Rapid detection of live methicillin-resistant *Staphylococcus aureus* by using an integrated microfluidic system capable of ethidium monoazide pre-treatment and molecular diagnosis

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is a bacterium resistant to all existing penicillin and lactam-based antimicrobial drugs and, therefore, has become one of the most prevalent antibiotic-resistant pathogens found in hospitals. The multi-drug resistant characteristics of MRSA make it challenging to clinically treat infected patients. Therefore, early diagnosis of MRSA has become a public-health priority worldwide. Conventionally, cell-culture based methodology and microscopic identification are commonly used for MRSA detection. However, they are relatively time-consuming and labor-intensive. Recently, molecular diagnosis based on nucleic acid amplification techniques, such as polymerase chain reaction (PCR), has been widely investigated for the rapid detection of MRSA. However, genomic DNA of both live and dead pathogens can be distinguished by conventional PCR. These results thus could not provide sufficient confirmation of an active infection for clinicians. In this study, live MRSA was rapidly detected by using a new integrated microfluidic system. The microfluidic system has been demonstrated to have 100% specificity to detect live MRSA with *S. aureus* and other pathogens commonly found in hospitals. The experimental results showed that the limit of detection for live MRSA from biosamples was approximately 10^2 CFU/ μ l. In addition, the entire diagnostic protocol, from sample pre-treatment to fluorescence observation, can be automatically completed within 2.5 h. Consequently, this microfluidic system may be a powerful tool for the rapid molecular diagnosis of live MRSA. © 2012 American Institute of Physics. [<http://dx.doi.org/10.1063/1.4748358>]

I. INTRODUCTION

Staphylococcus aureus (SA) is a Gram-positive coccal bacterium frequently found in the flora of normal skin and in nasal passages. It can cause a wide range of illnesses, from minor skin infections to life-threatening diseases such as bacteremia, endocarditis, meningitis, pneumonia, sepsis, and toxic shock syndromes. SA is considered as one of the most common outbreak pathogens in community and hospital infections.¹ Although penicillin was discovered to effectively inhibit streptococcal infections, the evolution of antibiotic-resistant pathogens has accelerated due to widespread antibiotics abuse from clinical treatments. Due to the excessive use of antibiotics, super bacteria have been easily produced that are multi-resistant to various kinds of antibiotics.² For instance, methicillin-resistant *S. aureus* (MRSA) was first reported in 1963 to have developed resistance to beta-lactam antibiotics and cephalosporins.³ The therapeutic problem of multiple

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drug-resistant bacteria in nosocomial infections has spread worldwide since then. Because MRSA is resistant to many types of antibiotics, only a very limited spectrum of antibiotics can be used for treatment of MRSA and, therefore, treatment of such an infection requires prolonged hospitalization and healthcare. Compared to the methicillin-sensitive SA (MSSA), the treatment of MRSA requires more expensive antibiotics, diagnostic kits, and a longer period of clinical care. The costs for MRSA treatments were estimated to be 6%–10% more than MSSA infections and the death rate for MRSA was 21% as compared with 8% with MSSA.⁴ The cefoxitin disk screen test, the latex agglutination test for PBP2a, and oxacillin E-test have been used as standard methods of laboratory diagnosis for MRSA, as indicated by the Clinical and Laboratory Standards Institute (CLSI).⁵ However, these diagnostic methods require a lengthy analysis period that is not suitable for early clinical treatment.

Recently, with the rapid development of molecular diagnosis techniques, nucleic acid amplification has become popular for use for pathogen diagnosis.⁶ These molecular diagnostic methods have been demonstrated to have the advantages of higher specificity, higher sensitivity and are even more rapidly than the traditional assays.⁷ For instance, various molecular diagnostic methods have been developed to detect the specific *mecA* gene, which confers methicillin (and oxacillin) resistance.^{8–10} Among them, the polymerase chain reaction (PCR) is a powerful tool for the diagnosis of MRSA in clinical samples and environments.^{11,12} However, the conventional large-scale apparatus required for the molecular diagnosis of MRSA has substantial tested sample and reagent consumption and is a lengthy and labor-intensive experimental processes that easily introduce systematic errors between operation steps. Furthermore, although PCR can quickly detect the presence of a specific DNA sequence, the DNA of dead bacteria is still stable in biological samples for weeks, which may cause false positive results. Thus, the viability of pathogens in a clinical sample cannot be easily estimated from PCR results. Consequently, this can lead to the overuse of antibiotic which may induce production of more multi-drug resistant bacteria after clinical treatment.

