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A versatile loop-mediated isothermal amplification microchip platform for *Streptococcus pneumoniae and Mycoplasma pneumoniae* testing at the point of care

Hua Wang[a,*,1], Zhan Ma[b,1], Juanxiu Qin[a], Zhen Shen[a], Qian Liu[a], Xiaoyin Chen[a], Hengliang Wang[c], Zhenghua An[c], Wu Liu[d], Min Li[a,*]

[a] Department of Laboratory Medicine, Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China
[b] Department of Laboratory Medicine, Shanghai Children's Hospital, Shanghai Jiaotong University, Shanghai, China
[c] Dept. of Physics, Fudan University, Shanghai, China
[d] Department of Micro/Nano Electronics, School of Electronic Information and Electrical Engineering, Shanghai Jiao Tong University, Shanghai, China

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ABSTRACT

Community-acquired pneumonia (CAP) is the leading cause of mortality in children under five years of age, globally. Given that *Streptococcus pneumoniae (S. pneumoniae)* and *Mycoplasma pneumoniae (M. pneumoniae)* are the most common pathogens associated with CAP requiring hospital admission, a simple, low cost, highly sensitive method is in great need for immediate and early diagnosis of CAP. Herein, we report a versatile microfluidic chip platform integrated with loop-mediated isothermal amplification (LAMP) for simultaneous *S. pneumoniae* and *M. pneumoniae* testing at the point of care. The platform includes a polymer/paper microfluidic chip and a portable device. On-chip magnetic particle-based nucleic acid extraction is used for concentration of pathogens' genomic DNA and is followed by LAMP. The portable device has the function of heating the microfluidic chip, and photographing and transmitting the result to a smartphone. Complete extraction of the DNA using the microfluidic chip took ~15 min versus > 1.5 h with a phenol-chloroform method. The analytical sensitivity of the assay was determined to be 20 fg by testing serial dilutions of target DNA ranging from 2 ng to 2 fg per reaction. We evaluated the clinical sensitivity and specificity of the IPμchip assay using 63 randomly selected oropharyngeal swabs and bronchoalveolar lavage fluid specimens from children. For comparison, these specimens were also tested against real-time PCR assay (*M. pneumoniae*), conventional PCR assay (*S. pneumoniae*), and culture tests (*S. pneumoniae*). These results yielded positive and negative predictive values for *M. pneumoniae* testing with the IPμchip platform of 96.9% and 100%, respectively. Compared with *S. pneumoniae* IPμchip, the clinical sensitivity of *S. pneumoniae* PCR and culture tests was 60% and 40%, respectively, while clinical specificity of the two tests was 100%. This versatile IPμchip platform has great potential for point of care testing of different kinds of pathogens, especially for developing nations.

1. Introduction

Community-acquired pneumonia (CAP) is the leading cause of morbidity and mortality worldwide. *S. pneumoniae* and *M. pneumoniae* are the most common pathogens associated with CAP requiring hospital admission (Viasus et al., 2017; Rogozinski et al., 2017). For several decades, bacterial culture has been a key approach to the laboratory diagnosis of bacterial pneumonia. However, this approach is time-consuming and the detection sensitivity is low. *M. pneumoniae* is very difficult to cultivate, with isolation from liquid culture requiring up to 8 weeks and often resulting in a low yield (She et al., 2010). Polymerase chain reaction (PCR) techniques have been shown to be reliable for diagnosing such pathogens. However, PCR methods demand sophisticated instruments, well-equipped laboratories, and specially trained technicians, which are not suitable for point-of-care (POC) applications, especially in resource-limited settings. Microfluidics-based devices stand out and have drawn intense attention from institutes to industries due to their various useful capabilities, such as low cost, simple operation, short turnaround, limited sample and reagents consumption, and capability for detection with high sensitivity (Sackmann et al.,

* Corresponding authors.
E-mail addresses: fdeduwh@126.com (H. Wang), ruth_limin@126.com (M. Li).
[1] These authors contributed equally to this work and should be considered co-first authors.

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2014). These advantageous features make them very suitable to create portable POC medical diagnostic systems. PCR-based portable microfluidic devices could thus have great impact on rapid detection. The design of portable and automated systems for low-resource settings is limited as a result of the high complexity of the reaction setup and the precise temperature control necessary to conduct the amplification (Kersting et al., 2014). Therefore, the development of a novel array is vital to address these limitations.

A possible alternative to PCR is loop-mediated isothermal amplification (LAMP), which is carried out at a single temperature throughout the entire reaction (Roy et al., 2016). LAMP relies on four to six primers and a strand-displacing DNA polymerase to drive auto cycling amplification of target DNA at a constant temperature. The final amplified product can then be easily detected by turbidity or fluorescence instead of gel electrophoresis (Notomi et al., 2015; Tomita et al., 2008). Microchips integrated with LAMP assays have the potential to provide urgently needed diagnostics for pathogens in resource-limited settings of both developed and developing countries. Recently, on-chip isothermal amplification methods have been developed for rapid pathogen detection including influenza A virus, *Neisseria meningitides, E. coli*, etc. (Fang et al., 2011; Luo et al., 2014; Oh et al., 2016; Dou et al., 2017, 2014). However, in these reports the sample was bacterial genomic DNA rather than complex physiological fluids. Therefore, the ability of the chip to perform pre-treatment of the "raw" samples was limited and it cannot accomplish the goal of POCT. Our group has also demonstrated an integrated LAMP microchip for rapid, sensitive, and instrument-free detection of tumor gene mutations (Wang et al., 2015., 2017). Herein, we describe a versatile microfluidic chip platform integrating LAMP for rapid detection of respiratory pathogens. The platform includes a polymer/paper microfluidic chip (Fig. 1A) and a portable device for temperature control and remote visualization of the results (Fig. 1B). This chip, with rapid sample-in-product-out capability for respiratory clinical specimens, can be utilized for genomic DNA extraction, amplification, and naked-eye read-out of results for respiratory pathogens. We call it IPμchip; namely integrated pathogens genomic DNA extraction, LAMP and detection microfluidic chip. As shown in Fig. 1A, the IPμchip has three distinct functional domains, one for pathogen lysis and DNA extraction (a), another for liquid control (b), and a third for LAMP (c). The pathogens' genomic DNA was extracted by magnetic beads method. A chromatography paper disk was placed in each microchamber to preload LAMP primers and the functionalized chip could recognize specific nucleic acid fragments of *S. pneumoniae* and *M. pneumoniae*.. Clinical diagnosis of *S. pneumoniae* and *M. pneumoniae* can be achieved by visual confirmation of green fluorescence under the UV light pen of the portable device. The portable device has a built-in heating module, a Bluetooth Wi-Fi camera module, and a dedicated app interface for a smartphone to communicate and receive results. Fig. 1C illustrates the principle of the pathogens LAMP detection (Tomita et al., 2008). Calcein is a metal indicator that yields strong fluorescence by forming complexes with divalent metallic ion. To begin with, the calcein is added to the LAMP reaction solution. The calcein in the reaction mixture initially combines with manganous ion (Mn^{2+}) so as to remain quenched under the UV light pen (365 nm). During the LAMP amplification process, pyrophosphate ions (PPi) are produced. When the amplification reaction proceeds, Mn^{2+} is deprived of calcein by the generated $Mn_2P_2O_7$, which results in the emission of fluorescence. And the free calcein is apt to combine with magnesium ion (Mg^{2+}) in the reaction mixture, so that it strengthens the fluorescence emission (510 nm). The generated fluorescence was recorded and wirelessly transmitted to a smartphone by the Wi-Fi camera. The

