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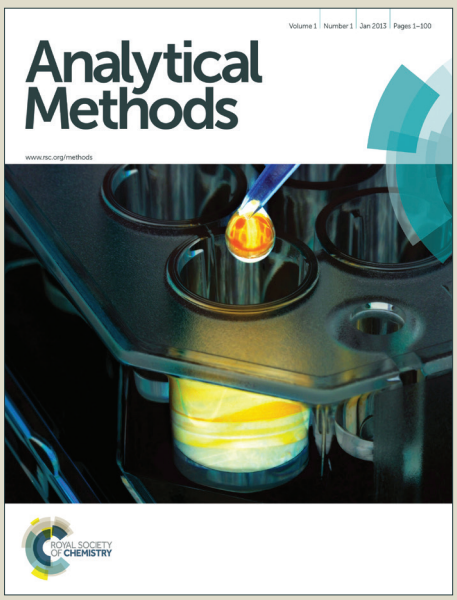

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Microfluidic Chip for rapid analysis of cerebrospinal fluid infected with *Staphylococcus aureus*

Cite this: DOI: 10.1039/x0xx00000x

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Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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Abstract: **A microfluidic assay based on loop mediated isothermal amplification was successfully developed to analyse *Staphylococcus aureus* in the clinical cerebrospinal fluid samples. A set of primers were designed showing high specificity and good sensitivity. The reagent consumption was reduced to 1.5ul and the total analysis time is about 60 minutes.**

Introduction

Staphylococcus aureus, which known as a major community-acquired pathogen, was also one of the most frequently encountered bacteria in hospitals. As a result, *S. aureus* is one of the important sources of infection in surgical procedure, such as cerebrospinal fluid (CSF) diversion process. Approximately 40 per 100,000 people suffered from hydrocephalus. Infections related with CSF diversion devices are always associated with high morbidity and mortality,[1] which often caused by *S. aureus*, *Pseudomonaas, Klebsiella pneumonia* and *Enterococcus faecals*.[2] Infections not only increased the costs (approximately 40,000 euro per episode), but also increased the incidence of seizures and neurological disturbances incidence.[3]Among all these infections, more than two thirds are caused by *Staphylococcus spp.* among which *S. aureus* is one of the most susceptible bacteria.[3] It can cause bacterial endocarditis by circulation.[3]

Previous studies have shown that, the higher rate of initial diagnosis, the lower morbidity and mortality rates.[4] So early identification is essential in *S. aureus* related CSF diversion infection and its treatments. At present, conventional identification methods used in most hospitals usually involves *S. aureus* sample pre-treatment and time consuming culture process. These methods always taken few days to complete and efficiency is relatively low. Generally, when the diagnostic results come out, the best treated time has passed. Antibiotic and antivirals are often used without knowing the species of pathogen and even the type of pathogen. Consequently, antibiotic and antivirals were often abused and the conditions of patients might get worse.

Several methods have been developed to identify *S. aureus* in recent years. Among these methods, molecular diagnostic methods are growing rapidly due to its high accuracy, sensitivity and specificity. Some molecular diagnostic techniques have been utilized on rapid pathogen detections, such as various polymerase chain reactions (PCRs). Because precise temperature control and power-intensive thermal cycling as well as complex optical inspection system are needed for the PCRs, [5] long time consuming and sophisticated instrument were still the major drawback.

Loop-mediated isothermal amplification (LAMP) is an attractive technique with high sensitivity and selectivity within less assay time .[6, 7, 8] Compared with traditional PCRs. This assay requires a set of four/six specially designed primers and *Bst* polymerase. [6, 7, 9] Without complex temperature control, specific nucleic acids are amplified under isothermal conditions (60-65℃) and produce stem-loop DNA structures with quality more than 500 μg/mL.[10] Because of the large amount of DNA product, the results can be visible with naked eyes by observing the white precipitate of magnesium,[11] or by observing the colour changing by adding calcein with manganous to the assay.[7] In recent years, LAMP method has been applied in detections of virus, bacteria, even in cancer diagnosis. Some of them has been developed as commercial kits and adopt as officially recommended methods.[12]

Microfluidics are promising technique dealing with fluid in micro sizes, characterized by low cost, easy operation and portability, and have been explored for molecular diagnose in the past decade.[13,14,15] Many chemical, biological and medical analysis processes have been integrated into a micro-scale microfluidic device. It is also a promising technique to perform LAMP assay.[12] [16,17,18] Fang et al. has fabricated a 5ul microfluidic system for different bacteria detection.[19,20,21] Huang et al. developed a AI

micro-nanofluidic chip, and realize single molecule diagnose with nanoliter reagent consumption.[22,23] Wu et al. integrated DNA extraction by glass solid phase extraction(SPE) bed, LAMP and online fluorescence detection into a single chip.[24] For *S. aureus* detection, Wang et al. recently reported a diagnostic LAMP microfluidic assay for detecting methicillin-resistant *Staphylococcus aureus* (MRSA).[25]