Recently, micro-electro-mechanical-systems (MEMS)-based biomedical systems have been widely applied to molecular diagnosis.^{13,14} In addition to reducing the required amount of reagents and samples, lab-on-a-chip (LOC) systems have been used in a variety of rapid diagnosis systems that avoid the danger of operators being infected by pathogens during the analysis process and decrease sources of errors due to automated sample handling. For instance, in order to simplify the experimental processes and to reduce the area of the microfluidic chip, serpentine-shape (S-shape) micro-pumps was used for fluidic transportation and sample mixing.¹⁵ The S-shape micro-channel was also equipped with a polydimethylsiloxane (PDMS)-based floating-block structure that prevented fluid backflow so that it could be used as a micro-valve as well. The PDMS membrane was peristaltically deflected by compressed air to drive the fluidic in the micro-channel. The fluid was transported forwards and backwards which was regulated by electromagnetic valves (EMV). This microfluidic control module was also applied to cell counting, sorting and nucleic acid amplification in microfluidic systems.^{16,17} In our previous works, the specific nucleotide probes were conjugated onto magnetic beads and were used to capture specific DNA by thermolysis and hybridization, for target nucleic acid extraction and purification.¹⁸ In order to shorten the whole operating process, the nucleic acid amplification results were measured by fluorescence signals in a microfluidic system which replaced slab-gel electropherograms. Therefore, integrated microfluidic systems have been developed to perform the entire analytical protocol including sample pre-treatment, sample transportation, nucleic acid amplification, and optical detection of pathogenic signals.^{19–21} However, it is still challenging to distinguish live or dead pathogens using these developed microfluidic-based protocols.

Ethidium monoazide (EMA) is a cell membrane impermeable, fluorescent, nucleic acid staining dye.²² The intermediate compound, nitrene, was produced by photolysis of EMA with visible light exposure that could be bound to DNA to form a stable monoadduct.²³ Free EMA in solution was photolyzed and converted to hydroxylamine that lost covalent bonding capability with DNA. Because the EMA cannot penetrate into integrity of live cellular membrane, it can be used to selectively label the DNA of dead cells within a population of viable and dead

cells. EMA has been previously applied for differentiating live and dead bacteria in the literature.^{24,25} The DNA of live bacteria from clinical samples was pre-treated by EMA under visible light exposure that was then captured by specific DNA probes conjugated onto the surface of magnetic beads after thermolysis. In this study, a new integrated microfluidic system using an EMA-PCR detection scheme was first developed for the detection of live MRSA pathogens. Then, any captured DNA, which would be released from live bacteria, was amplified by performing an on-chip PCR process. The PCR results were then quantified by fluorescence signals. The developed microfluidic system could detect the live MRSA after antibiotic treatment to verify the efficiency of this treatment and also to avoid overuse of antibiotic which decreased the risk of a multi-drug-resistant pathogen outbreak. This is the first time that the rapid diagnosis of live pathogens has been performed in a microfluidic system.

