Rapid identification of pathogens responsible for necrotizing fasciitis on an integrated microfluidic system

Ju-Ching Yu,^{1,a)} Pang-Hsin Hsieh,^{2,a)} Hsing-Wen Tsai,³ Wen-Hsin Chang,¹ Ting-Hang Liu,¹ Mel S. Lee,⁴ Kuo-Ti Peng,³ Kuo-Chin Huang,^{3,b)} and Gwo-Bin Lee^{1,5,6,b)}

¹Department of Power Mechanical Engineering, National Tsing Hua University, Hsinchu 30013, Taiwan

²Department of Orthopaedic Surgery, Linkou Chang Gung Memorial Hospital, Taoyuan 33305, Taiwan

³Department of Orthopaedic Surgery, Chiayi Chang Gung Memorial Hospital, Chiayi 61363, Taiwan

⁴Department of Orthopaedic Surgery, Kaohsiung Chang Gung Memorial Hospital, Kaohsiung 83301, Taiwan

⁵Institute of Biomedical Engineering, National Tsing Hua University, Hsinchu 30013, Taiwan

⁶Institute of NanoEngineering and Microsystems, National Tsing Hua University, Hsinchu 30013, Taiwan

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Necrotic fasciitis (NF) is a particularly aggressive and serious infection of the fascia that can penetrate into the musculature and internal organs, resulting in death if not treated promptly. In this work, an integrated microfluidic system composed of micropumps, microvalves, and micromixers was used to automate the detection of pathogens associated with NF. The entire molecular diagnostic process, including bacteria isolation, lysis, nucleic acid amplification and optical detection steps, was enacted on this developed system. Mannose binding lectin coated magnetic beads were first used as probes to isolate all bacteria in a sample. In this work, polymerase chain reaction assays featuring primers specific to genes from each of four NF-causing bacteria (*Vibrio vulnificus*, *Aeromonas hydrophila*, and methicillin-sensitive and resistant *Staphylococcus aureus*) were used to rapidly and exclusively verify the presence of the respective bacterial strains, and the limits of detection were experimentally found to be 11, 1960, 14, and 11 400 colony forming units/reaction, respectively; all values reflect improvement over ones reported in literature. This integrated microfluidic chip may then be valuable in expediting diagnosis and optimizing treatment options for those with NF; such diagnostic improvements could ideally diminish the need for amputation and even reduce the mortality rate associated with this life-threatening illness. *Published by AIP Publishing.*
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NOMENCLATURE

AH	<i>Aeromonas hydrophila</i>
bp	Base-pair
CFU	Colony forming unit
<i>E. coli</i>	<i>Escherichia coli</i>
gDNA	Genomic deoxyribonucleic acid

^{a)}J.-C. Yu and P.-H. Hsieh contributed equally to this work.

^{b)}Authors to whom correspondence should be addressed: kc2672@gmail.com and gwobin@pme.nthu.edu.tw.

LB	Luria-Bertani
LOD	Limit of detection
MBL	Mannose binding lectin
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
NCBI	National Center for Biotechnology Information
NF	Necrotizing fasciitis
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
SA	<i>Staphylococcus aureus</i>
VV	<i>Vibrio vulnificus</i>
16S rRNA	16S ribosomal ribonucleic acid

I. INTRODUCTION

The high morbidity and mortality rate associated with fascial infections known as necrotizing fasciitis (NF) has not diminished appreciably since the disease was first described over 60 years ago.^{1,2} The length of time required for accurate pathogen detection is partially responsible for such a poor prognosis, especially given the aggressive nature of most NF infections.³ Patient prognosis and survival can be improved by providing nutritional support and by directly treating the infected tissues (e.g., debridement) through surgical intervention, even by limb amputation, which is sometimes life-saving.⁴ High clinical vigilance (e.g., early diagnosis), in which fasciotomy combined with appropriate antibiotics at the earliest time, can thwart the need for such late-stage mutilation surgery.⁵ Although it can significantly increase the survival in NF patients, without the prompt knowledge of the causing pathogens and correct use of antibiotics, repetitive surgical debridement of necrotic and diseased soft tissues remains the only resort to save a patient's life.⁴ The infection would ideally be recognized and treated prior to such late-stage manifestations of severe infection.⁶

