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PAPER

A magnetic bead-based assay for the rapid detection of methicillin-resistant *Staphylococcus aureus* by using a microfluidic system with integrated loop-mediated isothermal amplification†

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This study reports a new diagnostic assay for the rapid detection of methicillin-resistant *Staphylococcus aureus* (MRSA) by combing nucleic acid extraction and isothermal amplification of target nucleic acids in a magnetic bead-based microfluidic system. By using specific probe-conjugated magnetic beads, the target deoxyribonucleic acid (DNA) of the MRSA can be specifically recognized and hybridized onto the surface of the magnetic beads which are then mixed with clinical sample lysates. This is followed by purifying and concentrating the target DNA from the clinical sample lysates by applying a magnetic field. Nucleic acid amplification of the target genes can then be performed by the use of a loop-mediated isothermal amplification (LAMP) process *via* the incorporation of a built-in micro temperature control module, followed by analyzing the optical density (OD) of the LAMP amplicons using a spectrophotometer. Significantly, experimental results show that the limit of detection (LOD) for MRSA in the clinical samples is approximately 10 fg μL^{-1} by performing this diagnostic assay in the magnetic bead-based microfluidic system. In addition, the entire diagnostic protocol, from bio-sample pre-treatment to optical detection, can be automatically completed within 60 min. Consequently, this miniature diagnostic assay may become a powerful tool for the rapid purification and detection of MRSA and a potential point-of-care platform for detection of other types of infections.

Introduction

An infectious disease, which is an illness resulting from the presence of pathogenic microbial agents in the host body, can be widely transmitted by micro pathogens from one person or species to another individual by replicating agents. In recent years, *Staphylococcus aureus* (*S. aureus*, SA) infection has been one of the most common infectious diseases that occur in hospitals and healthcare facilities. *S. aureus* usually colonizes in the anterior nares, opened wounds and even the urinary tract, such that a variety of clinical morbidities such as dermatitis, pneumonia, septicaemia, osteomyelitis and human meningitis may be induced by an infection of *S. aureus*.[1] More seriously, methicillin-resistance *Staphylococcus aureus* (MRSA), which is a bacterium resistant to all available penicillin and β-lactam antimicrobial drugs, has become one of the most prevalent antibiotic resistant pathogens in hospitals.[2] Additionally, community-associated MRSA (CA-MRSA) infections have caused more severe illnesses than traditional healthcare-associated MRSA (HA-MRSA) infections. Therefore, early prevention of the spread of MRSA among hospitals and community settings has become a worldwide, public-health priority issue in recent years.[3] Still, the infection rate of MRSA is increasing among hospitalized patients due to cross-infection and the lack of vaccines, such that people with weak immune systems may be infected easily. Furthermore, the mortality of patients with MRSA infections in the bloodstream would be two times higher than for patients infected by the methicillin-sensitive *Staphylococcus aureus* (MSSA).[4,5] Hence, there is an urgent need to develop the new strategy for the rapid detection of MRSA to limit the spread of these infections and to minimize the waste of public resources on ineffective treatments.

Conventionally, standardized detection and identification of MRSA is based on a cell culture-based methodology and microscopy to identify MRSA colonization in a cultured broth.[6] However, this time-consuming and labor-intensive protocol always limits its practical application for rapid clinical

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diagnosis.[7] Moreover, the accuracy and the sensitivity of MRSA detection are always critical issues for clinical diagnosis. Recently, molecular diagnostic techniques have been widely investigated for the rapid detection of MRSA-colonized patients with a higher sensitivity and specificity.[8] Such diagnostic protocols allow for *in vitro* pathogen-specific nucleic acid amplification such that the specificity and sensitivity of the diagnosis can be significantly improved. Among them, molecular diagnosis based on nucleic acid amplification techniques such as PCR has become well-developed methods for accurate detection of bacteria. Typically, target genes of bacteria can be specifically amplified by performing repeated thermal cycling with the incorporation of specific primer sets during the PCR process. For example, a PCR assay for accurate diagnosis of clinical isolates from patients infected by MRSA has been demonstrated.[9] A sensitivity as high as 82% for MRSA-positive clinical isolates has been achieved by using this PCR assay. Nevertheless, demanding requirements for PCR processing, such as precise temperature control during the thermal cycling and the time-consuming temperature ramping process, are normally required. Additionally, numerous bulky apparatus and power-intensive thermal cycling are always needed. Furthermore, sample pretreatment is a lengthy process, especially for extracting the target nucleic acids from clinical bio-samples prior to the PCR process. In addition, the lengthy and costly diagnostic process always needs to be performed by well-trained personnel and errors in the diagnosis may be caused by the manual processing steps.

Consequently, isothermal amplification, which allows for the exponential amplification of target nucleic acids in a constant temperature field, has attracted considerable interest for the rapid detection of pathogens in recent years.[10,11] For example, strand-displacement amplification (SDA) using a restriction endonuclease to nick the unmodified strand of a hemiphosphorothioate, then an exonuclease deficient Klenow to extend the 3′ end at the nick and to displace the downstream DNA strand, has been reported.[12] Another technique is rolling circle amplification (RCA), which generates tandemly linked copies of the complementary sequence of a circular template, has also been demonstrated.[13] However, these isothermal amplifications of target nucleic acids still have their limitations such as having complicated reaction schemes and the challenge of amplifying DNA targets of sufficient length to be useful in various applications and for diagnosis. Alternatively, LAMP has attracted considerable interest recently since it is a potentially rapid, accurate, and cost-effective nucleic acid amplification technique with high sensitivity and specificity. Target nucleic acid sequences of the bio-samples can be amplified by using four designated primers with the incorporation of *Bst* DNA polymerase under isothermal conditions (60–65 °C).[14] Three major steps involving an initial step, a cycling amplification step and an elongation step are conducted under a constant thermal condition and efficient amplification can be achieved since there is no time required for temperature ramping during the LAMP process.[15] As a consequence, new diagnostic schemes incorporating the LAMP technique for rapid diagnosis of pathogens such as *Campylobacter*, pathogenic *Escherichia coli*, *Salmonella*, *Staphylococcus aureus*, and noroviruses have been demonstrated in the literature in recent years.[16–20] For example, a LAMP-based detection of *Edwardsiella tarda*, from infected Japanese flounders, by targeting the haemolysin gene was reported.[21] Another two-step RT-LAMP protocol for the identification of G-protein of the infectious haematopoietic necrosis virus (IHNV) in fish has also been developed.[22] More recently, LAMP-based diagnostic techniques for the rapid identification of MRSA/SA by verifying the *mec*A gene of MRSA and the *spa* gene of SA have also been demonstrated.[23] The detection of MRSA in a blood culture with the incorporation of the LAMP process has been achieved with a sensitivity of 96.2% and a specificity of 100%.[24] Furthermore, the LAMP amplicons can be also optically detected by measuring the turbidity of the amplified products induced by the magnesium pyrophosphate and/or the colour change of the reaction mixture by the DNA-intercalating dye.[25,26]

Despite the attractiveness of the LAMP technique, there are still some potential shortcomings in adapting it for point-of-care (POC) testing for bedside healthcare and for clinical applications. The entire nucleic acid amplification process is still labor-intensive which requires lab-scale equipment and bulky thermo-heaters with a relatively large amount of bio-samples and reagents. More importantly, bio-sample pre-treatment processes prior to the LAMP process, such as nucleic acid extraction, are always required and should be performed by experienced personnel. Therefore, there is a great need to develop a complete "sample-to-answer" diagnostic assay by using a compact system to carry out the entire protocol within a short period of time. More importantly, the assay must have a high specificity and sensitivity and can be performed in an automatic manner.