II. MATERIALS AND METHODS

A. Experimental procedure

In this study, the experimental steps for detection of live MRSA included EMA pre-treatment, thermolysis and DNA denaturation, isolation of the *mecA* gene, PCR and fluorescent signal detection. The overall steps of the EMA-PCR protocol are illustrated in Fig. 1. Briefly, a pool of MRSA which included live and dead bacteria, specific-probe-conjugated beads and EMA were first co-incubated in a sample/washing-buffer loading chamber. The EMA could penetrate through the cellular membranes of dead bacteria under visible light exposure. The EMA would then irreversibly, covalently bind to the bacterial genomic dsDNA that inhibits DNA denaturation in the subsequent PCR process. However, live MRSA maintained the integrity of their cell membrane structure prevents the EMA from penetrating into the cell. All complexes were then transported into a PCR reaction chamber by activating the S-shape micro-pump. The on-chip micro-heaters activated thermolysis of the MRSA and denatured the bacterial dsDNA at 95 °C for 5 min. Then, the denatured DNA was captured by the specific-probe-conjugated magnetic beads at 58 °C for 10 min. Next, the hybridized

FIG. 1. Schematic illustration of the experimental procedures for EMA pre-treatment, thermolysis, DNA isolation, nucleic acid amplification, and optical detection of live MRSA.

DNA-probe conjugated magnetic complexes were isolated by using a permanent magnet placed underneath the microfluidic chip. All the other unbound reactants in the solution would be flushed into a waste chamber by washing twice with double distilled water (ddH₂O). Finally, the PCR reagent and SYBR Green I dye were added and thermocycling reactions were performed in the PCR reaction chamber. The excited fluorescence signals were collected by an optical detection module to determine the results of PCR amplification.

B. Chip design and fabrication

The integrated microfluidic system consisted of a microfluidic chip and an optical detection module. A schematic layout of the integrated microfluidic chip is shown in Fig. 2(a). The developed microfluidic chip was composed of several micro-devices that were all integrated into the chip to automate the entire assay process. The microfluidic chip was composed of two layers of PDMS layers and a glass substrate. The top PDMS layer was used as the fluidic channel layer comprising of a PCR reaction chamber, a sample/washing buffer loading chamber, a reagent loading chamber, and a waste chamber. The second PDMS layer was used as the air chamber layer containing air chambers and connection channels for the S-shape micro-pump. The PDMS membrane structure was made of a PDMS polymer and a curing agent (Sylgard 184A/B, Sil-More Industrial Ltd., USA) in a ratio of 10:1 by weight and formed by the PDMS casting technique.²⁶ Two PDMS layers and the glass substrate were bonded together to form the integrated microfluidic chip by using an oxygen plasma treatment. The details of this microfabrication process can be found in our previous study.²⁶ The peristaltic effect for liquid transposition was generated by compressed air that caused the time-phased deflection of successive s-shape PDMS membranes along a microchannel.¹⁵ EMVs (SMC Inc., S070 M-5BG-32, Japan) and a digital controller were used to regulate the pumping rate. Note that the reciprocating actuation between micro-chambers, connected to the pneumatic micro-pump, can be used as a micro-mixer in this developed microfluidic chip. The glass substrate patterned with metal electrodes was used as micro-heaters and along with a temperature sensor was used for performing thermolysis, hybridization, and PCR. Micro-heaters and a temperature sensor were used to control the temperature for EMA-PCR. The accuracy and reliability of temperature controlling using the micro-heater were investigated in the previous study.²⁷ Briefly, the temperature can be precisely controlled with a variation less than 0.1°. A photograph of this microfluidic chip is shown in Fig. 2(b). The dimensions of the chip were measured to be 31 mm × 21 mm.

FIG. 2. (a) A stacked view of the integrated microfluidic chip consisting of two PDMS layers and one glass substrate. A thick PDMS structure with air chambers and a thin PDMS membrane as a fluidic channel layer were used for flow control. The glass substrate was patterned with metal electrodes were used as micro-heaters and a temperature sensor for performing thermolysis, hybridization, and PCR. (b) A photograph of the integrated microfluidic chip consisting of micro-pumps, micro-valves, a waste chamber, a reagent loading chamber, a sample/washing buffer chamber, a micro-heater/temperature sensor, and a PCR reaction chamber.