Antimicrobial therapy is an important part of NF treatment but remains secondary to debridement.⁴ Given the potentially lethal nature of the disease, accurate antibiotic treatment should be started as soon as NF is suspected. However, the selection of the correct antibiotic regimen still relies on culture-based pathogen identification and antimicrobial susceptibility tests.⁷ Thus, definitive results may not become available until more than 72 h after biopsy collection.⁸ Not only is microbial culture-based diagnostics inherently slow but it is also error-prone and can result in the prescription of non-ideal or even ineffective antibiotics. Such misdiagnosis can also lead to inappropriate recommendations with respect to the timing and degree of debridement. Ideally, then, rapid identification of the causative pathogens would lead to better, more appropriate care and therefore an improved prognosis.⁹

Recently, microfluidic technologies have been adapted for a variety of biomedical applications, especially they have allowed for the miniaturization of diagnostics platforms.^{10,11} For instance, vancomycin-coated magnetic beads have been used to capture bacteria in complex samples and nucleic acid based amplification, and detection processes could be performed on integrated microfluidic system.¹¹ Microfluidic platforms possess several advantages over traditional diagnostic approaches in diagnostic applications, such as significantly decreased in sample and reagent consumption, higher sensitivity, higher portability, faster diagnosis, and relatively lower costs. Furthermore, functional microdevices such as micropumps, microvalves, micromixers, and microreactors can be integrated such that the entire diagnostic process is carried out in an automated fashion on miniaturized, disposable chips. In this work, we demonstrated that fast diagnosis of pathogens (bacteria) associated with necrotizing fasciitis could be performed automatically on an integrated microfluidic system within 90 min.

Mannose binding lectin (MBL)-coated magnetic beads used as a universal bacteria probe to isolate bacteria in samples was proposed in this work. Recently, an approach by using vancomycin as a universal probe to isolate bacteria was reported.¹¹ However, there is one drawback for using vancomycin as a probe to detect bacteria. For instance, vancomycin-resistant

Staphylococcus aureus (SA) cannot be detected because of the mutated peptides on the cell wall.¹²

In this work, an integrated microfluidic system composed of micropumps, microvalves, and micromixers was used to automate the diagnostic process for detection of pathogens associated with NF. Furthermore, the entire molecular diagnosis process including bacteria isolation, lysis, nucleic acid amplification, and optical detection could be automated on this developed system. This integrated microfluidic system could decrease the human errors due to the automatic sample pretreatment process and avoid operators to be infected by lethal pathogens during the diagnosis. Given the advantages of microfluidic chips and the need for more sensitive, rapid diagnostic assays for detection of NF-causing pathogens, we hypothesized that we could design a microfluidic system capable of detecting such bacteria, and then NF could conceivably be diagnosed in a minutes-hours timeframe. In order to demonstrate its capability, in this study, four important NF-associated pathogens, including *Vibrio vulnificus* (VV), *Aeromonas hydrophila* (AH), and methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* (SA and MRSA) were used as models. These bacteria constitute the major causes of NF and are regarded to be associated with high morbidity and mortality.^{13–15} Rapid identification of these four life-threatening pathogens on an integrated microfluidic system may be valuable in improving treatment outcomes of NF, thereby reducing the mortality rate and the need for amputation.

II. MATERIALS AND METHODS

A. Experimental procedures

The on-chip experimental procedure and the chip itself are schematically shown in Figs. 1 and 2, respectively. First, the bacterial cells were mixed with MBL-coated magnetic beads and incubated for 10 min by activating micromixers such that the target bacteria in the samples could be captured [Fig. 1(a)]. Then, the bacteria-magnetic bead complexes were collected at the bottom of the microfluidic chip by applying an external magnetic field in the integrated control system [Fig. 1(b)]. After bacteria-bead complex collection, washing buffer (phosphate buffered saline, PBS) was transported to wash out unwanted materials by activating micropumps. Then, the bacteria-bead complexes were re-suspended in a polymerase chain reaction (PCR) cocktail reagent (Table II) and thermally lysed to release genomic DNA (gDNA). Next, PCR amplification was performed by using a temperature control module located underneath the microfluidic chip [Fig. 1(d)]. Finally, a home-made detection system composed of a laser and a photomultiplier tube (PMT) was used for end-detection on fluorescent signals (SYBR® Green) emitted