To address the demanding diagnosis protocol for MRSA, micro-electro-mechanical-system (MEMS)-based biomedical systems integrated with functional microfluidic devices have been widely explored for molecular diagnostics in recent years.[27,28] Miniature microfluidic systems have been demonstrated to perform the entire diagnostic process from sample pre-treatment to signal analysis in clinical applications.[29–32] For example, a miniature real-time PCR assay for the detection of unpurified MRSA DNA was reported.[33] The detection limit of MRSA DNA in the proposed microfluidic system was found to be 11.2 pg per 3 μL. A microfluidic chip for the quantitative detection of purified MRSA DNA utilizing the LAMP process was also demonstrated.[34] Still, there has been no attempt at developing a diagnostic assay for the rapid pre-treatment of clinical samples and then accurate detection of pathogens by using the LAMP process in a miniature system. Therefore, this current study presents a new diagnostic assay for the rapid purification of DNA segments from three types of clinical samples (*i.e.* sputum, serum and milk) and the sensitive detection of MRSA by utilizing an integrated magnetic bead-based microfluidic system with the incorporation of a spectrophotometer. With the incorporation of specific probe-conjugated magnetic beads and an on-chip temperature control module, target DNA samples in clinical sample lysates can be specifically recognized and hybridized onto the surface of the magnetic beads, followed by purification of the target DNA-conjugated magnetic complexes from the clinical sample with the aid of a suction-type sample transportation module and a magnet. Moreover, target nucleic acids of MRSA can then be amplified by an isothermal LAMP process utilizing the built-in temperature control module, immediately followed

by analyzing the optical signals of the amplicons *via* the spectrophotometer.

Materials and methods

The diagnostic assay is performed *via* the magnetic-bead-based hybridization of the target DNA purified from clinical samples, an isothermal LAMP process for the amplification of the target genes, and then spectrophotometric analysis of the amplified target genes. The entire process can be accomplished automatically in an integrated microfluidic system within a short period of time. This is the first work that demonstrates the rapid identification of MRSA from various bio-mimetic clinical sample sources (*i.e.* milk, sputum and serum) with a high sensitivity and specificity in a less labor intensive manner. Moreover, efficient amplification *via* the LAMP process can be realized by utilizing a uniform temperature distribution within the reaction chamber generated by a built-in, self-compensated, temperature control module. In addition, all the external control components such as a control circuit board, electromagnetic valves (EMVs), a vacuum pump and a waste tank have been integrated into a hand-held portable system. As a consequence, the proposed microfluidic diagnostic assay incorporated with specific, probe-conjugated magnetic beads and the LAMP-on-a-chip assay may provide an integrated platform for the complete diagnosis of an infectious disease in a short period of time with less human intervention required.

Working principle of the diagnostic assay

Fig. 1(a) schematically illustrates the working principle of the magnentic-bead-based diagnostic assay in the microfluidic system for rapid purification and genetic detection of MRSA in the clinical bio-samples. First, clinical bio-samples such as sputum are loaded into the cell lysis/DNA hybridization/LAMP reaction chamber along with the specific nucleotide-coated magnetic beads. This is followed by loading the LAMP reaction mixture and washing buffer into the reagent chamber and washing buffer chamber, respectively (see the schematic layout of the microfluidic prototype in Fig. 1(b)). A temperature of 95 °C is then set within the cell lysis/DNA hybridization/LAMP reaction chamber to lyse the MRSA infected clinical bio-samples by utilizing the temperature control module, followed by hybridization of the released DNA with the oligonucleotide probe conjugated magnetic beads at constant temperature. Then, a permanent magnet is attached underneath the cell lysis/DNA hybridization/LAMP reaction chamber to collect the magnetic complexes, followed by the sucking out all the other biological interferents to the waste chamber with the aid of a vacuum pump. A washing process is then performed by pumping a washing buffer into the cell lysis/DNA hybridization/LAMP reaction chamber by using the sample transportation unit and removing the waste solution using the permanent magnet and the vacuum pump, such that the target DNA of the MRSA conjugated onto the magnetic beads can be successfully purified and concentrated from the clinical bio-samples. Next, the reaction mixture is transported into the cell lysis/DNA hybridization/LAMP reaction chamber to perform the LAMP process by utilizing the temperature control module.[30] Finally, the amplicons from the LAMP are optically analyzed by utilizing a spectrophotometer. With this approach, the target DNA of the MRSA can be isolated from the clinical bio-samples and then are used for the subsequent optical identification of genetic genes in a short period of time.

Preparation of bacterial samples

A total of 27 different bacterial strains were provided by Dr Wu, Department of Medical Laboratory Science and Biotechnology, National Cheng Kung University, Tainan, Taiwan (as listed in Table 1 in the Electronic Supplementary Information (ESI)†) for the verification of the specificity of the proposed microfluidic diagnostic assay. All the tested bacterial strains were cultured on TSAII 5% SB plate (BBL™, USA) at 37 °C, followed by suspending the bacterial colonies from the agar plates with PBS (Invitrogen, USA). Next, the bacterial sample solutions were stored in LB broth containing 30% (v/v) glycerol at −80 °C. Finally, the bacterial samples were re-suspended and mixed with the clinical bio-samples such as sputum, serum or milk to form a simulated sample, with a total volume of 100 μL, prior to the on-chip diagnostic assay.

Design of oligonucleotide probe and specific primer sets

A specific, inexpensive nucleic acid probe consisting of oligonucleotides with customized sequences were employed in the proposed microfluidic diagnostic assay for the hybridization of the streptococcal protein A (*spa*) gene of *S. aureus*. The staphylococcal sequences of protein A (*spa*) for the detection of common *S. aureus* (GenBank Accession No. BX571856) and the methicillin-resistance gene (*mec*A) (EU929079) for the identification of MRSA were obtained from the GenBank at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). The custom designed sequences of the oligonucleotide probe for the hybridization of MRSA DNA are listed in Table 2 in the ESI.†

In addition, two sets of primers including a forward inner primer (FIP), a backward inner primer (BIP) and two outer primers (F3 and B3) have been specifically designed for the detection of the *spa* gene and the *mecA* gene during the LAMP process, respectively (as shown in Table 2 in ESI†). All the primer sets were designed by utilizing the Primer Explorer V3 software (http://primerexplorer.jp/elamp3.0.0/index.html).