C. MRSA sample pre-treatment and PCR settings

The developed micro-thermal cycler was used in the EMA-PCR based microfluidic system for MRSA diagnosis.²⁷ The accuracy, temperature uniformity, and amplification efficiency of the temperature controller were verified and could be used in PCR. The overnight-cultured MRSA was boiled for 10 min. Then, 100 μ l of boiled MRSA was transferred onto a TSBY (3% Tryptic Soy Broth, 0.5% Yeast extract and 1.5% agar, BD Inc. USA) plate and incubated at 37 °C overnight. No bacterial colonies growing on the plate indicated that only dead MRSA was seeded on the plate. The appropriate amount of EMA was then added to 20 μ l of the live or dead bacterial sample and exposed to a visible light source at room temperature. The treated bacteria were incubated with specific DNA-probe-conjugated magnetic beads at 95 °C for 5 min to thermolysis the bacteria and to denature the bacterial genomic DNA. The sequences of the DNA probe and the forward/reverse primer pairs were designed from the *mecA* gene of MRSA and are listed in Table I.²⁰ A 262 base-pair (bp) fragment of *mecA* region was amplified for the EMA-PCR assay in this study. Detailed information about these methods, which the nucleotide probe conjugated onto the magnetic beads and specific DNA isolated from the MRSA strains, can be found in our previous work.²⁰ The unbound nonspecific DNA was then washed twice by ddH₂O and the captured DNA on the surface of the magnetic beads was collected by using a permanent magnet placed underneath the chip. The isolated DNA was dissolved by 5 μ l of ddH₂O and 15 μ l of the PCR reaction reagent was used, which contained 1 μ l of deoxyribonucleotide triphosphate (dNTP, 10 mM, Promega, USA), 2 μ l of 10 $\times$ PCR buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM 2-[2-(Bis (carboxymethyl) amino) ethyl-(carboxymethyl) amino] acetic acid (EDTA), 1 mM Dithiothreitol (DTT), 0.5% Tween, 0.5% Nonidet and 50% (v/v) glycerol, JMR Holdings, UK), 1 μ l of *mecA* specific primer pairs (0.5 μ l of each primer for the forward/reverse primers), 0.5 μ l of Superthermo Gold *Taq* DNA polymerase (5 U/ μ l, JMR Holdings, UK), and 10.5 μ l of ddH₂O. The thermocycling process for PCR was then performed under the following conditions: 95 °C for 5 min (initial denaturation), and 35 cycles of PCR at 95 °C for 20 s (denaturation), 58 °C for 20 s (annealing) and 72 °C for 20 s (extension) for each cycle; and then finally at 72 °C for 7 min.

D. Specificity of the EMA-PCR assay

The specificity of the EMA-PCR assay was determined by using MRSA, MSSA, and other hospital common outbreak bacteria. All tested bacterial strains that were provided from National Cheng Kung University Hospital, Tainan, Taiwan were listed in Table II. The specificity of the primer pairs was confirmed by slab-gel electropherograms to ensure whether specific *mecA* primers were specifically recognized by MRSA only. The mixture contents of the PCR reagent and the protocol were the same as the ones described previously. The specificity efficiency of the EMA-PCR assay was calculated as follows:

$$\text{specificity efficiency} = \frac{\text{the number of tested bacteria that } mecA \text{ amplified}}{\text{the total number of tested bacteria}} \times 100\%.$$

A 100% specificity efficiency indicated that the *mecA* product was successfully amplified in all of the tested bacteria.

TABLE I. Designed sequences of the primer pairs for *mecA* and the specific DNA probe for isolation of the *mecA* gene.

Primer	DNA sequences
Forward primer	5'-GCAACAAGTCGTAAATAAACACAT-3'
Reverse primer	5'-TCTCATATAGCTCATCATACACTT-3'
Specific probe	5'-TTTTTTTTTTTCGTTATTAGCTGGACGTCGTCGCGAACTATAA-3'

TABLE II. Thirty identified strains of bacterial samples were tested using the integrated microfluidic system.