FIG. 1. A schematic illustration of the experimental procedure performed on the integrated microfluidic system. (a) Incubation between samples and MBL-coated magnetic beads; (b) bacterial isolation and collection via external magnet, (c) after washing away unbound substances, bacteria were thermally lysed. (d) PCR process and end-point fluorescence detection.

FIG. 2. A schematic illustration and image of the integrated microfluidic chip.

during the PCR [Fig. 1(d)].^{10,11} All these steps described above were executed automatically on chip in a previously developed microfluidic system.¹¹

The detailed process performed on the integrated microfluidic chip could be described as follows. The four lab-cultured bacteria and MBL-coating beads were mixed and placed independently in four different chambers which were located in the central region of the chip. As shown in four identical regions in four red blocks in Fig. 2, the four tested bacteria samples were placed in the chamber (labeled as S) in the central region. Negative control (water) and positive control (gDNA) reagents were placed, respectively, in N and P chambers. After 10-min incubation of bacteria and beads, the bacteria-bead complexes were collected by using a permanent magnet placed under the chamber while the wash buffer (PBS) was transported by activating micropumps which were controlled by pneumatic pressure to wash away debris or interferents (blue arrow). Afterwards, the previously loaded PCR reagent cocktail with each specific PCR primer in the reagent chamber (chambers labeled as R in Fig. 2) was transported (green arrow) into each chamber individually to perform PCR.

B. Bacterial culture

Twenty-one bacterial species (Table I) were cultured at 37 °C in appropriate culture media, typically Luria-Bertani (LB) broth (Focus Bioscience Pty., Ltd, AU) or agar plates; in the case of the *Streptococcus* spp. cultures, cells were cultured on blood agar plates (Creative Life Sciences, Inc., Taiwan) and incubated at 37 °C for 16–18 h.

C. Preparation of recombinant MBL from *E. coli* expression system

A competent *Escherichia coli* (*E. coli*) strain BL21(DE3) (Sigma, USA) was used for expression of recombinant MBL. The target gene of bovine MBL with its open reading frame was cloned into a pET21b vector (Novagen, Germany) with a 6-His tag, and the MBL gene was expressed via codon optimization¹⁶ for the high expression efficiency in *E. coli* system.

MBL proteins in the prokaryotic expression system were expressed according to the following protocol. The transformed *E. coli* were cultured in LB media containing 50 mg/l ampicillin at 37 °C. When the optical density (OD) at a 600-nm wavelength in each culture reached a value of 0.4–0.5, MBL protein expression was induced with 0.5 mM isopropyl- β -D-thiogalactoside for 16 h at 25 °C. The cell pellets were then harvested by centrifugation at 6000 g and solubilized in a buffer comprised of 50 mM Tris-HCl, 50 mM NaCl, 2 mM MgCl₂, 14 μ M β -mercaptoethanol, and 0.2 mM phenylmethylsulfonyl fluoride (pH 7.8). Cells were then disrupted through sonication, and the supernatant was separated by ultracentrifugation (100 000 g) for 1 h at 4 °C. The supernatant was further treated with the Ni Sepharose 6 Fast Flow kit (GE Healthcare, England) containing a Tris-HCl buffer (50 mM Tris-HCl, 50 mM NaCl, 2 mM MgCl₂, 14 μ M β -mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.8) containing 20 mM

TABLE I. Bacterial strains used in the current study. The samples either originated from the Bioresource Collection and Research Center (“1” in the “Origin” column) or they were clinically isolated from the Division of Clinical Microbiology in Chang Gung Memorial Hospital’s Department of Laboratory Medicine, Chiayi (“2” in the “Origin” code = 2).