Preparation of specific probe-conjugated magnetic bead

The specific staphylococcal *spa* probe was conjugated with the carboxylated linkage, which is a functional group coated on the surface of the magnetic beads ($\varnothing = 4.5$ μm, concentration $= 4 \times 10^7$ beads per mL, MAGBEAD AGT-003-05, Applied Gene Technologies, USA).[35] 30 μL of oligonucleotide probe modified with an amine group (100 μM), 20 μL of EDAC (120 mg mL^{-1}, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, Invitrogen, USA) and 950 μL of the magnetic bead solution were mixed and incubated gently in a micro-tube at a rotation speed of 20 rpm for 18 h. Then, the well-mixed solution was washed twice by a PBS solution with 0.02% Tween-20 (Sigma, USA), followed by repeating the washing process to remove any unbound oligonucleotide probes utilizing the PBS solution with a 0.1% SDS

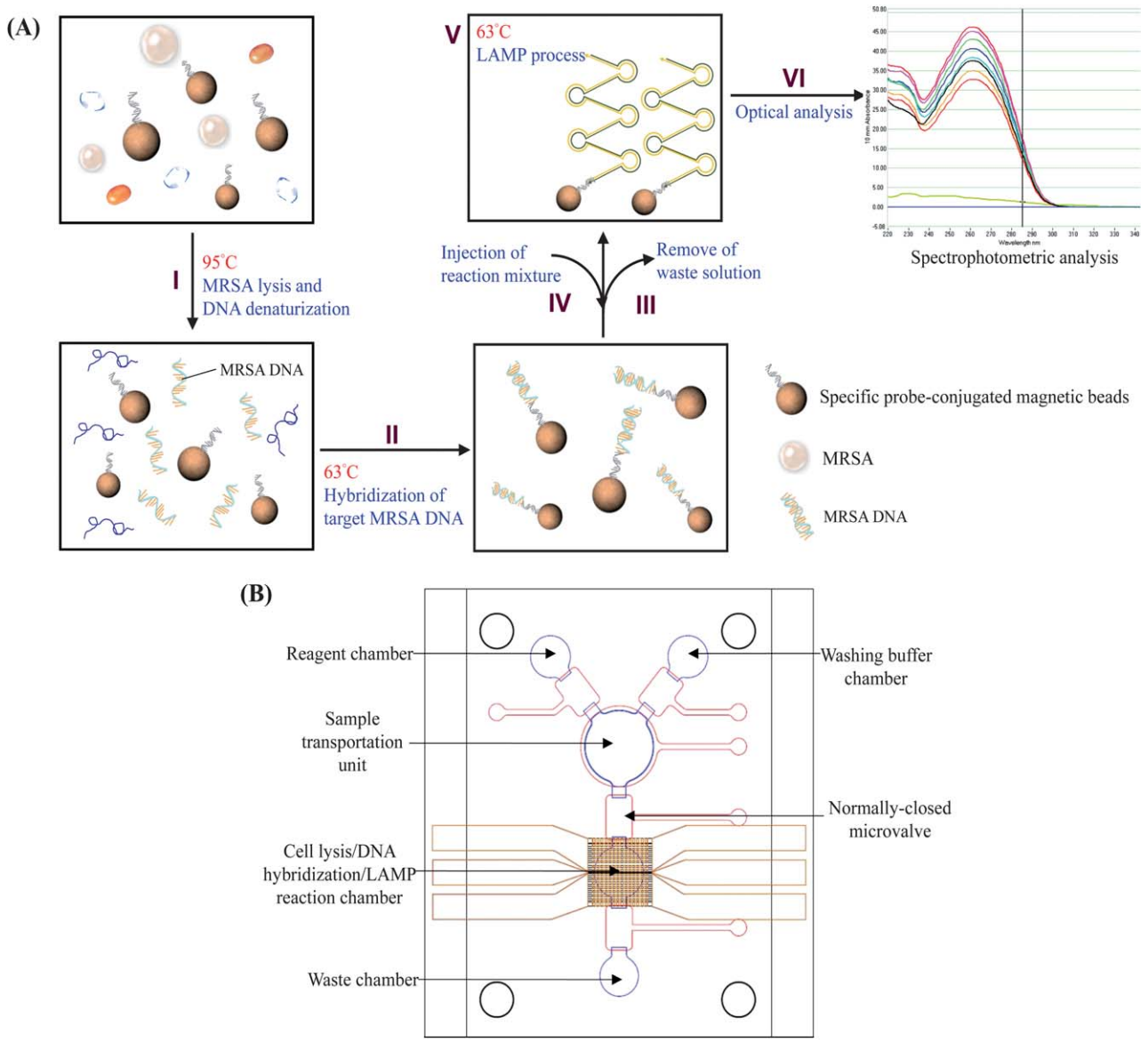

Fig. 1 (a) Schematic illustration of the experimental procedures for rapid sample pretreatment and detection of MRSA. (b) Layout of the proposed microfluidic chip consisting of a suction-type microfluidic control module for liquid delivery and a temperature control module for the LAMP process. The dimensions of the system are measured to be 32 mm × 30 mm × 10 mm.

solution (sodium dodecyl sulfate, Merck). Next, a blocking process was carried out by mixing 1 mL of ethanol amine (0.1 M, Sigma) with the probe-conjugated beads at a rotation speed of 20 rpm, at room temperature for one hour, such that any unbound coupling sites on the magnetic bead were then blocked. Finally, the washing process was performed again and the probe-conjugated magnetic beads were re-suspended into double-distilled water (ddH_2O) with a volume of 1 mL.

Nucleic acid amplification

Isothermal amplification of the target genes of MRSA is employed in the microfluidic diagnostic assay by performing the LAMP process utilizing the on-chip temperature control module. A total reaction volume of 30 μL containing 3.3 μM of each FIP and BIP, 0.3 μM of each F3 and B3, 1 μL of dNTPs (10 mM, Promega, USA), 3 μL of 10 × thermo-polymerase buffer (with 200 mM tris-HCl, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$ and 0.1% Triton X-100 (pH 8.8) at 25 °C), 10 μL of DNA template, 3 μL of ddH_2O and 1 μL of 8 U *Bst* DNA polymerase (large fragment; New England Biolabs Inc., USA) is employed in the LAMP process. Note that the reaction volume is proportionally decreased by half with a final volume of 15 μL in the microfluidic diagnostic assay. Optimal conditions, including the reaction temperature and time for both the LAMP and hybridization processes, have also been explored and will be discussed in the following sections.