Strains of bacteria	No.	Strains
Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA)	1 ~ 10	1519, 1568, 1569, 1570, 1571, 1572, 1573, 1601, 1602, 1603
Methicillin-sensitive <i>Staphylococcus aureus</i> (MSSA)	11 ~ 20	1201, 1202, 1203, 1204, 1205, 1206, 301, 1302, 1303, 25 923
<i>Streptococcus pneumoniae</i>	21	1645
<i>Escherichia coli</i>	22 ~ 23	57 911
<i>Enterobacter</i> sp.	24	1124
<i>Klebsiella pneumoniae</i>	25	112
<i>Pseudomonas aeruginosa</i>	26	138
<i>Proteus vulgaris</i>	27	380
<i>Haemophilus influenzae</i>	28	139
<i>Streptococcus pyogenes</i>	29	
<i>Streptococcus agalactiae</i>	30	

E. Optical detection of MRSA using SYBR Green I

An optical detection module was designed for the rapid detection of the fluorescent signals from the amplicons of the *mecA* genes. The module was composed of a photomultiplier (PMT, R928, Hamamatsu, Japan) device, a mercury lamp (MODEL C-SHG1, Nikon Corp., Japan), and a microscope (OLYMPUS BX43, OLYMPUS, Japan). The blue light from a mercury lamp was first directed through a band-pass (BP) filter (470/20BP, Nikon Corp., Japan) and was used to excite the fluorescence dye (SYBR Green I, AMRESCO, USA) in the amplified PCR products at an excitation wavelength of 488 nm. The excited fluorescence signals passed through the filters and were detected by the PMT. With this approach, highly sensitive detection of the target genes can be achieved by utilizing the optical detection module. As mentioned previously, the amplification of PCR was monitored for an increase in fluorescent signals that occurred when the amplified dsDNA were crosslinked with SYBR Green I. The thermal cycling process followed the protocol described in Sec. II C. The intensity of the fluorescence signals was determined by a software program (sisc chromatography data system, Scientific Information Service Corporation Inc., Taiwan). The statistical analyses were performed to determine the statistical difference by using the two-tail student *t*-test. The statistical significance was confirmed when $P < 0.05$.

F. Sensitivity of the EMA-PCR assay

In order to determine the limit of detection (LOD) of this developed microfluidic system, *mecA* gene amplification from the live MRSA using SYBR Green I fluorescence dye was performed. Significantly, a standard curve for live MRSA has been obtained by using the intensity of the fluorescence signals for the *mecA* amplicons from the serial dilution templates ranging from 10^5 to 10^1 colony forming unit/ μ l (CFU/ μ l). As a consequence, the fluorescence signal detected by the microfluidic chip could be immediately referenced to a standard curve for quantitative measurement of live MRSA.

III. RESULTS AND DISCUSSION

A. Characterization of pumping rate and mixing efficiency

The microfluidic chip was composed of S-shape micro-pumps and a micro-heater/temperature sensor and micro-chambers to provide storage and transport of reagents and amplified products. The S-shape pneumatic micro-pump was activated by compressed air to control the pumping rate. Figure 3(a) shows the relationship between the pumping rate of the micro-pump and the driving frequency of the EMV by using an applied air pressure of 15 psi. The

FIG. 3. (a) Relationship between the pumping rate and the driving frequency of the EMV at an operating air pressure of 15 psi for the micro-pump. (b) The relationship between the mixing index and the driving frequency of EMV at applied air pressures of 10 and 15 psi, respectively, for the micro-mixer.

experimental results demonstrated that the pumping rate increased with an increase in the driving frequency. In our previous work explored the pumping rate of the S-shape pneumatic pump. It increases with an increasing operating frequency (for a constant pneumatic pressure). However, the maximum pumping rate at a constant applied pressure is limited by the release time of the compressed air. If the operating frequency is too high, the air cannot be completely released and the pumping rate will not increase, but starts to fall.¹⁶ A maximum flow rate was measured to be 267 $\mu\text{l}/\text{min}$ at 35 Hz when using 15 psi of air pressure.