Herein we present a novel integrated diagnose assay for *S. aureus* detections of CSF samples. High species-specific primers for LAMP amplification of *S. aureus* was selected in this study. The assay could perform lysis to release DNA and LAMP amplification process in a single assay. The signal can be detected by naked eyes under 365nm UV lamp. The reaction time and sample and reagent consumptions were also greatly reduced.

Materials and methods

Bacteria

Staphylococcus aureus was obtained from China General Microbiological Culture Collection Center (CGMCC). Clinical bacteria *S. aureus* sample were obtained from Huashan Hospital and Shanghai Medical School. Extracted DNA of *α-Hemolytic streptococcus, β-Hemolytic streptococcus, γ-Hemolytic streptococcus, Corynebacterium diphtheriae, Pseudomonas perugino, Salmonella enterica, Hemophilus influenzae, Neisseria meningitides* and *Staphylococcus albus* were obtained from Shanghai medical school. *Pseudomonas aeruginosa, Klebsiella pneumonia, Enterococcus faecalis* and two clinical *S. aureus* were received from Huashan Hospital. All the clinical CSF samples were obtained from Huashan Hospital.

Bacteria cell lysis buffer was obtained from Tiangen Co. (China). LAMP reaction mixtures {contained Tris-HCl (pH 8.8), $(NH_4)_2SO_4$, KCl, $MgSO_4$, d NTP, BSA, betaine and so on} and celcein with manganous were obtained from Diaou Co. (China). Primers for LAMP amplification (0.8uM for FIP and BIP, 0.2uM for F3 and B3, 0.4uM for LF and BF) were synthesized by Invitrogen.

Chip fabrication

For the microfluidic chip fabrication, photoresist AZ-50XT (AZ Electronic Materials.), SU-8 2025 and SU 2150 (Microchem USA) were used to fabricate the mold. The chips were fabricated from PDMS (polydimethylsiloxane) (RTV 615, Momentive Performance Materials, NY, USA).

The three-layer chip (5 cm × 2.5 cm) was constructed by standard multilayer soft lithography.[26] Upper control silicon mold was made by a 30um thick AZ-50XT . The flow molds were fabricated using negative photoresist (SU8-2025) at 30um thickness and negative photoresist (SU8-2150) 300um thickness for the middle flow layer and the bottom LAMP layer respectively.

The upper control layer was made by pouring the mixture of PDMS (RTV 615A and B in a 10:1 ratio) onto the master, placed under vacuum for 1h to remove gas, then cured at 80℃ for 1h.. The middle flow layer was made by spin coating the control mold with PDMS (A:B=7:1) at 2,000 rpm and baking at 80℃ for 1h. Then the control layer structure was peeled off the mold and aligned to the flow layer. Following a 1h bake at 80℃, the bonded control and flow structure was gently peeled from the flow mold, and 0.75mm-diameter holes were drilled. The bottom LAMP layer was prepared the same method as the upper control layer. The bonded control and flow structure was then mounted onto the LAMP layer and baked at 80℃ for 12h before utilization.

A home-made heating system was used for temperature control. It includes a heating membrane (Length, 60mm; Width, 40mm; Thickness, 0.2mm, Jiari Electronic Materials Co., China) located beneath the chip as thermal source. A temperature sensor (Willhi electron Co., China) was used for accurate temperature feedback control.

Result and discussion

Due to the large amount of pyrophosphate ions which produced by LAMP reaction, the results can be read out by adding calcein with Manganous ions to emit fluorescence. Initially the fluorescence from calcein was quenched due to the combination between Manganous ions (Mn^{2+}) and calcein. In the DNA amplification process, pyrophosphate ions are produced as a by-product and it will bind with Manganous ions to release the calcein, resulting the fluorescence emission from calcein under excitation at 365nm. The free calcein could combine with magnesium ion (Mg^{2+}) in the reaction mixture to make the fluorescence stronger. [7] Consequently, the positive result will emit green fluorescence, while the negative results do not show fluorescence. In the proposed study, method by adding calcein with manganous ions was used and all the fluorescence were detected under UV excitation at 365nm.