In addition to the isothermal amplification of the target nucleic acids, a conventional PCR assay targeting the *mec*A gene was also performed for comparison by utilizing the aforementioned

outer primer sets (F3/B3). 30 μL of PCR reaction mixture consisting of 0.5 μL of Supertherm Gold polymerase (5 U μL^{-1}, JMR Holdings, UK), 0.3 μM of each F3 and B3, 10 μL of purified DNA samples and 1 μL of 10 mM dNTP. The PCR process was started with an initial denaturization at 94 °C for 5 min, followed by performing 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s for 35 cycles and an additional extension step at 72 °C for 7 min. Finally, the PCR/LAMP products can be visualized on 2% agarose gels stained with ethidium bromide (EtBr, 50 μg mL^{-1}, Sigma, USA), followed by image acquisition utilizing an ultraviolet (UV) analysis system (302 nm, 3UV™ transilluminator, UVP, Canada).

Microfluidic diagnostic assay

A microfluidic diagnostic assay including a magnetic-bead-based DNA extraction process, isothermal amplification of the target gene, and spectrophotometric analysis of the amplified genes, has been implemented on a chip for rapid identification of MRSA from the clinical samples. The entire diagnostic procedure from bio-sample pre-treatment to isothermal amplification is accomplished automatically in the developed microfluidic platform. Fig. 1(b) shows a schematic diagram of the microfluidic chip consisting of a microfluidic control module and a temperature control module. A photograph of the proposed microfluidic chip is shown in Supplementary Fig. 1 in the ESI.† Three structural layers including a glass substrate patterned with metal electrodes, a thick polydimethylsiloxane (PDMS) layer for the air chambers and a thin-film PDMS membrane with the structures for the microfluidic channels are bonded together to form the integrated microfluidic system by using an oxygen plasma treatment. A suction-type, pneumatic microfluidic control module has been integrated for the manipulation of fluidic samples by utilizing a sample transportation unit (see Fig. 1(b)). The details and the optimal design parameters of the microfluidic control module can be found in our previous work.[36] Notably, the clinical samples are not damaged during transportation since a lower shear force is generated by the suction-based microfluidic control module. Furthermore, a self-compensated temperature control module that is comprised of two sets of array-type micro-heaters and a micro temperature sensor is fabricated to generate a uniform temperature distribution within the thermal cell lysis/DNA hybridization/LAMP reaction chamber. Details of the self-compensated temperature control module and the optimal design can be referenced in a previous work.[30] The improvement in the amplification efficiency of the LAMP process within a small reaction volume is attributed to the high thermal uniformity (±0.2 °C) within the reaction chamber. Significantly, all the components, including the microfluidic control module and the temperature control module, are precisely controlled by a hand-held application specific integrated circuit (ASIC) controller, such that the entire microfluidic diagnostic assay including the sample transportation process and the isothermal amplification of the target genes can be completed in an automatic manner.

Consequently, the proposed diagnostic assay for rapid isolation of target DNA and nucleic acid amplification *via* LAMP can be realized in the microfluidic system with a high sensitivity, in a short period of time. At first, 15 μL of the clinical samples along with 5 μL of the probe-conjugated magnetic beads are loaded into the cell lysis/DNA hybridization/LAMP reaction chamber, followed by loading 20 μL of the LAMP reaction mixture and 50 μL of the PBS buffer into the reagent chamber and washing buffer chamber, respectively. Then, a temperature distribution of 95 °C is achieved within the cell lysis/DNA hybridization/LAMP reaction chamber to lyse the clinical bio-samples for 10 min. The hybridization process is then performed by keeping the temperature at 63 °C within the reaction chamber for 10 min, such that the released DNA of the MRSA can be hybridized onto the surface of the probe-conjugated magnetic beads. Afterwards, all the interferent substances in the biological solution are sucked out to the waste chamber by an external vacuum pump along with the use of a permanent magnet. A washing process is then carried out by pumping the phosphate buffered saline (PBS) washing buffer into the cell lysis/DNA hybridization/LAMP reaction chamber at a flow rate of 150 μL min^{-1} by utilizing the sample transportation unit, followed by transporting 20 μL of the LAMP reaction mixture from the reagent chamber to the LAMP reaction chamber at a flow rate of 300 μL min^{-1}. The isothermal amplification is then performed by maintaining the temperature distribution at 63 °C within the reaction chamber for 40 min. Finally, the amplicons from the LAMP process are optically analyzed utilizing a spectrophotometer.

Spectrophotometric analysis

In addition to a conventional slab-gel electrophoretic analysis of the DNA amplicons, a spectrophotometer has been employed in the current microfluidic diagnostic assay to quantify the amplicons. Several researchers have reported that the turbidity of the amplified products increased due to the precipitation of magnesium pyrophosphate during the amplification process.[14,15] Therefore, optical analysis of the LAMP process incorporated with DNA staining dyes or fluorescent-labeled primers has been developed in recent years.[37,38] Still, various bulky apparatus such as optical analyzers with complicated setups and sophisticated chemical synthesis of dye-labeled primers are normally required. Alternatively, absorption spectrophotometry has been commonly employed in liquid chromatography and optical analysis is used to monitor the progression of bio-molecules, since nucleic acids and proteins generally absorb most intensely in the UV range. Hence, a spectrophotometric analysis by measuring the absorbance values of the optical density at a wavelength of 260 nm (OD_{260}) for the LAMP amplicons has been utilized in this study. Significantly, a standard curve for semi-quantitative analysis of MRSA has been investigated by calculating the difference in absorbance values (ΔOD_{260}) for the LAMP amplicons from the isolated templates with concentrations ranging from 100 pg μL^{-1} to 10 fg μL^{-1}. As a consequence, the optical signal from the microfluidic diagnostic assay can be immediately detected and referenced to a standard curve for the semi-quantitative detection of MRSA.

Results and discussion

In the developed microfluidic diagnostic assay, an optimization of the diagnostic protocol is first performed. Then, optimum operating conditions for the isolation of the target DNA of the MRSA and the LAMP reaction are explored in the integrated

microfluidic system. This is followed by analysis of the OD_{260} of the amplified products utilizing a spectrophotometric analysis. Successful demonstration of the resulting specificity and sensitivity of the microfluidic diagnostic assay, as compared to the bench-top diagnostic system, is also presented.

Optimization of hybridization and LAMP reaction

In order to optimize the operating conditions, including the reaction temperature and the reaction time of the diagnostic assay, a standardized staphylococcal genomic DNA is collected and is used as the template for the optimization of the diagnostic protocol. Detailed information about the preparation of the standardized staphylococcal genomic DNA can be referenced in the ESI.† The standardized staphylococcal genomic DNA with a concentration of 10 pg μL^{-1} is used for both the optimization of the hybridization process and the LAMP reaction. Note that the experimental verification of these optimum conditions during the diagnostic process is first carried out by utilizing a conventional bench-top thermo-cycler (MyCycler™ thermal cycler, Bio-Rad, USA). 10 μL of standardized DNA of the MRSA strain 1601 is first hybridized with the specific *spa* probe-conjugated magnetic beads at different reaction temperatures (from 55 °C to 68 °C) and reaction times (from 0 min to 20 min) during the hybridization process, followed by purification of the DNA-conjugated magnetic complexes and performing the subsequent LAMP process. Fig. 2(a) and 2(b) show the experimental results for the hybridization at different reaction temperatures and reaction times, respectively. It is clearly observed that the optimal hybridization process for the reaction temperature and the reaction time are 63 °C (lane 4 in Fig. 2(a)) and 10 min (lane 3 in Fig. 2(b)), respectively.