Species	Origin	Lane # in Fig. 4
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> (SA) BCRC10780	1	1
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> (MRSA) BCRC15211	1	2
<i>Aeromonas hydrophila</i> subsp. <i>hydrophila</i> BCRC13018	1	3
<i>Vibrio vulnificus</i> BCRC12905	1	4
<i>Staphylococcus epidermidis</i>	2	5
<i>Streptococcus pyogenes</i>	2	6
<i>Streptococcus agalactiae</i>	2	7
β -streptococcus Gr. non ABD	2	8
<i>Streptococcus viridans</i>	2	9
<i>Enterococcus</i>	2	10
Gram-positive bacilli	2	11
<i>Escherichia coli</i>	2	12
<i>Klebsiella pneumoniae</i>	2	13
<i>Enterobacter cloacae</i>	2	14
<i>Proteus vulgaris</i>	2	15
<i>Serratia marcescens</i>	2	16
<i>Pseudomonas aeruginosa</i>	2	17
<i>Shewanella putrefaciens</i>	2	18
<i>Vibrio cholerae</i>	2	19
<i>Vibrio parahaemolyticus</i>	2	20
<i>Vibrio alginolyticus</i>	2	21

imidazole, and the resin was loaded onto a column with slow rotation overnight. The purified MBL protein was then eluted in a Tris-HCl buffer containing 250 mM imidazole. The target protein was dialyzed to remove excessive imidazole with the Amicon kit (Merck Millipore, Germany) according to the manufacturer’s recommendations.

D. Preparation of MBL-coated magnetic beads

100 μ l (1 mg) of Dynabeads[®] (bead diameter: 1.05 μ m, MyOne[™] carboxylic acid, Thermo Fisher Scientific Inc., USA) was first washed with PBS twice and re-suspended in 950 μ l PBS. Then, 30 μ l (2.5 μ g) of prokaryotic MBL and 20 μ l of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (120 mg/ml, Invitrogen Corp., USA) were added and the mixture was gently rotated at 4 °C in dark for 18 h. The uncoated MBL was washed out with 1 ml of 0.02% Tween 20 (Sigma) and 1 ml of 0.1% sodium dodecyl sulfate (Sigma). Then 1 ml of 0.1 M ethanol amine (Sigma) in PBS buffer was further added to block the magnetic bead surfaces at 4 °C for 1 h with slow tilt rotation to avoid non-specific binding. Finally, 1 ml of 1X PBS (cat#70011, Thermo Fisher Scientific, Inc., USA) was used to wash away excessive ethanol amine, and the MBL-coated magnetic beads were re-suspended in 1 ml of PBS. The MBL-coated magnetic beads should be kept at 4 °C since proteins do not maintain stability at 4 °C for long durations. MBL-coated magnetic beads were used soon after generation.

E. DNA extraction and PCR

The gDNA extracted from twenty-one bacterial species were used for the PCR assays aimed at verifying the specificity of the NF related pathogens. Bacterial cells were prepared from single colony cultured in LB media at 37 °C overnight, and then $\sim 10^8$ cells were harvested by centrifugation at 10 000 g for 5 min. The gDNA was extracted from the collected

TABLE II. Primers of PCR amplification for bacteria identification.

Name ^a	Gene	Target species	Sequence (5' to 3')	Length ^b	Gene reference
Universal-F	16S rRNA	Bacteria	TTTTTTTATTAGATACCCTGGTAGTCCACGCC	387	Reference 17
Universal-R			TTTTTTTTCGTCATCCCCACCTTCCTCC		
SA _{nuc} -F	SNc	<i>S. aureus</i>	GTGGTTCTGAAGATCCAACAGT	99	WP_001641381.1
SA _{nuc} -R			TAACCGTATCACCATCAATCGC		
SA _{mecA} -F	mecA	Methicillin-resistant <i>S. aureus</i>	TATGGCTCAGGTACTGCTATCC	185	KC243783.1
SA _{mecA} -R			TGAGTTGAACCTGGTGAAGTTG		
VV _{hA} -F	vvhA	<i>V. vulnificus</i>	ATGGGCGTGAACTCAACTATC	144	KC821520.1
VV _{hA} -R			AAACAGTGGATGATTCCAGTCG		
AH _{lip} -F3	Act	<i>A. hydrophila</i>	GTACACGACAGCGCAATTT	110	KC687134.1
AH _{lip} -R3			ACGTCCATGTCTTCACCGAC		