The reciprocating actuation between micro-chambers connected to the pneumatic micro-pump can be used as a micro-mixer. A mixing index (σ) was used to quantify the mixing effect,^{28,29}

$$\sigma(A) = \left(1 - \frac{\int_A |C^+ - C_\infty^+| dA}{\int_A |C_0^+ - C_\infty^+| dA} \right) \times 100\%,$$

where $\sigma(A)$ was defined as the mixing index for the normalized species concentration (C^+) distributed within the area of the mixing chamber (A). C_0^+ was the initial condition under the unmixed state, and C_∞^+ was the completely mixed condition of the normalized concentration. The mixing index was 100% when the samples were mixed completely and 0% indicated that the samples were completely separated. The mixing index of the micro-mixer was characterized and is shown in Fig. 3(b). Two air pressures, 10 and 15 psi were tested at different driving frequencies. In our previous work has demonstrated that the applied pressure was suitable for clinical applications or pathogen detection by using the microfluidic device.^{17,18} A maximum mixing efficiency of 96.96% was achieved with an applied pressure of 15 psi and a driving frequency of 9 Hz for 20 s.

B. Detection of live MRSA

The primer sets and the probe sequences of *mecA* are listed in Table I. In order to optimize the operating conditions for EMA pre-treatment, including the reaction concentration and the reaction time, live and dead MRSA were prepared and used for the optimization of the diagnostic protocol. The CFUs were determined from their optical density at 600 nm (OD_{600}) for MRSA using a spectrophotometer (NanoPhotometerTM Pearl, IMPLER, Germany).³⁰ These optimal conditions for EMA pre-treatment were performed in the integrated microfluidic system and confirmed by slab-gel electrophoresis. The electrophoretic results for optimal conditions of

FIG. 4. Determining optimum conditions for EMA pre-treatment for the live MRSA diagnostic assay. (a) Optimization of the EMA concentration to distinguish the live and dead MRSA after the PCR process. Lane 1 presents the dead MRSA and lane 2 is for live MRSA. Both of them were pre-treated by 10, 1, 0.1 $\mu\text{g/ml}$ of EMA for 30 min, respectively. (b) Optimization reaction time for the EMA pre-treatment for live and dead MRSA. Lane 1 indicates the dead and lane 2 is for live MRSA. Both of them were treated by 1 $\mu\text{g/ml}$ EMA for 25, 20, 15, 10, and 5 min, respectively.

the EMA pre-treatment assay are shown in Fig. 4. Figure 4(a) shows the results for live/dead MRSA treated with different EMA concentrations (10, 1, and 0.1 $\mu\text{g/ml}$). Both live and dead MRSA could be amplified with *mecA* products by using 0.1 $\mu\text{g/ml}$ of EMA treatment. However, the *mecA* products could be amplified only in live MRSA under 1 $\mu\text{g/ml}$ of EMA. The experimental results verified that 1 $\mu\text{g/ml}$ of EMA was the minimum concentration that could be distinguished for the survival state of MRSA after 25-min of visible light exposure (18 W, 1200 lm).

Furthermore, the difference in *mecA* amplified products for live and dead MRSA was used to test the minimum operating time, using 1 $\mu\text{g/ml}$ of EMA. The operating time of the EMA pre-treatment was explored and the results are shown in Fig. 4(b). Both live and dead MRSA could be used to amplify the PCR product of *mecA* after a 5-min treatment. However, after 10-min of treatment of EMA, only the *mecA* could be amplified in live MRSA. The results indicated that the optimum operating time was 10 min in the EMA-PCR assay for live MRSA diagnosis.

The DNA-probe-conjugated magnetic beads and *mecA* primers were used to double-check for specificity in the tests of MRSA in this study. The live MRSA, MSSA, and other bacteria that have been commonly found in hospital outbreaks were tested to confirm the specificity of the *mecA* gene. The results showed that the DNA probe conjugated onto the surface of the magnetic beads and the *mecA* primers have a high specificity to capture live MRSA (Fig. 5(a)). Furthermore, the gel electrophoresis graphs indicated that MSSA and other bacteria, such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Enterobacter* sp. cannot be amplified with the 262 bp of *mecA* PCR products (Figs. 5(b) and 5(c)). This indicated that the *mecA* probe had a high specificity for MRSA diagnosis.