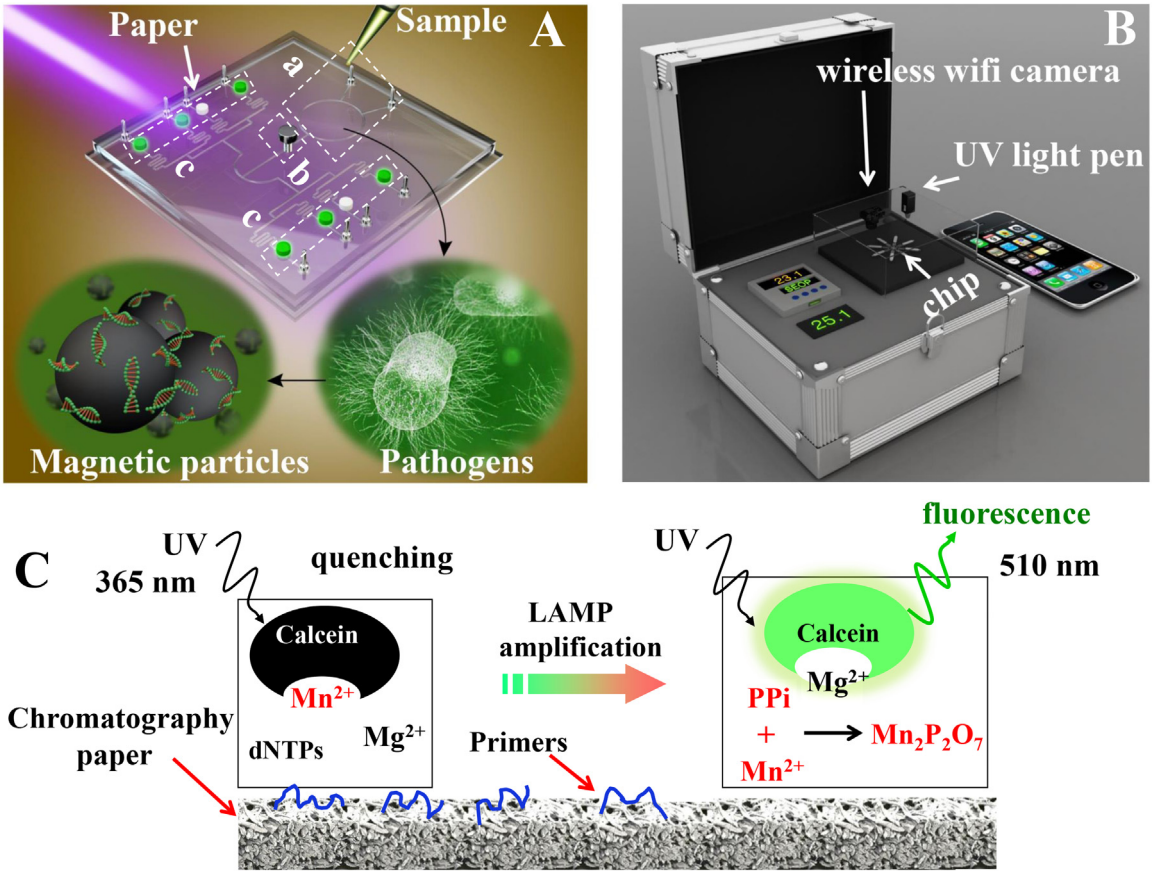

Fig. 1. 3D schematics of the IPμchip platform. (A) 3D illustration of the schematic of the chip. The IPμchip has three distinct functional domains. (a), DNA extraction region, (b) fluid control region, (c) the LAMP region. The pathogen's genomic DNA was extracted by magnetic beads method. A chromatography paper disk is placed in each microchamber to preload LAMP primers. (B) 3D schematics of the portable device. (C) The schematic diagram of the LAMP detection principle.

IPμchip platform will be used for point-of-care identification of *S. pneumoniae* and *M. pneumoniae*.

2. Materials and methods

2.1. Materials

A total of 63 clinical specimens (45 oropharyngeal swabs and 18 bronchoalveolar lavage fluids) were collected from children admitted to the Children's Hospital, Shanghai Jiaotong University from 1 September 2017 to 31 March 2018. This study was approved by the ethics committees of Renji Hospital of Shanghai Jiao Tong University School of Medicine. LAMP amplification (Loopamp® DNA Amplification kit) and Loopamp® fluorescence detection reagents (calcein) were all purchased from Beijing Loopamp Co. Ltd., China. LAMP primers were synthesized by Sangon Biotech Co. Ltd. Shanghai, China. Poly (dimethylsiloxane) (PDMS) used as the material for the microfluidics was purchased from Dow Corning Shanghai Co. Ltd. AZ 50XZ and SU-8 3050 used as the positive surface patterns was purchased from MicroChem Corp., MA, USA. Four LAMP chambers were fabricated with poly(methylmethacrylate) (PMMA) through mechanical microfabrication (Wenchang Chip Technology Co., Ltd. Shanghai, China). Magnetic beads and pathogens genomic DNA extraction kit were purchased from Shanghai Haoyuan Biotechnology Co., LTD.