^aAll primers have an optimal annealing temperature of 60 °C.^bAmplicon size in base pairs.

cells with the Presto Mini gDNA Bacteria Kit (Geneaid Biotech, Ltd., Taiwan) according to the manufacturer's recommendations. All gDNA samples were stored at −20 °C until used as templates in the PCR assays.

All primers used in this study for bacteria identification are listed in Table II. The PCR primer set of universal 16 S ribosomal ribonucleic acid (16 S rRNA) was adopted from a previous study.¹⁷ The DNA sequences for each target genes of the respective four bacteria (VV, SA, MRSA, and AH) commonly found in necrotizing skin and soft tissue infections were obtained from the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov>) (NCBI, USA) and the specific primer sets were designed on website (MPprimer, <http://biocompute.bmi.ac.cn/MPprimer/>)¹⁸ and Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). For each gene, PCR amplification was performed in a 20 µl of mixture (Table III). After an initial denaturation at 95 °C for 5 min, samples were thermocycled for 35 cycles in the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) at 95 °C for 15 s and 60 °C for 30 s. It is worth noting that the microfluidic chip was placed on top of a thermoelectric (TE) cooler for controlling the thermocycling process of PCR. The annealing temperatures of four specific primers for each bacterium were optimally designed such that the same PCR thermocycling could be used by using the same TC cooler.

TABLE III. Constituents of PCR reagent cocktail.

PCR	Volume (µl)		
ddH ₂ O	6.2	5.2	6.2
primer F(10 µM)	0.4	0.4	0.4
primer R(10 µM)	0.4	0.4	0.4
KAPA Fast master mix ^a	10	10	10
Sybr Green ^b	3	3	3
Genomic DNA for positive control	0	1	0
TOTAL	20	20	20
	Sample	Positive control	Negative control

^aKAPA Fast: KAPA Biosystems, Inc., USA.^bSybr Green: AppliChem GmbH, Germany.

F. Capture ability test of the MBL-coated magnetic beads on-chip

The capture ability of the MBL-coated magnetic beads towards four kinds of NF-causing-related bacteria was explored by mixing 20 μ l of serial dilutions of bacteria culture with concentrations ranging from 10^9 to 10^2 colony forming units (CFU) per ml of each of the four targets, NF-causing bacteria (SA, MRSA, AH, and VV) with 2 μ l of MBL-coated magnetic beads (concentration = 1 mg/ml) for 10 min. All the PCR reagents, washing buffer, the bacteria, and MBL-bead mixtures were loaded onto respective chambers in the chip. All the following procedures can be executed on chip automatically by the programmed procedures controlled by computer, which is described in Sec. II A. After that, the PCR products of each bacterial dilution were analyzed by slab-gel electrophoresis.

G. Electrophoresis analysis

After detecting fluorescent signals by the home-made detection system,¹¹ PCR products were also analyzed by slab-gel electrophoresis to validate the performance of the entire diagnostic procedure carried out on the integrated microfluidic system. Ten μ l of PCR products with 2 μ l of DNA loading dye (Promega, USA) as well as 100 base-pair (bp) DNA ladders (108 ng/ μ l, 5 μ l; DM003-R500, GeneDireX, USA) were electrophoresed on 2% TBE agarose (MB755-0100, GeneDireX) gel at 100 V for 30 min and post-stained with ethidium bromide (0.5 μ g/ml, Sigma). The PCR products were observed with an exposure time of 2 s at 302 nm with a UVTM transilluminator (UVP, Canada).

