Vancomycin-resistant gene identification from live bacteria on an integrated microfluidic system by using low temperature lysis and loop-mediated isothermal amplification

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Vancomycin-resistant *Enterococcus* (VRE) is a kind of enterococci, which shows resistance toward antibiotics. It may last for a long period of time and meanwhile transmit the vancomycin-resistant gene (*vanA*) to other bacteria. In the United States alone, the resistant rate of *Enterococcus* to vancomycin increased from a mere 0.3% to a whopping 40% in the past two decades. Therefore, timely diagnosis and control of VRE is of great need so that clinicians can prevent patients from becoming infected. Nowadays, VRE is diagnosed by antibiotic susceptibility test or molecular diagnosis assays such as matrix-assisted laser desorption/ionization/time-of-flight mass spectrometry and polymerase chain reaction. However, the existing diagnostic methods have some drawbacks, for example, time-consumption, no genetic information, or high false-positive rate. This study reports an integrated microfluidic system, which can automatically identify the vancomycin resistant gene (*vanA*) from live bacteria in clinical samples. A new approach using ethidium monoazide, nucleic acid specific probes, low temperature chemical lysis, and loop-mediated isothermal amplification (LAMP) has been presented. The experimental results showed that the developed system can detect the *vanA* gene from live *Enterococcus* in joint fluid samples with detection limit as low as 10 colony formation units/reaction within 1 h. This is the first time that an integrated microfluidic system has been demonstrated to detect *vanA* gene from live bacteria by using the LAMP approach. With its high sensitivity and accuracy, the proposed system may be useful to monitor antibiotic resistance genes from live bacteria in clinical samples in the near future. *Published by AIP Publishing.* [<http://dx.doi.org/10.1063/1.4977439>]

NOMENCLATURE

AST	antibiotic susceptibility test
bp	Base-pair

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CDC	Centers for Disease Control and Prevention
CFU	Colony formation unit
DNA	Deoxyribonucleic acid
EMA	Ethidium monoazide
LAMP	Loop-mediated isothermal amplification
LOD	Limit of detection
MALTI-TOF	Matrix-assisted laser desorption ionization/time-of-flight mass spectrometry
PCR	Polymerase chain reaction
VRE	Vancomycin resistance <i>Enterococcus</i>
16S rRNA	16S ribosomal ribonucleic acid

I. INTRODUCTION

Antibiotics were first discovered in 1928 and revolutionized the field of medicine in the 20th century (Tan and Tatsumura (2015)). However, misuse of antibiotics creates a group of antibiotic-resistant bacteria. Among them, vancomycin-resistant *Enterococcus* (VRE) has caused serious concerns for public health. *Enterococci* are Gram-positive cocci that often exist in the gastrointestinal tract (Murray (1990)). There are a variety of *Enterococcus* species that have been identified, but only few are related to human infections, namely, *E. faecalis* (90%), *E. faecium* (5% to 15%), and *E. gallinarum* (less than 5%) (Gordon *et al.* (1992); Lewis and Zervos (1990); Moellering (1992); Patterson *et al.* (1995); and Ruoff *et al.* (1990)). Traditionally, penicillin or ampicillin in combination with aminoglycoside is used to treat *Enterococci* infections. Furthermore, vancomycin is further used in hospitals for treating *Enterococci* with penicillin or ampicillin or aminoglycoside resistance (Tsau *et al.* (1993)). This may lead to a more serious issue, i.e., the rise of VRE because there is few or no proper drug that can be used to cure VRE. VRE was first identified in Europe in 1986 (Leclercq *et al.* (1988) and Uttley *et al.* (1988)) and was found to be extremely fatal for patients in the intensive care units (Uttley *et al.* (1988)). The bacteremia mortality rate of patients with VRE reaches 40%, which is much higher than that of patients with vancomycin susceptible *Enterococcus* (Edmond *et al.* (1996)). Not only is VRE difficult to treat but it is also easily transmitted due to its intrinsic resistance to several commonly used antibiotics and its tolerance to the extreme environment (Cetinkaya *et al.* (2000)). It can survive in a dry environment from at least 7 days up to 4 months. Centers for Disease Control and Prevention (CDC) reported that in the United States the percent of all *Enterococcus* health-associated infections resistant to vancomycin increased from 0.3% to 40% in the past two decades (CDC (1993) and CDC (2013)). In other words, one out of three hospitalized patients suffers from VRE infections. To curb this trend, The World Health Organization (WHO) has announced antibiotic resistance a “global threat” to arouse global attention and CDC also calls for the development of “Identification/Antimicrobial Susceptibility Test” (Mendelson and Matsoso (2015)).