2.2. Microchip design and fabrication

As shown in Fig. 2, the microchip comprises two layers, a PDMS layer on the top of a glass slide. The top PDMS layer has three distinct functional domains, one for pathogen lysis and DNA extraction, and another for liquid control and the third for LAMP. The cell lysis and DNA extraction region contains one sample inlet and one waste liquid outlet (diameter 1.5 mm, depth 150 μm), two channels (width 500 μm, depth 150 μm), and one chamber (diameter 10 mm, depth 1 mm). The liquid control region contains one channel (width 100 μm, depth 50 μm) that is controlled by a PMMA screw (diameter 2.5 mm, height 5 mm, pitch of screws, 0.7 mm). The LAMP region contains one reagent inlet reservoir (diameter 2.0 mm, depth 150 μm); 8 wells as LAMP zones (diameter 3.0 mm, depth 2.0 mm) were connected to the corresponding serpentine channel (width, 300 μm; depth, 150 μm). A chromatography paper disk (diameter 2.0 mm) was placed inside each LAMP zone as a storage substrate for LAMP primers. Microchamber 1/1′ and 2/2′ were coated with *S. pneumoniae* primer sets and *M. pneumoniae* primer sets, respectively. Positive control (PC) template DNA and its primer mix (PM) were provided by the Loopamp DNA amplification kit. Microchamber P1 and P2 were coated with PC template DNA and PM as positive controls. Microchamber N1 and N2 contained no primers as the negative control. The bottom layer is a glass slide mainly for structural support. PDMS/glass hybrid microchip was produced by molding a PDMS silicone elastomer against a microfabricated master. It should be noted that an AZ layer with a thickness of approximately 50 μm could be achieved by spin-coating AZ 50XZ at 1000 rpm for 20 s. A thickness of 150 μm could be achieved by spin-coating SU-8 3050 at 1000 rpm for 30 s (MA6, Karl Suss Corp., GER). The wells were adhered to the patterned surface with UHU PLUS 300 epoxy resin adhesive and then the chip master mold was fabricated. The PDMS precursor mixture, prepared at a weight ratio of base to curing agent of 10:1, was poured carefully on the master, and placed under a vacuum for 0.5 h to remove the bubbles. A PMMA screw was put vertically into the PDMS precursor mixture. The distance between the bottom of the screw and the top of the microfluidic channel was about 0.5 mm. After curing at 65 °C for 2 h, the screw was taken out from the PDMS precursor mixture. The cured PDMS replica was gently peeled off the master, and a conical inlet/outlet was drilled manually using a punch. After slabs of PDMS embossed with microfluidic channels were treated with oxygen plasma, a chromatography paper disk (diameter 2.0 mm) was placed inside each LAMP zone and then the PDMS was sealed irreversibly to the glass slides.

2.3. Developing a portable device

A portable device with a built-in Bluetooth module was developed according to procedures described by our previous work with minor modifications (Wang et al., 2017). This handheld device has the function of heating the IPμchip and transmitting the experimental results to a smartphone. Wi-Fi cameras (the filter type: ultraviolet mirror) and a UV pen tube lamp (wavelength: 365 nm, intensity: 1300 mW/cm2) have been incorporated into the portable unit. The device has a dedicated smartphone app interface to communicate and receive the results. The main technical parameters for the battery-powered device were:

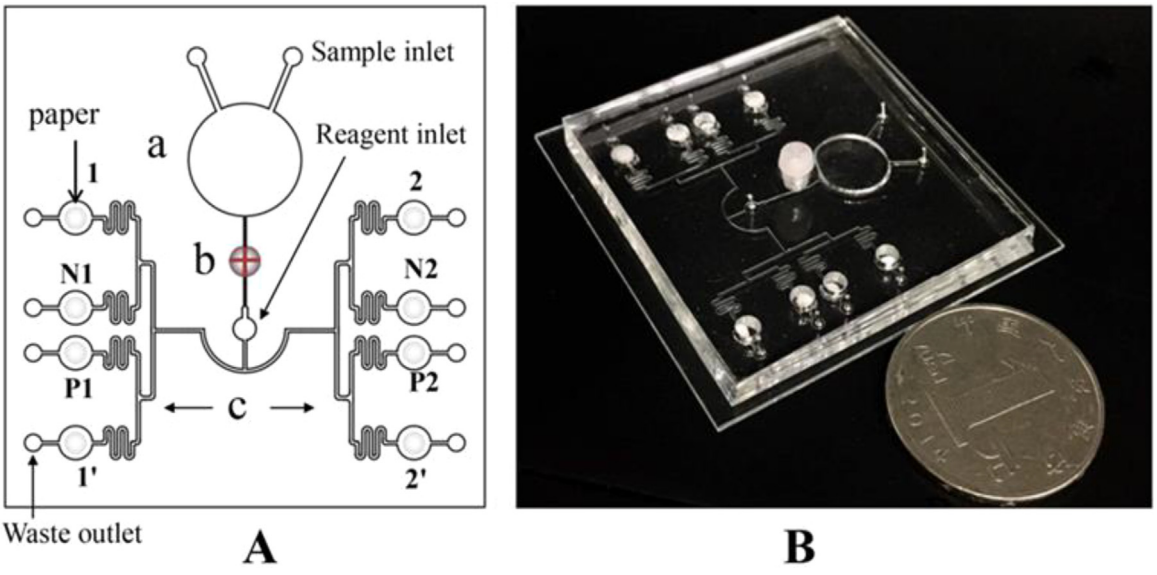

Fig. 2. Structure diagram of the IPμchip (A) and photograph of the chip (B). (a) Chamber for DNA extraction. (b) PMMA screw, (c) LAMP zones. A chromatography paper disk was placed inside each LAMP zone. Microchamber 1/1′ and 2/2′ were loaded with *S. pneumoniae* primer sets and *M. pneumoniae* primer sets, respectively. Microchambers P1 and P2 were loaded with template DNA and its primer mix from the Loopamp DNA amplification kit as positive controls. Microchambers N1 and N2 contained no primers as the negative control.