III. RESULTS AND DISCUSSION

A. Bacteria capture ability via MBL-coated magnetic beads

PCR amplification on-chip with 16S rRNA primer sets in bacterial samples bound to MBL-coated magnetic beads was successful for four types of NF-causing bacteria [Fig. 3(a)]. These four kinds of bacteria cells were captured by MBL-coated beads and can be observed under the microscopy [Fig. 3(b)]. The capture rates for MRSA, SA, VV, and AH were experimentally found to be 10.3, 14.0, 2.9, and 0.2%, respectively (Table IV). The reason why capture rates for VV and AH were relatively low is because these two bacteria may not have expressed MBL-specific binding sites on their membranes. Nevertheless, these capture rates are higher than those obtained upon using vancomycin-coated beads.^{10,11}

B. Specificity tests for specific genes in necrotizing fasciitis-related bacteria

Since knowing which bacteria are the cause of infection allows for the prescribing of the most appropriate antibiotics, four specific PCR primer sets were designed to specifically amplify genes from each of the four NF-causing bacteria. The PCR processes were then used to distinguish four different kinds of bacteria commonly found in NF, including SA, MRSA, AH, and VV. To validate the specificity of the four primer sets, PCRs were conducted with 21 bacterial strains (Table I). For the VV-specific PCR assay [Fig. 4(a)], the expected 144-bp *vvHa* gene fragment was observed only upon using VV as template. Similarly, Figs. 4(b) and 4(c) show the results of the primer sets used to identify genes specific to SA and MRSA strains, respectively, and the 99-bp *SNc* and 185-bp *mecA* fragments were observed only when the target strains were used as templates. The *SNc* primers were actually able to amplify this gene from both SA and MRSA samples [Fig. 4(b)]. In contrast, since *mecA* gene is a drug-resistance gene that is only present in MRSA, it was not amplified in SA [Fig. 4(c)]. Finally, the 110-bp AH-specific *Act* fragment was only amplified when AH cells were used as template [Fig. 4(d)]. These PCR-derived findings gave us confidence in integrating the four, strain-specific PCR assays into our microfluidic chip for NF-causing pathogen detection. All of the above fluorescent signals of PCR amplification (data not shown) were detected under CFX96 TouchTM Real-Time PCR Detection System, which meets the results analyzed by slab-gel electrophoresis.

FIG. 3. Capture ability of MBL-coated magnetic beads towards NF-associated bacteria (a). Four kinds of NF-causing bacteria could be captured by these MBL-coated beads. Lane M: 100-bp ladder. P: positive control (1 μ l of genomic DNA, 10 ng/ μ l). N: negative control (distilled water). Lane 1: methicillin-resistant *Staphylococcus aureus* (MRSA). Lane 2: *Staphylococcus aureus* (SA). Lane 3: *Aeromonas hydrophila* (AH). Lane 4: *Vibrio vulnificus* BCRC12905 (VV). (b) Microscopic images of bacteria captured by MBL-coated magnetic beads. The upper left and right panels depict MRSA and SA cells, respectively, and the lower left and right panels depict AH and VV cells, respectively.

TABLE IV. Capture rates of magnetic beads coated with either mannose binding lectin (MBL) or vancomycin. Values represent capture rate = $\text{CFU}_{\text{bead}}/(\text{CFU}_{\text{unbond}} + \text{CFU}_{\text{bead}})$.

	MRSA	SA	VV	AH
Original conc. (cfu/ml)	5.7×10^9	6.9×10^9	2.3×10^9	4.5×10^9
MBL-coated beads	10.3% (117.5/1140)	14.0% (193/1380)	10.3% (13.5/460)	0.2% (2/900)
Original conc. (cfu/ml)	7.8×10^6	9.1×10^6	4.8×10^6	1.4×10^6
Vancomycin-coated beads	1.1% (17.3/1560)	0.77% (14/1920)	1.2% (11.3/960)	2.8% (7.8/280)

