterial content was more than 7.4×10^5 CFU/ml, at which concentration the fluorescence signal can be hardly observed. Further reduction of the cells to 7.4×10^4 CFU/ml did not yield detectable signals. Since 0.1 μl of the bacterial suspension was served as PCR template, samples as little as 74 CFU of *E. coli* by 16S rDNA from on-chip PCR and hybridization could be detected.

4. Conclusion

In this study, a microfluidic device was successfully demonstrated for high-throughput analysis of five clinically significant bacterial species using on-chip PCR and DNA hybridization, without electrophoresis process. Universal primers were chosen targeting the conserved region of bacterial 16S rDNA so as to amplify approximately 310 bp fragment from a wide range of bacterial species. Furthermore, oligonucleotide probes were designed for species-specific identification of five frequently clinically encountered pathogens. The detection can be achieved within 2 h 30 min, of which the detection limit is 74 CFU of *E. coli*. Based on the mechanism of the device, high-throughput identifications can be performed with a large amount of species continuously amplified and hybridized simultaneously, which is promising for rapid and high-throughput disease diagnosis.

Acknowledgment

This work was funded by NSFC (21377026 and 20975025), Cultivation Fund of the Key Scientific and Technical Innovation Project, Ministry of Education of China (708031). The authors also thank Mr. Bo Yao for the help in electronics.

References

[1] Y. Liu, J.X. Han, H.Y. Huang, B. Zhu, Exp. Biol. Med. 230 (2005) 587–591.
[2] Y. Liu, J.X. Han, B. Zhu, H.Y. Huang, Chin. J. Microbiol. Immunol. Infect. 2 (2004) 210–217.
[3] K. Sen, D.M. Asher, Transfusion 41 (2001) 1356–1364.
[4] W.W. Jing, W. Zhao, S.X. Liu, L. Li, C.T. Tsai, X.Y. Fan, W.J. Wu, J.Y. Li, X. Yang, G.D. Sui, Anal. Chem. 10 (2013) 5255–5262.
[5] C.S. Zhang, J.L. Xu, W.L. Ma, W.L. Zheng, Biotechnol. Adv. 24 (2006) 243–284.
[6] M.U. Kopp, A.J. de MelloA. Manz, Science 280 (1998) 1046–1048.
[7] T. Nakayama, Y. Kurosawa, S. Furui, K. Kerman, M. Kobayashi, S.R. Rao, Y. Yonezawa, K. Nakano, A. Hino, S. Yamamura, Y. Takamura, E. Tamiya, Anal. Bioanal. Chem. 386 (2006) 1327–1333.
[8] Y. Liu, C.B. Rauch, R.L. Stevens, R. Lenigk, J. Yang, D.B. Rhine, P. Grodzinski, Anal. Chem. 74 (2002) 3063–3070.
[9] C. Zhang, D. Xing, Nucl. Acids Res. 35 (2007) 4223–4237.
[10] Y. Zhang, P. Ozdemir, Anal. Chim. Acta 638 (2009) 115–125.
[11] J.H. Kim, A. Marafie, X.Y. Jia, J.V. Zoval, M.J. Madou, Sens. Actuators B: Chem. 113 (2006) 281–289.
[12] G. Mitterer, M. Huber, E. Leidinger, C. Kirisits, W. Lubitz, M.W. Mueller, W.M. Schmidt, J. Clin. Microbiol. 42 (2004) 1048–1057.
[13] N.C. Cady, S. Stelick, M.V. Kunnavakkam, C.A. Batt, Sens. Actuators B: Chem. 107 (2005) 332–341.
[14] J. Petersen, L. Poulsen, S. Petronis, H. Birgens, M. Dufva, Nucleic Acids Res. 36 (2008) e10.
[15] I. Schneegaß, J.M. Ќohler, J. Biotechnol. 82 (2001) 101–121.
[16] Y. Li, S.J. Guo, N. Shao, S. Tu, M. Xu, Z.R. Ren, X. Ling, G.Q. Wang, Z.X. Lin, S.C. Tao, Lab Chip 11 (2011) 3609–3618.
[17] G.L. Lukacs, P. Haggie, O. Seksek, D. Lechardeur, N. Freedman, A.S. Verkman, J. Biol. Chem. 275 (2000) 1625–1629.