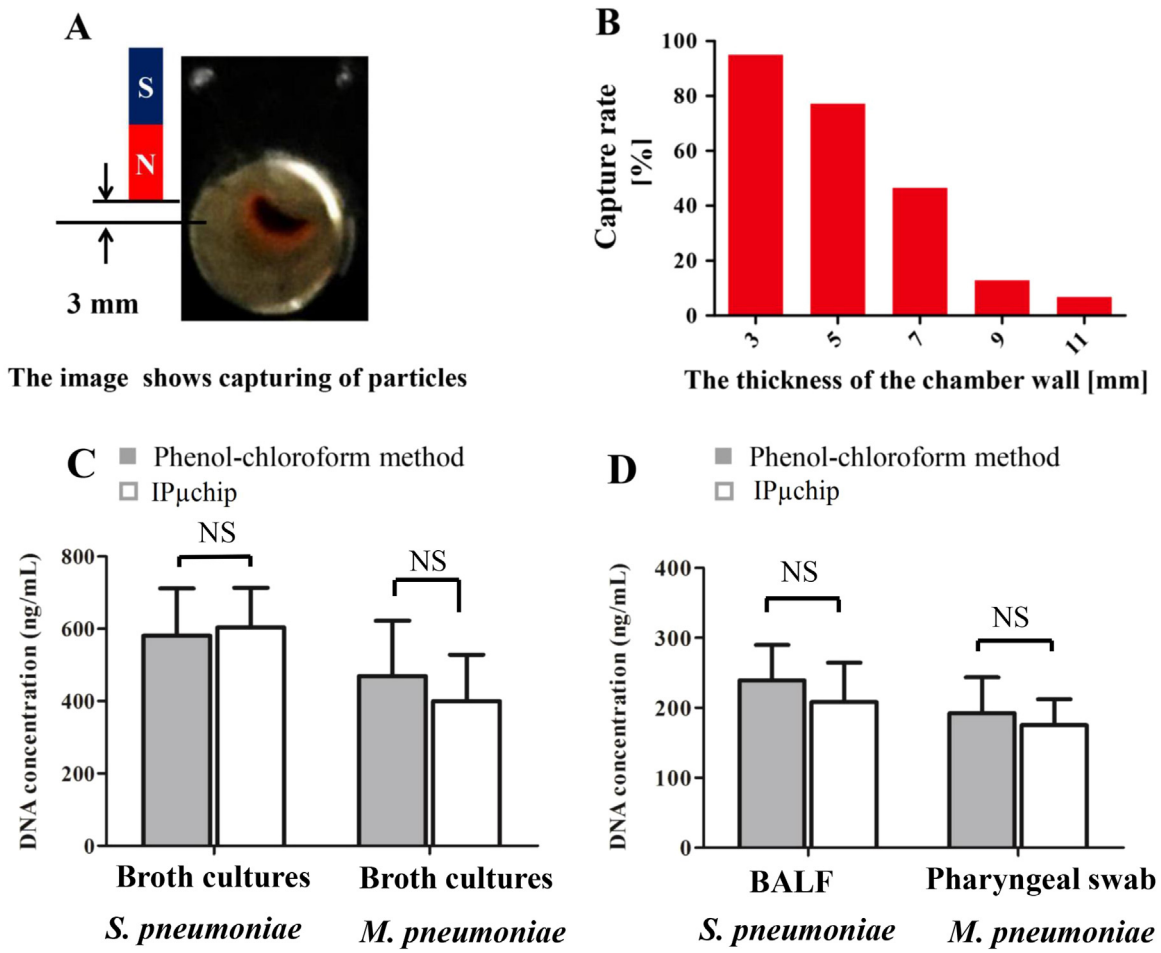

Fig. 3. The image shows capturing of particles in the chip (A). Particle capture was demonstrated at 95.0% at the 3 mm chamber wall thickness (B). Average concentrations of nucleic acid obtained from broth bacterial cultures of *S. pneumoniae* and *M. pneumoniae* following lysis on the chip and phenol-chloroform method (C). Average concentrations of nucleic acid obtained from BALF with *S. pneumoniae* and pharyngeal swab with *M. pneumoniae* after sample preparation on the chip compared to the phenol-chloroform method (D). On-chip and phenol-chloroform experiments were performed in quintuplicate and triplicate, respectively. Error bars depict 95% CI. NS: Non-significant differences.

temperature range, 37–100 °C; temperature fluctuation (°C), ± 0.2. A temperature control based on PID microcontroller was realized by regulating the input power of a Peltier heater. The internal thermometer used was calibrated with precision of 0.01 °C and, with carefully programmed PID values; the reaction temperature was controlled with an accuracy of ± 0.2 °C. The device is low-cost and its power consumption is low, making it portable to carry out analyses in resource-limited settings.

2.4. Integrated IP μchip operation

The assay was first optimized in a tube, and then translated to the chip format. After the screw valve was closed, samples (5 μL pure broth culture or 30 μL sediment of the real sample) were injected into the release chamber by a micropipette. Then, 30 μL DNA extraction liquid was injected into the chamber and mixed well with the micropipette for 5 min. Magnetic particles (0.5 μL, about 200,000 particles) were injected into the chamber and mixed well with a micropipette for 2 min. After capturing the target DNA, the DNA-magnetic particles were collected at the top of the chamber using a Neodymium magnet, and supernatant was gently collected and discarded. Then, the magnet was removed and 50 μL 100% ethyl alcohol was injected into the chamber and suspended the DNA-magnetic particles. The DNA-magnetic particles were recollected at the top the chamber using the magnet and then ethyl alcohol was gently discarded. After a wash step (50 μL 70% ethyl alcohol), nucleic acids were eluted in 40 μL deionized water. Then, the magnetic particles were captured and the inlet of the IP μchip was sealed with epoxy. The IPchip was placed on the portable device and heated at 65 °C for 5 min. The screw valve was opened and nucleic acids were pipetted out. Then the screw valve was closed to isolate the DNA release chamber and LAMP reaction chamber. Both 60 μL of the LAMP mix and 8 μL of mineral oil were subsequently introduced at the reagent inlet to fill the various different LAMP zones. After the inlet and outlet reservoirs were sealed with epoxy, the microfluidic device was placed on the portable device at 65 °C for 60 min for the LAMP reactions. After the LAMP reactions, the portable UV pen light was applied to illuminate the LAMP products. The generated fluorescence was recorded and wirelessly transmitted to a smartphone by the Wi-Fi camera. The final results were analyzed by the naked eye and confirmed by agarose gel electrophoresis.