In addition, the optimal conditions of the LAMP process for the detection of MRSA/MSSA, including the reaction temperature and the reaction time, are also explored and are shown in Fig. 2 (c)–(f). Fig. 2(c) and 2(d) show the LAMP reactions at different temperatures of 68 °C, 65 °C, 63 °C and 60 °C for the detection of MRSA and MSSA, respectively. All the LAMP processes in the optimization of the reaction temperature are performed for 60 min. From the experimental results, the nucleic acid amplification can be accomplished at a temperature of 63 °C (lanes 3 in both Fig. 2(c) and 2(d)). Similarly, the LAMP processes with different reaction times of 120, 90, 60, 50, 40, 30, 20 and 10 min for the detection of MRSA and MSSA are shown in Fig. 2(e) and 2(f), respectively. The LAMP reactions for the verification of the optimum reaction time are realized with

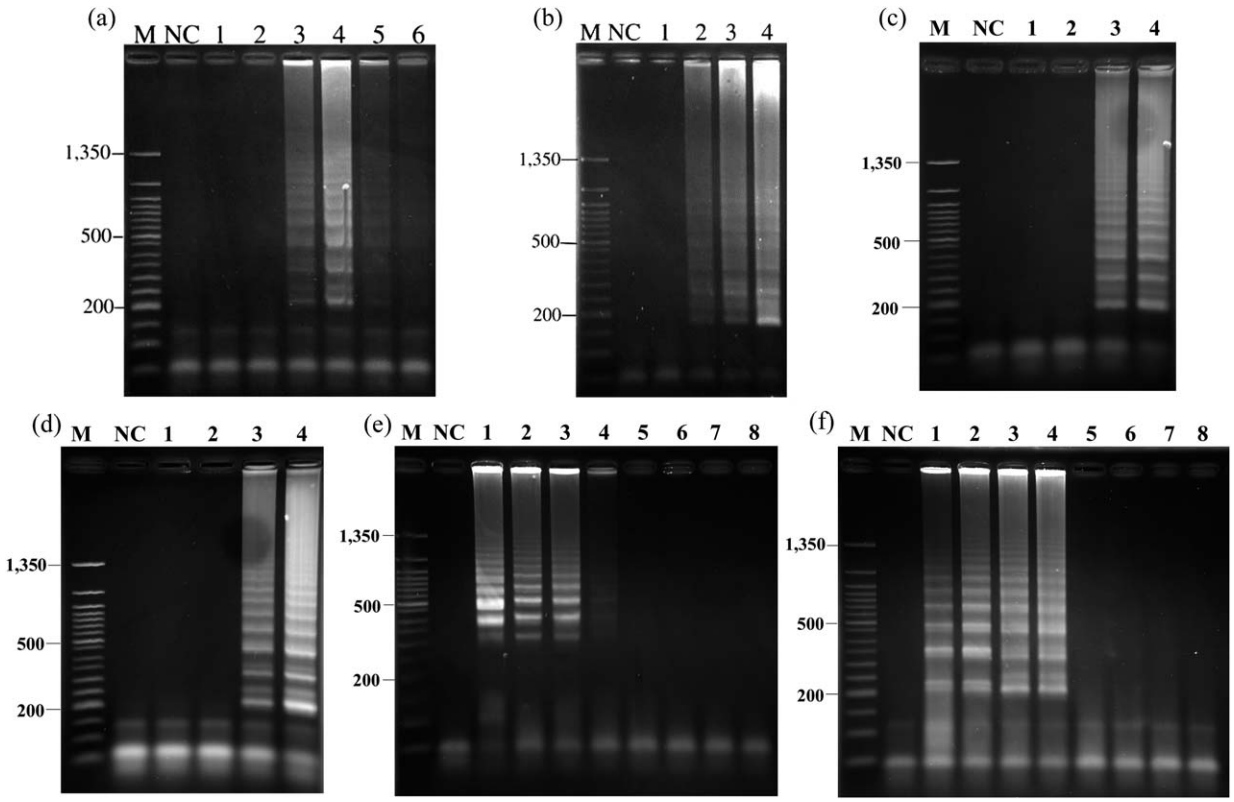

Fig. 2 Determining optimal conditions for the MRSA/MSSA diagnostic assay. (a) Optimization of the temperature during the hybridization between the specific probe-conjugated magnetic beads and the released DNA of the MRSA. Lanes 1–6 present the slab-gel results from the LAMP amplicons by performing the isothermal process with the extracted DNA of the MRSA strain 1601 at 55, 58, 60, 63, 65 and 68 °C for 60 min, respectively. (b) Determination of the optimized time during the hybridization between the specific probe-conjugated magnetic beads and the released DNA of the MRSA. Lanes 1–4 indicate the LAMP results carried out at 63 °C for 0, 5, 10 and 20 min, respectively. (c) and (d) show the optimal reaction temperature of LAMP for the detection of MRSA and MSSA, respectively. Lanes 1–4 indicate the results for the LAMP performed at 68, 65, 63 and 60 °C, respectively. (e) and (f) show the optimal reaction time of LAMP for the detection of MRSA and MSSA, respectively. Lanes 1–8 are the results for the LAMP conducted for 120, 90, 60, 50, 40, 30, 20 and 10 min, respectively. Note that Lane M represents the 50-bp DNA ladders; and Lane NC: ddH_2O.

a reaction temperature of 63 °C. The optimum reaction time is determined to be 60 min from successful amplification of the *mec*A and the *spa* genes of MRSA (lane 3 in Fig. 2(e)) and MSSA (lane 3 in Fig. 2(f)), respectively. As a result, the proposed microfluidic diagnostic assay for the rapid detection of MRSA/MSSA can be realized by following three optimized steps in the bench-top thermo-cycler: (1) thermal lysis at 95 °C for 10 min, (2) DNA hybridization at 63 °C for 10 min, and (3) the LAMP process at 63 °C for 60 min.

The developed diagnostic assay for target DNA isolation and the LAMP process has also been carried out automatically in the integrated microfluidic system. Note that the LAMP process with a final volume of 15 μL is employed by proportionally decreasing the amount of the LAMP reagents described previously (50%). Fig. 3(a) shows a comparison of the amplification efficiency between the conventional diagnostic assay performed by the bench-top thermo-cycler and the integrated microfluidic system with different reaction times (from 20 min to 60 min) when the total reaction volume is 15 μL. Note that the positive control (PC) case is the amplification from the DNA templates with the optimal conditions described above and the negative control (NC) case represents the amplification with the ddH_2O. All the LAMP reactions are carried out at a temperature of 63 °C. It is clearly observed that successful amplification can be realized in the integrated microfluidic system with an optimal reaction time of 40 min while the conventional system requires more than 60 min. This indicates that the reaction time for the LAMP process can be reduced to 40 min by utilizing the integrated microfluidic system with the microfluidic diagnostic assay. Therefore, the optimized experimental conditions for the microfluidic diagnostic assay are (1) thermal lysis at 95 °C for 10 min, (2) DNA hybridization at 63 °C for 10 min, and (3) the LAMP process at 63 °C for 40 min.