Primer design and specificity measurement

The target *nuc* gene which encodes thermostable nuclease (Gene Accession No. V01281) was selected, and specificity was tested by BLAST. The gene was reported to be found only in *S. aureus* strains, not in other *Staphylococcus* species.[27] Primers in our assay were designed by LAMP designer (Biosoft) shows in table 1, and the specificity was evaluated by BLAST.

In order to confirm its specificity, *Pseudomonas aeruginosa, Klebsiella pneumonia* and *Enterococcus faecalis* were used which frequently infected patients during CSF diversion operation process. 2 clinical *S. aureus* samples were used for testing conservation of the *nuc* gene.

All the images were taken under the wavelength of 365nm UV light.[7] As shown in Fig. 1a, the high specific primers can be used to distinguish *S. aureus* from other pathogen which frequently infect patient during CSF diversion operation process successfully. So the primer selected can be used to diagnose whether the CSF of patient was infected with *S. aureus.*

For further confirming the specificity, 9 reference strains which were the most well-known pathogens have been used, including *α-*

Hemolytic streptococcus, β-Hemolytic streptococcus, γ-Hemolytic streptococcus, Corynebacterium diphtheriae, Pseudomonas perugino, Salmonella enterica, Hemophilus influenzae, Neisseria meningitides and *Staphylococcus albus*.

As shown in Fig. 1b, in all reaction assays, only the assay with *S. aureus* presented positive result with fluorescence emission. The experiment showed high specificity for *S. aureus* detection. In Fig. 1c, all assay with *S. aureus* revealed positive results, which proved that the selected *nuc*-gene has high conservation and the selected primers can be used in clinical detection.

Table 1 Sequence of primers

Primer	Sequence (5' to 3')
S. aureusF3	CAAGTCTAAGTAGCTCAGCAA
S. aureusB3	CCAAGCCTTGACGAACTAA
S. aureusFIP	TCTGAATGTCATTGGTTGACCTACATAAAGAA
S. aureusBIP	AATATGGTCCTGAAGCAAGTGCGCTAAGCCAC
S. aureusLF	CCGTATCACCATCAATCGCT
S. aureusLB	GAAGTCGAGTTTGACAAAGGTC

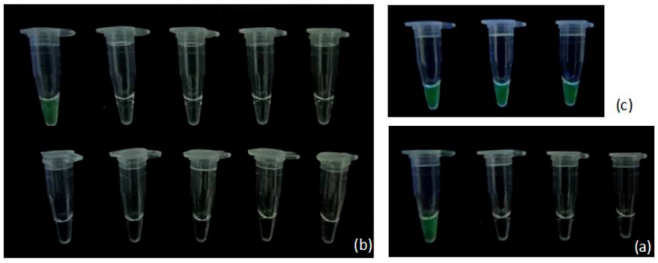

Figure 1(a) From left to right, the template for LAMP reactions are *S. aureus, Pseudomonas aeruginosa, Klebsiella pneumonia* and *Enterococcus faecalis*, all the pictures were taken under 365nm UV lamp.

Figure 1 (b) From left to right, the template for LAMP reactions are *S. aureus, α-Hemolytic streptococcus, β-Hemolytic streptococcus, γ-Hemolytic streptococcus, Corynebacterium diphtheriae, Pseudomonas perugino, Salmonella enterica, Hemophilus influenzae, Neisseria meningitides* and *Staphylococcus albus* , all the pictures were taken under 365nm UV lamp.

Figure 1(c) From left to right, the template for LAMP reactions are *S. aureus* and two clinical *S. aureus* strain, all the pictures were taken under 365nm UV lamp.

Chip design

In this study, the LAMP microfluidic chip was fabricated from 3 layers PDMS (shown in figure 2). The upper layer was the pneumatic control layer that dictated the status of the fluidic channels located in the middle layer, and four LAMP reaction channels, were located in the bottom layer and connected to the fluid channels in the middle layer.

The picture of the fabricated microfluidic assay shown in figure 3. 4 units were contained for 4 samples detection. Each unit consist one injection channels for loading sample (200um width and 30um depth), one lysis chamber(200um width, 30um depth) for extract DNA and one reaction chamber (400um width, 330um depth) for LAMP reaction. The volume of each reaction was about 1.5ul. Valve1, 2, 3and valve 4 were "oil valve", which were filled with oil instead of nitrogen. Valve 1, 2, 4 were used to seal lysis chamber during lysis process and LAMP chamber during LAMP reaction. And valve 3 was used to control the injection of CSF sample. To prevent cross-contamination, each sample was injected through different microchannel respectively.