FIG. 4. The specificity of the PCR assays designed for specifically amplifying genes from each of four NF-associated bacteria. The specificity of each set of primers for its respective target gene was determined by PCR amplification of purified genomic DNA from *Vibrio vulnificus* BCRC12905 (VV), *Staphylococcus aureus* (SA), methicillin-resistant *S. aureus* (MRSA), *Aeromonas hydrophila* (AH), and 17 other pathogenic bacterial strains. (a) Specificity of *vvhA* gene primer for identifying VV with an expected size of 144 bp. (b) Specificity of *SNe* gene primer for identifying SA with an expected size of 99 bp. (c) Specificity of *mecA* gene primer for identifying MRSA with an expected size of 185 bp. (d) Specificity of *Act* gene primer for identifying AH with an expected size of 110 bp. Lane M: 100-bp DNA ladder; the tested bacteria from lanes 1–21 as followed: *V. vulnificus* BCRC12905, *S. aureus* BCRC10780, *S. aureus* BCRC15211, *A. hydrophila* BCRC13018, *S. epidermidis*, *S. pyogenes*, *S. agalactiae*, β -strepto. non ABD, Viridans group streptococci, *Enterococcus*, Gram-positive bacilli, *E. coli*, *K. pneumonia*, *E. cloacae*, *P. vulgaris*, *S. marcescens*, *P. aeruginosa*, *S. putrefaciens*, *V. cholera*, *V. parahaemolyticus*, *V. alginolyticus*, respectively; lane 22 was negative control (distilled water).

C. Limit of detection for identification of NF-causing bacteria on bench

The stock concentrations of MRSA, SA, VV, and AH were 5×10^9 , 1×10^{10} , 3.8×10^8 , and 5.6×10^9 CFU/ml, respectively, which were measured by the plate count method, and their limit of detection (LOD) when using MBL-coated magnetic beads was experimentally found to be 112, 200, 76, and 1120 CFU/reaction, respectively (Fig. 5). The LOD for MRSA, SA, and VV is superior to those reported in the literature,¹¹ and the LOD of AH is comparable to that of a previous study.¹¹ It shows that the proposed assay could be carried out on bench with comparable or even superior performance.

D. Limit of detection for identification of NF-causing bacteria on the integrated microfluidic chip

Furthermore, LOD on the MBL-coated magnetic beads performed on the integrated microfluidic chip was further investigated in this study. The concentrations of MRSA, SA, VV, and AH used in LOD tests of the integrated microfluidic system were measured to be 5.4×10^9 , 9.8×10^9 , 6.8×10^8 , and 5.7×10^9 CFU/ml, respectively, which were measured by the plate count method. Their respective LODs (Fig. 6) were experimentally found to be 11, 1960, 14, and 11400 CFU/reaction, respectively. The LODs performed on the integrated microfluidic system for MRSA and VV were found to be superior to those reported in the literature,¹¹ and the LOD for SA was comparable to the previous study.¹¹ However, the LOD for AH was not as low as the other three bacteria. A recent study reported that a crucial component of the lectin pathway, the mannose binding lectin-associated serine protease-1 gene, could mediate innate immunity against AH in *Ctenopharyngodon idella*.¹⁹ Therefore, AH might require more lectins

FIG. 5. Limit of detection (LOD) for genes specific to four types of NF-associated bacteria. (a) LOD for the *mecA* gene specific to MRSA captured by MBL-coated magnetic beads. Lane M: 100-bp ladder; lanes 1–8: 10-fold serial dilutions of MRSA with an initial concentration of 5.5×10^9 CFU/ml (1.1×10^8 CFU/reaction). P: positive control (1 μ l of genomic DNA, 10 ng/ μ l). N: negative control (distilled water). (b) LOD for the *SNe* gene specific to SA. Lane M: 100-bp ladder. Lanes 1–8: 10-fold serial dilutions of SA with an initial concentration of 1×10^{10} CFU/ml (2×10^8 CFU/reaction). P: positive control (1 μ l of genomic DNA, 10 ng/ μ l). N: negative control (distilled water). (c) LOD for the *vvhA* gene specific to VV. Lane M: 100-bp ladder. Lanes 1–8: 10-fold serial dilutions of VV with an initial concentration of 3.8×10^8 CFU/ml (7.6×10^6 CFU/reaction). P: positive control (1 μ l of genomic DNA, 10 ng/ μ l). N: negative control (distilled water). (d) LOD for the *Act* gene specific to AH. Lane M: 100-bp ladder. Lanes 1–4: 10-fold serial dilutions of AH with an initial concentration of 5.6×10^9 CFU/ml (1.1×10^6 CFU/reaction). P: positive control (1 μ l of genomic DNA, 10 ng/ μ l). N: negative control (distilled water).