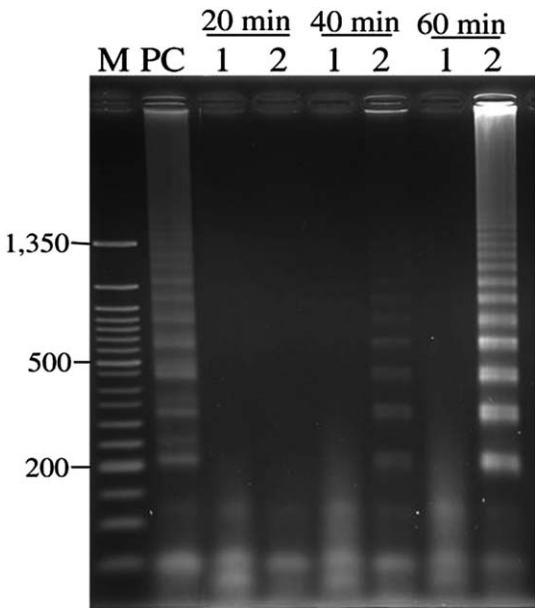

Fig. 3 Comparison of the LAMP amplification efficiencies between the bench-top system and the microfluidic diagnostic assay for the detection of MRSA with different reaction times. Lane M: 50-bp DNA ladders, lane NC: negative control without DNA templates, lanes 1: the results performed by the bench-top system, lanes 2: the results amplified by the integrated microfluidic system.

Furthermore, it is clearly observed that successful amplification is carried out in the microfluidic assay with less sample and reagent consumption when compared to the conventional bench-top system, indicating that efficient amplification of target genes can be accomplished with the incorporation of the integrated microfluidic system. It is also concluded that the high amplification efficiency is achieved due to the uniform temperature field generated by the integrated microfluidic system equipped with a self-compensated, temperature control module. Consequently, a microfluidic diagnostic assay with high amplification efficiency is achieved in a shorter period of time.

Specificity of the microfluidic diagnostic assay

A high specificity for the proposed microfluidic diagnostic assay has been verified since only target genes associated with MSSA/MRSA are recognized and adhere onto the surface of the probe-conjugated magnetic beads. This is followed by amplifying them with the incorporation of specific primer sets during the LAMP process. The specificity of the developed microfluidic diagnostic assay is verified by utilizing different bacterial samples including 10 strains of MRSA (as listed in Table 1 in ESI†), 10 strains of MSSA (as shown in Table 1 in ESI†), and 7 common infections of bacteria including *Streptococcus pneumoniae* (No. 1645), *Escherichia coli* (No. 57), *Enterobacter* sp. (No. 1124), *Klebsiella pneumoniae* (No. 112), *Pseudomonas aeruginosa* (No. 138), *Proteus vulgaris* (No. 380) and *Haemophilus influenzae* (No. 139). The bacterial sample is loaded into the integrated microfluidic system where the probe-conjugated magnetic beads, the washing buffer and the reaction mixture are pre-loaded. Then the entire diagnostic process including the DNA hybridization and the isothermal amplification is automatically performed. Fig. 4(a) and 4(b) show the high specificity for the detection of MSSA/MRSA isolated from the different bacterial strains, respectively. 10 strains of MSSA have been used to verify the specificity of the diagnostic assay *via* two specific primer sets, namely the *spa* gene (Fig. 4(a-1)) and the *mec*A gene (Fig. 4(a-2)). Experimental results show that the successful amplifications of MSSA can only be accomplished by utilizing the primer sets of the *spa* gene while there are no amplicons during the LAMP process by using the primer sets of the *mec*A gene. Similarly, 10 strains of MRSA have been employed to validate the specificity by using both the specific *spa* primer sets (Fig. 4(b-1)) and the *mec*A primer sets (Fig. 4(b-2)). From these results, amplification of both of these two genes has been carried out successfully, indicating that the identification of MRSA can be completed in the microfluidic diagnostic assay with a high specificity.

Moreover, the specificity of the microfluidic diagnostic assay is also verified by performing a cross-reactivity assay with the aforementioned different sources of DNA with the primer sets of the *spa* gene (Fig. 4(c-1)) and the primer sets of the *mec*A gene (Fig. 4(c-2)). Several different common types of bacteria with antibiotic resistance have been used in the microfluidic diagnostic assay. It is clearly observed that only the sample extracted from the MRSA is amplified successfully (lane PC in both Fig. 4(c-1) and 4(c-2)) without any false-positive or false-negative results. The experimental data, therefore, demonstrates that a high