2.5. LAMP amplification

The LAMP reaction was carried out in a tube using the Loopamp® reaction mixture. The total volume of the system was 25.0 μL, which contained 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH4)_2SO_4$, 0.1% Tween20, 0.8 M Betaine, 25 mM calcein, 0.5 mM $MnCl_2$, 1.4 mM dNTPs, 0.2 mM each of the outer primer (F3/B3), 1.6 mM each of the inner primer (FIP/BIP), 1.6 mM each of the inner primer (LF), 8U Bst Polymerase, and 2 μL of DNA sample as a template.

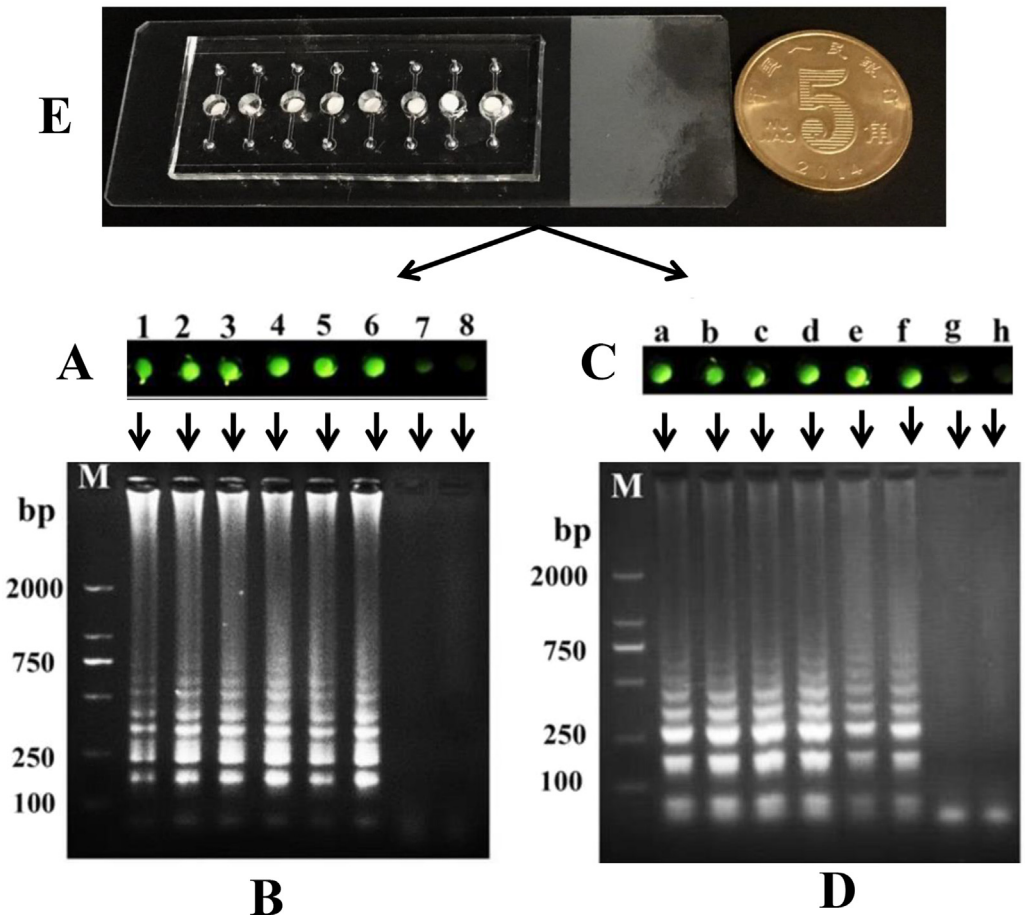

Fig. 4. The detection limit of *S. pneumoniae* and *M. pneumoniae* genomic DNA in microchambers. Serial dilutions of *S. pneumoniae* genomic DNA and *M. pneumoniae* M129 genomic DNA at concentrations of 2 ng/μL, 200 pg/μL, 20 pg/μL, 2 pg/μL, 200 fg/μL, 20 fg/μ1, 2 fg/μL%, and 0 fg/μL (deionized water) were analyzed by the microchip (E), respectively. A, Numbers (1–8) indicate the corresponding concentrations of the *S. pneumoniae* genomic DNA in the given sample. B, Agarose gel electrophoresis of the LAMP amplified products from (A). C, Letters (a-h) indicate the corresponding concentrations of the *M. pneumoniae* genomic DNA in the given sample. D, Agarose gel electrophoresis of the LAMP amplified products from (C).

The amplification was performed at 65 °C on a thermostat water bath for 1 h. The primers were designed manually according to the primer design software Primer Explorer V5. The details of the LAMP primers used in this study are listed in Tables SI 1 and SI 2.

2.6. Real-time PCR analysis for M. pneumoniae

Real-time PCR targeting the CARDS toxin gene of *M. pneumoniae* was used as a comparison for IPμchip. Real-time PCR analysis for *M. pneumoniae* was performed as Thurman *et al.* have described previously (Thurman et al., 2011). Amplification was monitored in real-time on an ABI7500 Fast thermal cycler, with an initial denaturation at 95 °C for 5 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.7. PCR analysis for S. pneumoniae

PCR analysis for the *S. pneumonia* was performed as Llull *et al.* have described previously (Llull et al., 2006). The PCR conditions included an initial denaturation at 95 °C for 5 min, followed by a 25 cycle amplification, with each cycle consisting of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 1 min. Products were visualized by resolution on a 2% agarose gel followed by staining with Goldview.