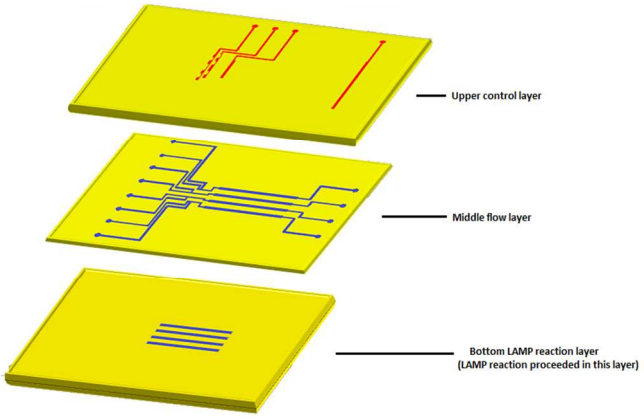

Figure2 Schematic diagram of the multilayered structure.

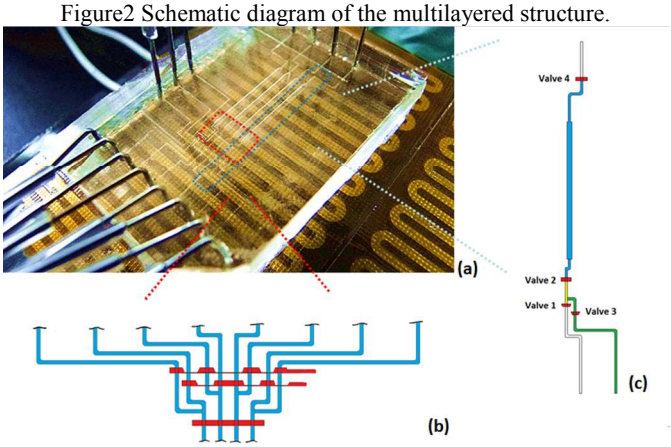

Figure 3a Picture of LAMP microfluidic assay
Figure 3b Illustration of the 4 parallel sample control system
Figure 3c Illustration of one individual LAMP assay, the green, the yellow and blue part showed sample injection channel, lysis chamber and LAMP reaction chamber respectively. Different valves showing in red.

Chip operation

As shown in figure 4, the sample and lysis buffer at a volume ratio of 100: 1 were first injected into the lysis chamber. While the upstream (valve 1), downstream (valve 2) micro valves of the chamber maintained and valve 3 were closed (figure 4a). After the injection of sample, the chamber was heated to 70℃ for 30min. Subsequently, the valve1, 2 were open, the LAMP reaction mixtures, *Bst* polymerase, primers, and calcein with manganous were loaded into the amplification chamber with valve 3 closed (figure 4b). Then both the upstream and the downstream valves of the LAMP chambers (valve 2 and valve 4) were closed. The temperature of the chamber was adjusted to 65°C to perform LAMP amplification. The final fluorescence image was detected after 40 min, under 365nm UV lamp (figure 4c).

The LAMP microfluidic in this study was performed with the CSF sample directly instead of conventional DNA extraction or purification process. Extra amount of *Bst* polymerase and BSA (0.1ug/ul) was added into reaction mixture, so as to make up for the adsorption of DNA and protein onto PDMS.

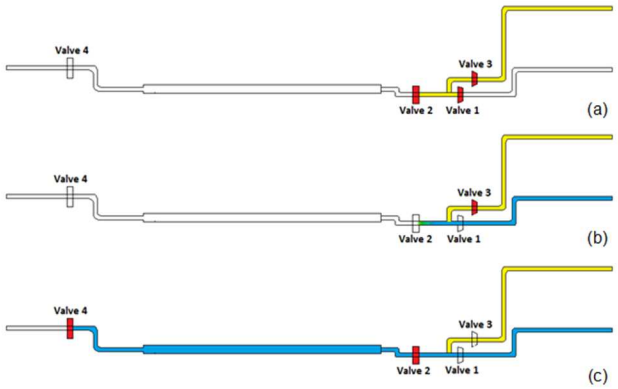

Figure 4 Schematic illustration of chip operation process. (a) lysis process with valve 1, 2, 3 closed; (b)LAMP reagents injection with valve 1,2 opened and valve 3 closed; (c)LAMP amplification process for 40 minutes with valve2,4 closed.