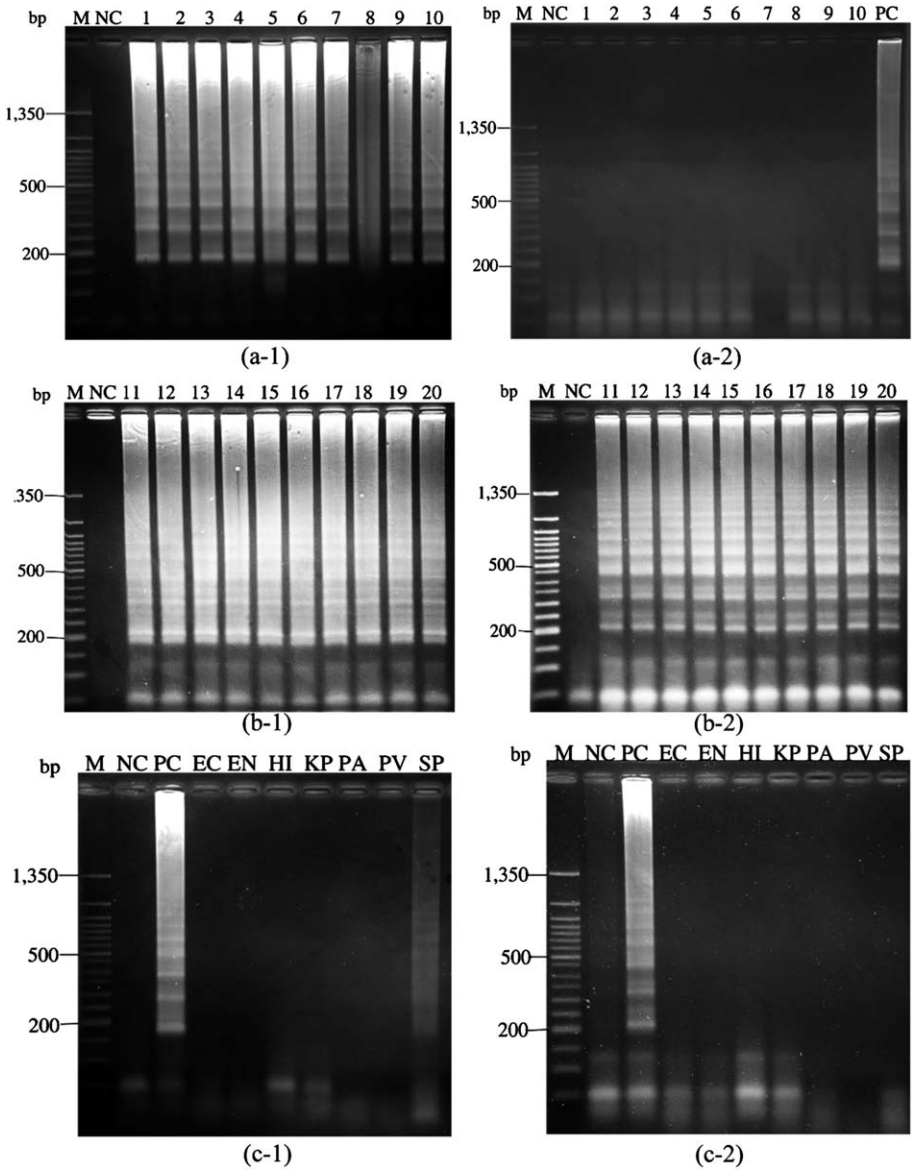

Fig. 4 Specificity of the microfluidic diagnostic assay. (a) 10 strains of MSSA are verified by primer sets of the *spa* gene (a-1) and primer sets of the *mec*A gene (a-2), respectively. (b) 10 strains of MRSA verified by primer sets of the *spa* gene (b-1) and primer sets of the *mec*A gene (b-2), respectively. (c) 7 common strains of bacteria with antibiotic resistance verified by primer sets of the *spa* gene (c-1) and primer sets of the *mec*A gene (c-2), respectively. Lane M: 50-bp DNA ladders, lane NC: negative control using ddH_2O, lane PC: positive control using MRSA strain 1601, lane EC: *Escherichia coli*, Lane EN: *Enterobacter* sp., Lane HI: *Haemophilus influenzae*, Lane KP: *Klebsiella pneumoniae*, Lane PA: *Pseudomonas aeruginosa*, Lane PV: *Proteus vulgaris*, Lane SP: *Streptococcus pneumoniae*.

specificity for the proposed microfluidic diagnostic assay can be verified since the target DNA can be separated from the bacterial sample by utilizing the magnetic beads and the successfully amplification of target genes can also be realized.

Limit of detection (LOD) compared with the conventional PCR technique

The LOD of the proposed microfluidic diagnostic assay is also investigated and compared with conventional nucleic acid amplification in the bench-top thermo-cycler, as shown in Fig. 5. 10 ng μL^{-1} of DNA from the MRSA strain 1601 are used to perform a ten-fold serial dilution and are tested in both the conventional PCR process (Fig. 5(a)) and the microfluidic LAMP assay (Fig. 5(b)) by utilizing the primer sets of the *mec*A gene. The detection limits of the conventional PCR and the microfluidic diagnostic assay are found in lane 4 in Fig. 5(a) and lane 7 in Fig. 5(b), respectively. Namely, 1–10 pg μL^{-1} of DNA for the conventional PCR process in the bench-top thermo-cycler and 1–10 fg μL^{-1} of DNA for the LAMP process in the microfluidic diagnostic assay are successfully detected. The sensitivity of the microfluidic diagnostic assay is experimentally found to be 1000-fold higher than that of the conventional PCR in the bench-top system. Moreover, the LOD of the proposed microfluidic

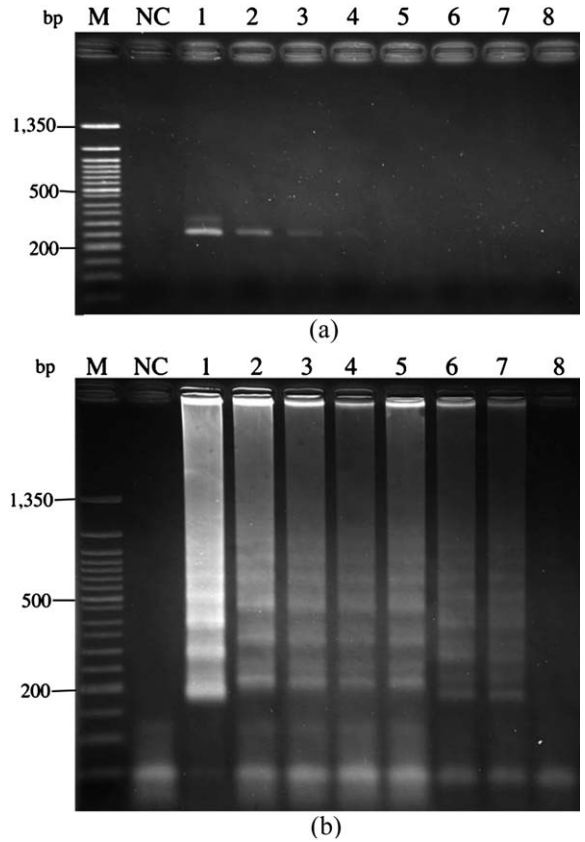

Fig. 5 Comparison of the detection sensitivity for MRSA. Slab-gel electropherograms of the amplicons from a conventional PCR in the bench-top thermo-cycler (a) and the proposed microfluidic diagnostic assay (b). Lane M: 50-bp DNA ladders, lane NC: negative control using ddH_2O, lanes 1–8: 10-fold serial dilutions of DNA of MRSA strain 1601 ranging from 10 ng μL^{-1} to 1 fg μL^{-1}.