3. Results and discussion

3.1. Magnetophoretic-based micro chamber for pathogen DNA extraction

In this work, we utilized a neodymium magnet on a microfluidic chip for magnetic particles collection and DNA concentration. An advantage of using magnetic nanoparticles is the ease of isolation of DNA from cellular components. Kamat et al. (2018) have employed magnetic nanoparticles and a permanent magnet for effective bacterial lysis and DNA extraction. The chamber of 10 mm diameter was fabricated for collection of magnetic particles. A neodymium magnet was placed at the top of the chamber to control the motion of magnetic particles in microfluidic flow. The PDMS thickness of the chamber wall was carefully chosen for optimal magnetic force. An image of magnetic particles collection at the top of the chamber using a neodymium magnet is shown in Fig. 3A. A thickness 3 mm was chosen for the chamber wall to have sufficient magnetic force in capturing and holding magnetic particles in the microfluidic chip. To measure the magnetic particle collection rate, the magnet was moved away from the chamber and the entire chamber was flushed with TE buffer solution. The number of magnetic particles in flushed solution was counted using flow cytometry (Fig. SI1). The magnetic particle capture rate, which decreases as the chamber wall thickness increases, is shown in Fig. 3B. The capturing of particles was demonstrated at 95% at a 3 mm chamber wall thickness. On-chip DNA extraction from bacterial cultures was studied. Results were compared to the concentrations obtained from the same cultures with the phenol-chloroform method (SI1). Purified nucleic acids obtained from real samples and pure broth cultures were quantified by fluorometry (Qubit™ dsDNA Assay kit, Invitrogen) using a fluorospectrometer (Fluorometer Qubit 3.0). On-chip lysis resulted in similar DNA yields compared to the phenol-chloroform method (Fig. 3C). Non-significant differences were found between the two procedures for the two tested bacterial populations ($P > 0.05$). DNA extraction from the *S. pneumoniae* of a bronchoalveolar lavage fluid (BALF) and the *M. pneumoniae* of a pharyngeal swab were also tested on the chip. Differences between the chip assay and the phenol-chloroform procedure were not significant ($P > 0.05$) (Fig. 3D). These results demonstrated that the chip assay required less time, but the DNA

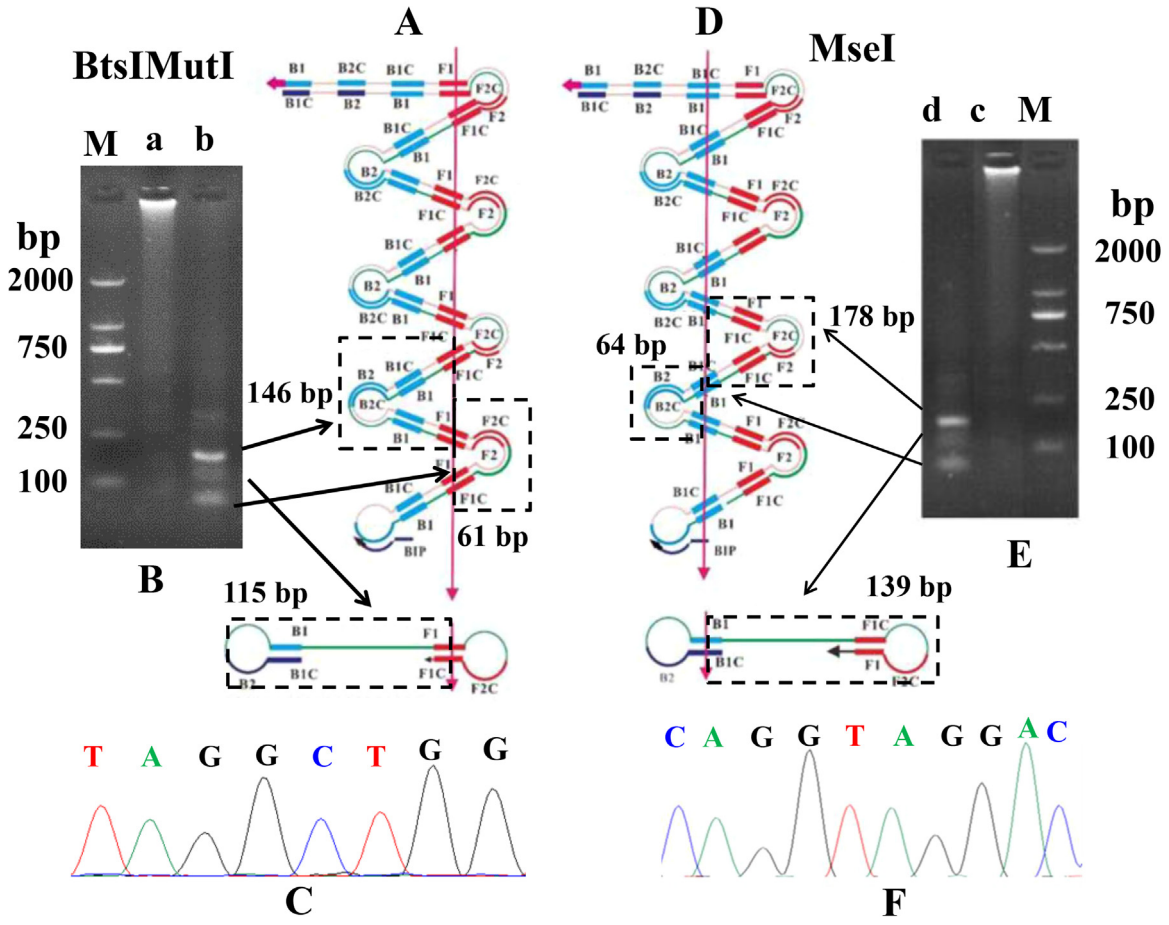

Fig. 5. Restriction analysis of the amplified *S. pneumoniae* and *M. pneumoniae* genomic DNA. (A) BtsImutI cuts F1. (B) The specific amplification confirmed by BtsImutI; lane a: ladder-like bands; lane b: three bands of the predicted sizes of approximately 146 bp, 115 bp, and 61 bp produced by the BtsImutI enzyme. (C) Sequencing results of the *S. pneumoniae* target sequence. (D) MseI cuts B1. (E) The specific amplification was confirmed by using MseI. lane c: ladder-like bands. Lane d: three bands of the predicted sizes of approximately 178 bp, 64 bp, and 139 bp products produced by MseI. (F) Sequencing results of the *M. pneumoniae* target sequence. Lane M: 2000 bp ladder size markers.

extraction efficiency of the chip assay was consistent with the conventional method. Furthermore, downstream applications such as LAMP are compatible with the microfluidic DNA extraction.