Antibiotic susceptibility test (AST) provided by the Clinical and Laboratory Standards Institute (CLSI (2013)) is the gold standard for antibiotics-resistant bacteria monitoring in most hospitals. However, AST involves complicated and time-consuming processes (20–72 h), which makes timely monitoring impossible (Dalgaard *et al.* (1994)). Moreover, AST might not diagnose bacteria with resistance to antibiotics accurately when the number of antibiotics-resistant bacteria is far less than that of bacteria with susceptibility to antibiotics so that the AST results may demonstrate “susceptible” and the potential threat of antibiotic resistance may be easily ignored (Hiramatsu (2001)). As alternatives, rapid molecular diagnostic assays with high sensitivity and specificity are used to acquire the genetic information of antibiotics-resistant bacteria (Clark *et al.* (2013); Patel *et al.* (1997); and Strommenger *et al.* (2003)).

Six different types of vancomycin-resistant *Enterococcus* including *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, and *vanG* have been reported (Murray (1997)). Only *vanA*, *vanB*, and *vanC* have been observed in general clinical practice so far (Pootoolal *et al.* (2002)). Among them, *vanA* is the most predominant one. Furthermore, laboratory data have demonstrated that *vanA* can be transmitted to *Staphylococcus aureus* (Noble *et al.* (1992)). If *vanA* gene is transmitted to

methicillin-resistant *Staphylococcus aureus* spontaneously in clinical operations, it may become a super-bacterium with no drug to treat. Therefore, timely monitoring of VRE carrying *vanA* is urgent.

Matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF) and polymerase chain reaction (PCR) (Clark *et al.* (2013); Patel *et al.* (1997); and Strommenger *et al.* (2003)) have been reported to identify the antibiotic-resistant gene in 2–6 h. However, the above-mentioned methods have some disadvantages. For example, in addition to human operational errors and potential contamination, samples have to be pre-treated for obtaining deoxyribonucleic acid (DNA) with high quality so that the assays may not be interfered. Another disadvantage is that these methods cannot distinguish live or dead bacteria because DNA remains intact even after mortality in the bacteria. Therefore, MALDI-TOF and PCR which use DNA as detection targets still obtain positive results from dead bacteria. Thus, clinicians might treat dead bacteria with antibiotics. Not only does it increase medical cost and prolong the treatment period, but it also might give rise to a new class of antibiotic-resistant bacteria. Previously, our group has developed an integrated microfluidic systems for live bacteria detection by using vancomycin-coated magnetic beads for sample pre-treatment, ethidium monoazide (EMA) for live or dead bacteria distinction, and PCR or nanogold probes for gene identification (Chang *et al.* (2014), (2015); and Liu *et al.* (2012)) with detection limits of about 10^2 colony formation units (CFUs) per reaction. However, these systems cannot be used to detect VRE because vancomycin-coated magnetic beads have no affinity to VRE due to the mutated peptides on the cell wall (Cetinkaya *et al.* (2000)). In addition, the detection limits should be pushed to lower concentrations so that the developed system can be used in extreme cases of infection such as bacteremia or root canal procedure during which the number of bacteria should be as less as possible. Therefore, an integrated microfluidic system including a microfluidic biochip and an integrated control system capable of detecting the *vanA* gene from live VRE was developed in this study. New nucleotide probes capable of isolating genomic DNA from live VRE were developed and tested for sample pre-treatment. Loop-mediated isothermal amplification (LAMP) (Kim *et al.* (2014) and Notomi *et al.* (2000)), which has high sensitivity than its counterpart PCR, was used to amplify the target gene. As VRE is one of the major antibiotic-resistant bacteria found in periprosthetic joint infections (Legout and Senneville (2013); Rasouli *et al.* (2012); and Zimmerli *et al.* (2004)), joint fluids were used in this study to validate the performance of the developed system under clinical environment. However, there are too many proteins in the joint fluid samples. The previously employed thermal lysis (Chang *et al.* (2013)) cannot perform sample pre-treatment because proteins in the joint fluid samples will be denatured at 95 °C. Therefore, we also developed a low temperature chemical lysis assay to lyse bacteria in protein-rich samples so that the denaturation and clotting of proteins could be prevented. The experimental results showed that the proposed system is highly specific to VRE carrying *vanA* gene with a detection limit as low as 10 CFU. All processes including live or dead bacteria distinction by using EMA, sample pre-treatment, nucleotide acid amplification, and fluorescent signal detection and analysis can be completed on an integrated microfluidic platform within 1 h. This is the first time that a microfluidic system capable of detecting *vanA* gene from live VRE by using the LAMP assay has been demonstrated. It might be useful for timely monitoring VRE carrying *vanA* gene in clinical setting in the near future.