S. aureus detection of CSF sample by powerful LAMP microfluidic assay

In this study, clinical CSF samples with infection were used to evaluate the assay. 24 clinical CSF samples were tested using the LAMP microfluidic assay. Because CSF contains some proteins which may affect the LAMP reaction, high temperature treatment was adapted in LAMP microfluidic assay, and false positive results reduced (data not show). 70 ℃ was used for preventing false positive in LAMP microfluidic assay. For improving efficiency, lysis buffer at the ratio of 1:100 (lysis buffer: sample) was used. In this assay, 24 clinical CSF samples were respectively first mixed with lysis buffer at the ratio of 100: 1, and then loaded into the lysis chamber at 70℃ for 20 min. Afterwards, LAMP reaction mixtures, *Bst* polymerase, primers and calcein with manganous were injected in and then mixed with clinical samples and lysis buffer. The LAMP reaction was carried out at 63℃ for 40 minutes.

As shown in figure 5, 24 clinical samples results carried out in 6 different LAMP microfluidic assay and detected under 365nm UV lamp. 4 results in reactions chambers contained in one assay shown in each picture. Green fluorescence indicated positive results which were clearly distinguished from background, and negative results were without fluorescence. The fluorescence images illustrated clearly the difference between the positive results and the negative result. All the results in each assay were the same as traditional method.

The results shown LAMP microfluidic assay successfully carried out with high specificity, without cross-contamination. The infected CSF sample can be detected in 60 minutes within LAMP microfluidic assay by detecting under 365nm fluorescence.

Conventional *S.aureus* diagnosis normally takes 1-2 days and consumes 25ul. The LAMP microfluidic assay can detect *S. aureus* infected CSF with high accuracy and the whole reaction volume was just 1.5ul which was much deduced. To our knowledge, the volume can be further decreased by further improve the size of reaction chamber.

Since the LAMP reaction is carried out in aqueous solution, evaporation will often lead to the failure of LAMP microfluidic assay. To overcome this problem, reports have been represented by using plungers, or injecting mixed PDMS into inlets and outlets [19-21, 28]. Such methods were labor intensive and cannot integrate into fully automatic operating platform.

Due to high temperature environment condition inside the microfluidic chip, common nitrogen-filled pneumatic valve was improper in LAMP microfluidic assay. Thus, a prerequisite sealing method was developed in LAMP microfluidic assay. Oil was chose to seal the reaction system due to its high boiling point instead of nitrogen. Oil was purged into valve in control layer and tested under 65℃ and 70℃. The results showed no evaporation during amplification and lysis process. So not only the "oil valve" can be used in LAMP microfluidic devices within easy fabrication and control, but also used in other microfluidic devices with heating system.

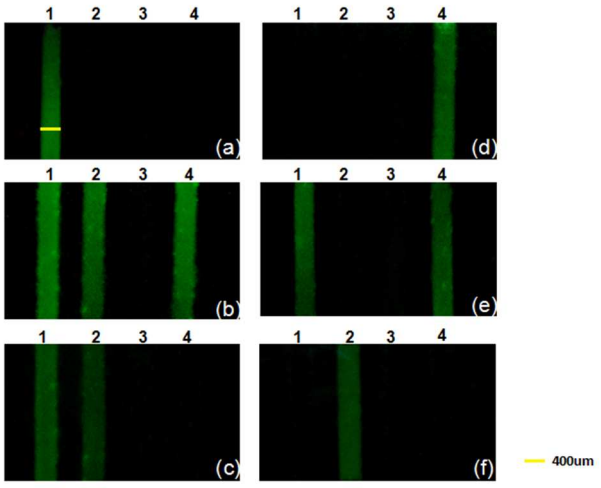

Fig 5 Fluoresces images of clinical samples detection results in reaction chambers.

Conclusions

Herein a simple microfluidic assay was successfully developed for rapid and accurate detection of clinical CSF sample infected with *S. aureus*. The whole chip was made of PDMS by standard multilayer soft lithography. It was capable of parallel detection with sample consumption of 0.12ul which was much lesser than other reported LAMP microfluidic assay. Total analysis time for 4 samples detection was about 60 minutes including pre-treatment and amplification part. Finally, the results can be detected by naked eyes under UV lamp without any expensive or complicate machine.

Leaking or the bubble problem was solved by using a simple "oil valve" which was modified from common pneumatic valve using oil for substitute instead of nitrogen. Clinical samples were used to validate the assay. 24 samples have been tested; same as conventional clinical tests which results were the same as the tests in our LAMP assay. It can be used in medical diagnose with high accuracy since high specificity primers was designed in this study after verified by 12 kinds pathogens which are frequently infect patients. It has potential in other pathogens detection by designing new primers and realizing different clinical sample detection such as blood by integrating the enrichment part we are focus on now.

Acknowledgement: This work was funded by NSFC (21377026, 20975025), Cultivation Fund of the Key Scientific and Technical Innovation Project, Ministry of Education of China (708031).

Notes and references

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Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/c000000x/

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