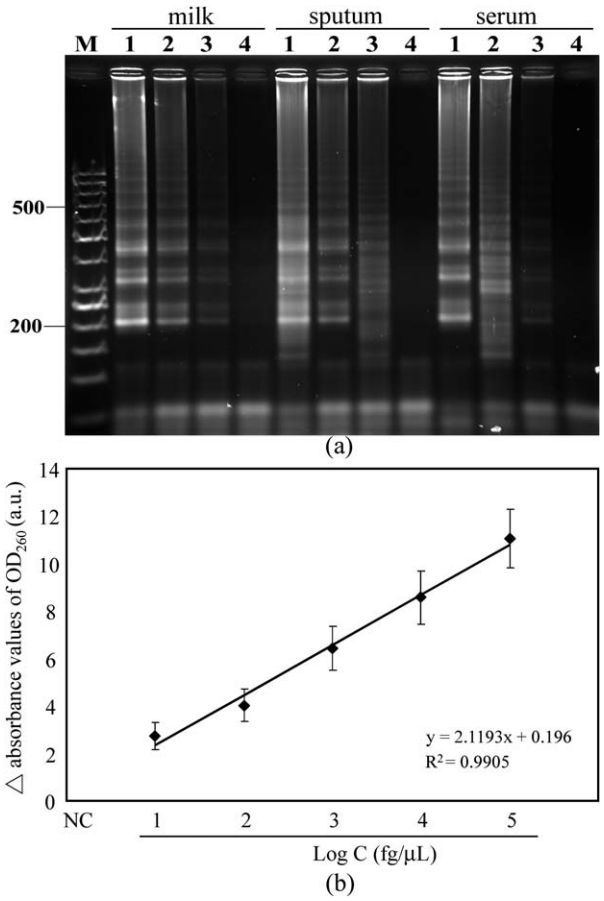

Fig. 6 (a) Detection of MRSA spiked in three types of simulated clinical samples, namely milk, sputum and serum. MRSA with different concentrations ranging from 10^3 CFU/μL (lane 1) to 1 CFU/μL (lane 4) are used to verify the performance of the microfluidic diagnostic assay. The detectable concentration of 10 CFU/μL can be found in lane 3 for each clinical bio-sample. Lane M: 50-bp DNA ladders. (b) The standard curve of spectrophotometric signals from the LAMP amplicons with different initial DNA concentrations ranging from 100 pg μL^{-1} to 10 fg μL^{-1}. Raw data for the absorbance of OD260 of MRSA LAMP amplicons can be found in Table 3 in ESI.†

diagnostic assay is also comparable with the detection limit of the real-time PCR-based centrifugal microfluidic platform.[39] Hence, the high sensitivity of the proposed microfluidic diagnostic assay without using an external, bulky, thermo-block for the PCR process is verified.

Detection of clinical bio-samples

S. aureus has been widely reported as the etiologic agent that causes more than half of the food poisoning epidemics worldwide.[40] More importantly, the bacterial particles of *S. aureus* often circulate in the blood vessels of the body, resulting in the rapid spread of the bacteria in the infected host.[41] A significant improvement in disease control could be made if the target bacteria in the clinical bio-samples can be accurately and automatically detected by this proposed microfluidic diagnostic assay. Hence, stimulated clinical bio-samples mixed with the MRSA are used to verify the performance of the developed microfluidic diagnostic assay in this study. Three different types of clinical bio-samples, namely milk, sputum and serum, are mixed with MRSA with concentrations ranging from 10^4 colony-forming units (CFU)/μL to 1 CFU/μL. The sputum and serum samples were freshly prepared from healthy volunteers and purchased milk was used with food safety certification. This is followed by loading the clinical bio-samples into the integrated microfluidic system to explore the capability of the microfluidic diagnostic assay. Fig. 6(a) shows the slab-gel electropherograms for the detection of MRSA in the clinical bio-samples. It is found that MRSA with a concentration of 10 CFU/μL (lanes 3 in each clinical bio-sample) in the simulated bio-samples is successfully detected by utilizing the proposed microfluidic diagnostic assay. In comparison, the LAMP-only micro-chip is only capable of detecting pure DNA extracted from MRSA.[34] The proposed microfluidic diagnostic assay integrated with magnetic-bead-based extraction of target DNA and the LAMP process is the first miniature system reported for the rapid detection of bacteria in these various simulated types of clinical samples.

Optical detection of microfluidic diagnostic assay

Instead of using the conventional slab-gel electrophoresis or capillary electrophoresis, the proposed microfluidic diagnostic

assay also presents an end-point, semi-quantitative spectrophotometric analysis of the LAMP process for rapid detection of MRSA with the incorporation of a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific Inc., USA). UV–visible absorption spectrophotometry is a well-established technique in macroscale analytical chemistry and laboratory diagnostics.[42] The diagnostic procedure for MRSA in the clinical samples can be, therefore, completed automatically by utilizing the microfluidic diagnostic assay. This is followed by immediately measuring the OD_{260} absorbance of the amplicons *via* the spectrophotometer for a semi-quantitative analysis. Fig. 6(b) shows a standard curve of the spectrophotometric signals from the LAMP amplicons with different initial DNA concentrations ranging from 100 pg μL^{-1} to 10 fg μL^{-1}. The DNA template is purified and extracted from MRSA utilizing the protocols described above, followed by mixing 10 μL of purified DNA with the clinical samples and loading the mixture into the reaction chamber to perform hybridization, LAMP and the optical detection. The experimental results show that the specific optical signal of OD_{260} is emitted and detected by the spectrophotometer and a standard curve between the absorbance values of OD_{260} and the initial concentration from the DNA templates is also calculated as follows.

$$y = 2.1193x + 0.196 \quad (1)$$

where y represents the absorbance values of the OD_{260} of the tested bio-samples and x represents the logarithm of the concentration (C) of DNA templates. Note that the negative control (NC) uses ddH_2O as the template for the microfluidic diagnostic assay and this optical signal can be regarded as a noise level. The correlation coefficient (R^2) for the regression analysis between the concentration of DNA templates and the absorbance values of the OD_{260} is found to be 0.9905, indicating that the clinical samples with MRSA can be diagnosed automatically with a high sensitivity using the developed microfluidic diagnostic assay. In addition, the entire diagnostic procedure including the sample pre-treatment, the LAMP and the optical detection can be completed within 60 min.

Conclusions

The rapid identification and detection of *S. aureus* in clinical samples such as food or sputum always poses a challenge. The entire diagnostic protocol, such as a time-consuming agar-based bacterial culture or tedious centrifugal processes, is technically demanding and labor-intensive.[43,44] Recently, several commercial kits such as BBL® Crystal™ MRSA ID System (Becton Dickinson Microbiology Systems, Maryland, USA), MRSA-Screen™ Assay (Denka Seiken Co., Ltd., Tokyo, Japan), EVIGENE MRSA Detection Kit (EVIGENE; Statens Serum Institute, Copenhagen, Denmark), and Velogene Rapid MRSA Identification Assay (ID Biomedical Corp, Vancouver, BC) have become available for the clinical identification of MRSA, especially for detecting the spread of MRSA in hospitals and communities. The diagnostic time of these most common real-time PCR kit for the detection of MRSA from clinical specimens were within a few hours.[45] Nevertheless, the cost of these bulky systems is still relatively expensive. They also require more reagents, samples and more power consumption. As a consequence, a LAMP process with its high sensitivity and specificity and a magnetic-bead-based hybridization for the isolation of target DNA from clinical samples are integrated in this study to achieve the rapid identification of MRSA. Two specific primer sets, including the inner and the outer primers for recognition of six distinct sequences of MRSA, have been designed and verified. Moreover, the LAMP amplicons can be optically measured by analyzing the absorbance or optical density of the products. Experimental data shows that the LOD of this microfluidic diagnostic assay is 10 fg μL^{-1}, which is 1000-fold higher than the sensitivity of the conventional PCR in the bench-top system. Significantly, the entire diagnostic protocol can be completed in approximately 60 min (namely, 95 °C for 10 min for thermal lysis, 63 °C for 10 min for DNA hybridization, and 63 °C for 40 min for the LAMP process) with less human intervention required. Therefore, this microfluidic diagnostic assay may provide a promising protocol for the rapid isolation and detection of infectious diseases in future clinical applications.

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