About the sensitivity testing, we have provided the LOD of the developed integrated microfluidic system by using the results of electrophoresis and the endpoint detection of fluorescent signals. To determine the detection limit of the microfluidic system, a serial dilution of live MRSA samples with concentrations ranging from 10^5 to 10^1 CFU/ μl were measured by the intensity of their fluorescence signal and compared with results from slab-gel electrophoresis. The LOD of the two methods were comparable (Figs. 6(a) and 6(b)). Therefore, the fluorescent dye was used in the integrated microfluidic system to determine the detection limit. Only a serial dilution of the controlled sample was used to determine the detection limit. A standard curve for detection limit could then be determined from the intensity of the fluorescence signals. According to the resulting standard curve, the LOD for live MRSA detection was 10^2 CFU/ μl (Fig. 6(b)). The LOD for MRSA was reported to be 10 CFU per reaction from a blood sample by using real-time PCR in a centrifugal microfluidic platform.³¹ The sensitivity of the EMA-PCR based microfluidic system was not as sensitive as the ones using PCR, real-time PCR, or loop-mediated isothermal amplification (LAMP) for MRSA

FIG. 5. Specificity tests for the detection of live MRSA on the microfluidic chip. (a) 10 strains of MRSA (lanes 1-10), (b) 10 strains of SA (lanes 11-20), and (c) 10 common strains of bacteria with antibiotic-resistance (lanes 21-30) were verified by using primer pairs of the *mecA* gene, respectively. Lane L: 100-bp DNA ladders, lane NC: negative control using ddH₂O, lane PC: positive control using MRSA strain 1601. Detailed information about these tested non-SA bacteria are listed in Table II.

diagnosis (about 10 to 100 times). However, these molecular diagnosis methods cannot be differentiate between live and dead bacteria. In clinical treatment, the diagnosis of live bacteria in tested samples is more important than the limitation of detection. The detection limit of the proposed method may not as good as the conventional PCR, however, the entire process, including sample pre-treatment can be performed on a single chip.

The results from measurement of fluorescence signals were found to be consistent with those from the slab-gel electropherogram (Fig. 6(a)). This demonstrated that the integrated microfluidic system had a high specificity with reasonable sensitivity and may be a promising diagnosis method for the detection of live MRSA. Furthermore, it only took 2.5 h to

FIG. 6. (a) The detection sensitivity for live MRSA was verified by using slab-gel electropherograms. Lane L: 100-bp DNA ladders, lane NC: negative control using ddH₂O. The tested MRSA with different CFU ranging from 10⁵ to 10¹ CFU/μl were used to verify the performance of the developed microfluidic system. (b) The standard curve of fluorescence signals for samples with different initial CFUs of live MRSA ranging from 10⁵ to 10² CFU/μl.

automatically perform the entire diagnosis protocol and only 20 μl of bacterial samples were consumed.

IV. CONCLUSION

Rapid and accurate detection of methicillin resistance in *S. aureus* is important for the appropriate use of antimicrobial therapy and to control the nosocomial spread of MRSA strains. The U.S. Food and Drug Administration approved a test kit, KeyPath MRSA/MSSA Blood Culture Test (MicroPhage Inc. USA) that had a 98.8% specificity to determine whether bacteria growing in a patient's positive blood culture sample were MRSA or MSSA. The other common laboratory test protocols also take 5 to 10 h for identification of MRSA/MSSA infections. Compared with these popular methods or the certified diagnosis kit, our developed microfluidic systems can successfully purify and detect live MRSA directly from clinical samples. The specificity was 100% (10 tested samples of MRSA) by using this microfluidic system. The LOD of the microfluidic system was 10² CFU/μl. The entire process from clinical sample pre-treatment to fluorescence detection only took 2.5 hs. More importantly, this is the first time that the rapid diagnosis of live pathogens has been performed in a microfluidic system. It may provide a promising tool to offer accurate clinical information for the selection of an appropriate antibiotic regimen will also reduce the outbreak of multi-drug-resistant bacteria.

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