3.2. The detection limit of pathogens in microchambers

To evaluate the ability of the LAMP microchamber to detect low amounts of *S. pneumoniae* and *M. pneumoniae*, we constructed a PDMS/paper microchip with eight 14 μL LAMP zones (diameter 3.0 mm, depth 2.0 mm) (Fig. 4E). A chromatography paper disk (diameter 2.0 mm) loaded with *S. pneumoniae* or *M. pneumoniae* LAMP primers was placed inside each LAMP zone. Operation of the microchip was simple and did not require the use of any precise valves or pumps. We used 10-fold serial dilutions of genomic DNA to evaluate the sensitivity of the assay. Serial dilutions of *S. pneumoniae* DNA and *M. pneumoniae* M129 genomic DNA at concentrations of 2 ng/μL, 200 pg/μL, 20 pg/μL, 2 pg/μL, 200 fg/μL, 20 fg/μL, 2 fg/μL, and 0 fg/μL (deionized water) were analyzed by microchamber-based LAMP, respectively. The sample containing 1 μL of nucleic acid was first introduced via the inlet. A reaction mixture for LAMP of 11.5 μL was drawn slowly into the microchamber by a micropipette. The inlet and outlet were sealed with epoxy to form an integral microchamber for the LAMP reaction. The entire microchip was incubated at 65 °C for 1 h, using the portable device. Numbers (1–8) and letters (a-h) indicate the corresponding concentrations of the *S. pneumoniae* and *M. pneumoniae* genomic DNA in the given samples (2 ng/μL to 0 fg/μL), respectively. The detection limit of the *S. pneumoniae* and *M. pneumoniae* were identical when measured by direct visual inspection or gel electrophoresis. As shown, the detection limit of the *S. pneumoniae* chip assay (Fig. 4A, B) and the *M. pneumoniae* chip assay (Fig. 4C, D) were 20 fg/μL.

3.3. Accuracy of the LAMP assay

To further confirm the accuracy of the LAMP assay, the amplified products were digested with restriction endonucleases, and their sizes were analyzed using gel electrophoresis. The final products were a mixture of stem-loop DNAs with various stem lengths and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand. The products of the *S. pneumonia* DNA amplification were digested with BtsImutI. As shown in Fig. 5B, BtsImutI cut F1. Consequently, if the amplification products were to have exactly the same structures, the products would be expected to be cut to 61, 115 and 146 bp fragments by BtsImutI. The sizes of the fragments generated by BtsImutI digestion were consistent with the predicted sizes (Fig. 5A, lane b). The amplified products of *M. pneumoniae* DNA were digested by MseI. As shown in Fig. 5E, MseI cuts B1. Theoretically, the products would be cut to 64, 139 and 178 bp fragments by MseI. The sizes of the fragments generated by the MseI digestion were consistent with the predicted sizes (Fig. 5D, lane d). The amplified products digested with these enzyme were completely resolved into the expected identical component parts, indicating that the amplified products were derived from the intended