to be captured by the MBL-coated magnetic beads. Despite this relatively high LOD, we nevertheless feel that the developed microfluidic system may be promising for detection of pathogens associated with NF; furthermore, it could be modified for use in microbial diagnostics of other bacteria or viruses.

The developed microfluidic chip aimed for end-point detection of these four bacteria. It is not designed for quantitative PCR and therefore could not be used for quantification of bacteria. However, even if that the capture rate is relatively low, the well-designed PCR primer is still sensitive enough to identify the respective bacteria and get fluorescence signals in this microfluidic system.

It is worth noting that once the bacteria were captured by magnetic beads, a washing process could be performed to wash away these PCR inhibitors (like Ca^{+2} or heparin) that must be removed prior to PCR by activating a micropump while applying an external magnetic field. Later, PCR reagents could be injected by activating another micropump. Therefore, the developed technology could be used for detecting four different necrotic fasciitis causing bacteria from clinical samples in the near future. Furthermore, in this study, we only used the lab-cultured bacteria to carry out the experiments to demonstrate that the bacteria isolation and detection can be integrated in single chip. According to our previous experience,¹¹ the LOD for

FIG. 6. Limits of detection (LOD) for genes specific to four strains of NF-associated bacteria on an integrated microfluidic system. (a) LOD for the *mecA* gene specific to MRSA. Lane M: 100-bp ladder. Lanes 1–4: 10-fold serial dilutions of MRSA with an initial concentration of 5.4×10^9 CFU/ml (1.1×10^4 CFU/reaction). P: positive control (1 μ l of genomic DNA, 10 ng/ μ l). N: negative control (distilled water). (b) LOD for the *SNe* gene specific to SA. Lane M: 100-bp ladder. Lanes 1–4: 10-fold serial dilutions of SA with an initial concentration of 9.8×10^9 CFU/ml (2×10^4 CFU/reaction). P: positive control (1 μ l of genomic DNA, 10 ng/ μ l). N: negative control (distilled water). (c) LOD for the *vvhA* gene specific to VV. Lane M: 100-bp ladder. Lanes 1–4: 10-fold serial dilutions of VV with an initial concentration of 6.8×10^8 CFU/ml (1.4×10^3 CFU/reaction). P: positive control (1 μ l of genomic DNA, 10 ng/ μ l). N: negative control (distilled water). (d) LOD for the *Act* gene specific to AH. Lane M: 100-bp ladder. Lanes 1–8: 10-fold serial dilutions of AH with an initial concentration of 5.7×10^9 CFU/ml (1.1×10^4 CFU/reaction). P: positive control (1 μ l of genomic DNA, 10 ng/ μ l). N: negative control (distilled water).

using real samples may be degraded (about 20%–30% higher) depending on the capture rates of the magnetic beads in the real samples.

IV. CONCLUSIONS

The automatic diagnosis of four pathogens associated with necrotizing fasciitis has been demonstrated by using an integrated microfluidic system composed of micropumps, microvalves, and micromixers. The entire molecular diagnosis process including bacteria isolation, lysis, nucleic acid amplification, and optical detection could be automated on this developed system. Mannose binding lectin-coated magnetic beads were demonstrated as an efficient universal bacteria probe to isolate bacteria in samples. Rapid identification of four life-threatening pathogens (including *Vibrio vulnificus*, *Aeromonas hydrophila*, and methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*) has been successfully demonstrated. The limits of detection for these four bacteria were experimentally found with comparable or even superior performance when compared with manual operation. The developed microfluidic system may

be promising for detection of pathogens associated with necrotizing fasciitis and could be extended for other applications for fast diagnosis of viruses and bacteria.

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