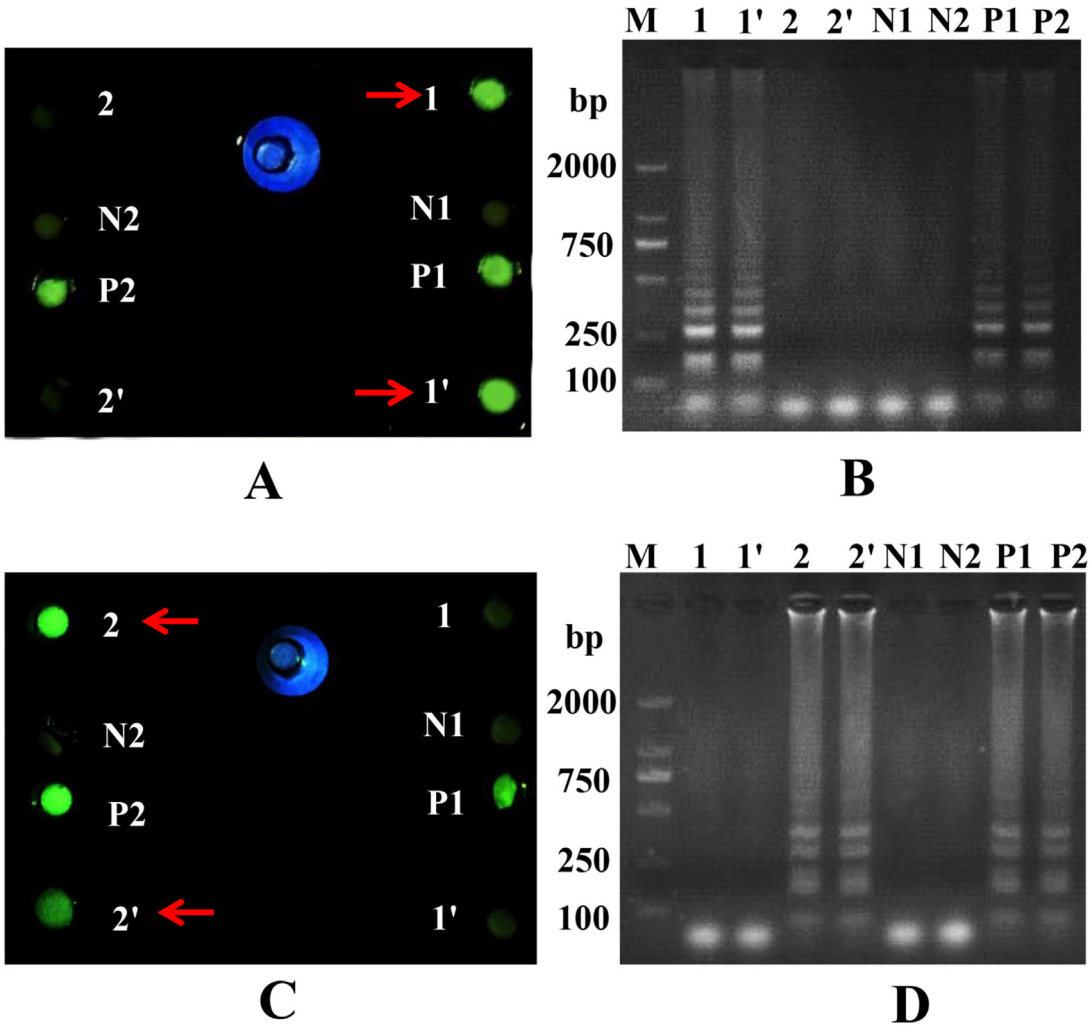

Fig. 6. Performance of IPμchip for analyzing clinical samples. The results were further confirmed by gel electrophoresis of all LAMP products. A, B: Patient with *S. pneumoniae* pneumonia. C, D: patient with *M. pneumoniae* pneumonia. Numbers 1/1′ and 2/2′ represent the *S. pneumoniae* and *M. pneumoniae* chambers, respectively. N1/N2 and P1/P2 represent negative and positive control, respectively. Lane M, 2000 bp ladder size markers.

M. pneumoniae and *S. pneumoniae* sequence and thereby confirming the specificity of the reaction. These results demonstrate that microchip-based LAMP is a highly accurate method for screening for *S. pneumoniae* and *M. pneumoniae*. To verify the structures of the amplified *S. pneumoniae* and *M. pneumoniae* LAMP products, the amplified products were sequenced (Sangon Biotech Co. Ltd., Shanghai, China). The sequencing results of the *S. pneumoniae* and *M. pneumoniae* target sequence is shown in Fig. 5C and F, respectively.

3.4. IPchip analysis of clinical specimens

A subset of clinical specimens comprised of oropharyngeal swabs and BALF (n = 63), was subjected to direct specimen testing with the IPμchip assay. Of these specimens, 32 cases were shown to be *M. pneumoniae* positive and 10 cases were shown to be *S. pneumoniae* positive based on testing by IPμchip. Of these, 33 cases were known to be *M. pneumoniae* positive based on testing by real-time PCR. One of the specimens was positive in real-time PCR but not by IPμchip, resulting in an analytical sensitivity of 96.9% and a specificity of 100% compared to the real-time PCR. (Table SI3). These results demonstrate that the sensitivity of the *M. pneumonia* IPμchip assay is nearly identical to that of real-time PCR. Among the 10 *S. pneumonia* IPμchip-positive specimens, 6 cases were PCR positive and 4 cases were culture positive. All 4 cases of the *S. pneumoniae* culture-positive specimens were positive by both *S. pneumoniae* IPμchip and PCR assays. In addition, 53 IPchip-negative specimens were also negative by PCR and culture methods (Table SI4). Compared with *S. pneumoniae* IPμchip, the clinical sensitivity of *S. pneumoniae* PCR and culture tests was 60% and 40%, respectively; while the clinical specificity of the two tests was 100%. These results demonstrate that the IPμchip assay was more sensitive than the *S. pneumoniae* PCR and culture methods. The robust performance of the IPμchip assay suggests that chip-based detection of pathogens could be feasible in a wide variety of clinical settings. Representative results of the assay are presented in Fig. 6. Fig. 6A shows the results of a patient with *S. pneumoniae* pneumonia. *S. pneumoniae* triggered the green fluorescence signal in chamber 1/1′. None of these samples could induce the fluorescence signal in chamber 2/2′ or the negative control chamber. Fig. 6C shows the results of a patient with *M. pneumoniae* pneumonia. *M. pneumoniae* triggered the green fluorescence signal in chamber 2/2′. None of these samples could induce the green fluorescence signal in chamber 1/1′ or the negative control chamber. Microchamber P1/P2 showed bright green fluorescence under the UV light pen of the portable device. Subsequently, the results were further confirmed by conventional gel electrophoresis of all LAMP products (Fig. 6B, D). The results show that the IPμchip platform will enable rapid, low-cost detection of *M. pneumoniae and S. pneumoniae* cases and the inclusion of an unambiguous visual readout via WiFi and smartphone app further simplifies the assay already amenable to point-of-

care use. Recently, lab-on-a-chip systems have been developed for rapid pathogens detection (Oh et al., 2016; Roy et al., 2017). However, all the analysis steps could not be carried out on those chip system. Integration of the steps required for POCT via real-time PCR has been developed in the Cepheid GeneXpert (Cohen et al., 2018; Moore et al., 2017) and the FilmArray Respiratory Panel test (Song et al., 2016; Leber et al., 2018) and the Roche cobas® Liat® system. However, these instruments are expensive to purchase, which may limit the availability and utility of POCT via real-time PCR, and the assays are complex to perform in resource-limited laboratories in developing countries. Compared to other microchip systems, all the assays include sample pretreatment, pathogens lysis, DNA extraction, LAMP, and visual detection could curried out on the IPchip platform. Additionally, the IPchip require easier fabrication. The best part of the platform is that the IPchip cost a total of $5, a far cry from the $200–400 of FilmArray Respiratory Panel test or GeneXpert. Although the platform has many advantages, the assay protocol is complicated. Therefore, we will develop an IPchip automation system instead of manual operation in the future.

4. Conclusion

Altogether, we developed an IPchip platform for point-of-care detection of S. pneumoniae and M. pneumoniae. This chip was functionalized with rapid sample pretreatment, pathogens lysis, DNA extraction, LAMP, and direct naked eye. These results demonstrate that the assay was more sensitive than the PCR and culture methods and could be feasible in a wide variety of clinical settings. Even though the assay protocol is complicated, further optimization and improvements to this automation platform may lead to the availability of a rapid, cost-efficient laboratory test for detection in resource-limited settings,

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2018.11.011.

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