II. MATERIALS AND METHODS

A. Experimental procedures

The experimental procedures of the proposed approach are illustrated in Figure 1. Before detection, distilled water as wash buffer and reagents for LAMP positive and negative controls were pre-loaded in the corresponding chambers on the microfluidic chip. Note that mineral oil (Sigma-Aldrich Corp. LLC, USA) has to be loaded on the top of reagents for LAMP positive and negative controls to avoid possible evaporation during the thermal process. The constituents and volumes of reagents for LAMP positive and negative controls are listed in Table I. Then, the sample was first treated with 1 μ l of 1 mg/ml EMA (Molecular Probes[®], USA) in the

FIG. 1. The experimental procedure of the integrated microfluidic system, including (a) ethidium monoazide (EMA) treatment, (b) low temperature chemical lysis and nucleotide probe hybridization, (c) target DNA isolation by using nucleotide probes coated magnetic beads and washing step, and (d) loop-mediated isothermal reaction (LAMP) and fluorescence detection.

reaction chamber and exposed under visible light (18 W, 1200 lm) for 5 min (Figure 1(a)). According to our previous study (Chang *et al.* (2014)), EMA can distinguish live bacteria from dead ones after 5-min of light exposure. EMA can be employed to distinguish between live and dead bacteria as it can penetrate into the broken cell walls of dead bacteria and intercalate into DNA irreversibly (Chang *et al.* (2014); (2015); and Liu *et al.* (2012)). Then, reagents for low temperature chemical lysis (detail information for low temperature chemical lysis was described in Section II D) and 5 μ l of 16S ribosomal ribonucleic acid (16S rRNA) nucleotide probe-coated magnetic beads (7×10^5 beads/ μ l) (Bergin *et al.* (2010)) were added into the reaction chamber (Figure 1(b)). In this step, the EMA-intercalated double-stranded DNA (dsDNA) of dead bacteria cannot be captured by 16S rRNA nucleotide probe-coated magnetic beads. Therefore, there will be not any LAMP product resulting from dead bacteria. On the contrary, non-EMA-intercalated dsDNA in live bacteria could be successfully captured by 16S rRNA nucleotide probe-coated magnetic beads so that it can be amplified by the following LAMP procedure. After low temperature chemical lysis at 37 °C for 2.5 min and magnetic beads capturing at 56 °C for 15 min, a magnet was placed underneath the microfluidic chip to collect DNA-magnetic bead complexes (Figure 1(c)). After washing for two times by using 100 μ l distilled

TABLE I. Constituents of LAMP reagents. Betaine: Sigma-Aldrich Corp. LLC, USA. *Bst* polymerase and buffer: New England Biolabs, USA. dNTP: New England Biolabs, USA. Sybr Green: AppliChem GmbH, German.

Van A LAMP		Volume (μ l)		
		Negative LAMP reagents	Positive LAMP reagents	Sample LAMP reagents
ddH ₂ O		12	11	12
5M Betaine		4	4	4
10 $\times$ <i>Bst</i> buffer		3	3	3
10 mM dNTP		1	1	1
Primer	F3 (10 μ M)	1	1	1
	B3 (10 μ M)	1	1	1
	FIP (20 μ M)	3	3	3
	BIP (20 μ M)	3	3	3
<i>Bst</i> polymerase		1	1	1
Sybr Green		1	1	1
Plasmid for positive control		0	1	0
Total		30	30	30

water (Figure 1(d)), 30 μl of LAMP reagents for *vanA* detection with fluorescent dye was pumped into the reaction chamber (Figure 1(e)). Note that the reagents for positive and negative controls were also pumped into their corresponding chambers simultaneously at this step for quality control. The fluorescence signals resulting from LAMP products were then detected by a photomultiplier tube sensor in the integrated control system and the fluorescence signals were shown on a touch screen during the LAMP process for user monitoring (Figure 1(f)). At the end, it showed the detection results so that the user could easily identify if there is any *vanA* gene in the samples.

B. Chip design

A microfluidic chip designed to perform the entire process mentioned in Section II A is shown in Figure 2(a). This microfluidic chip was fabricated by using a hot-embossing method in collaboration with Jabil Circuit Inc., Ltd., Taichung, Taiwan. It was composed of two silica-gel layers to create functional micro-components for liquid handling such as pneumatically driven micro-pumps, micro-mixers, normally closed micro-valves, and micro-chambers. These two silica-gel layers were bonded together by oxygen plasma treatment and sealed with a glass substrate at the bottom for preventing liquid leakage and providing support. Please note that some parts on metal template molds corresponding to the normally closed micro-valves and thin film membranes were with surface roughing treatment. Therefore, these micro-components will not be bonded with the glass substrate during the oxygen plasma treatment process so that they can be uplifted for liquid handling. Besides, the microfluidic chip was framed by a plastic chip for two reasons. First, the plastic frame is helpful for positioning the microfluidic chip in the integrated control system. Second, clear labeling for each chamber was printed on the plastic frame so that the user will not load the wrong reagents. As shown in Figure 2(b), WB, SLR, PLR, NLR, BS, PC, and NC stand for wash buffer chamber, sample LAMP reagent, positive control LAMP reagent, negative control LAMP reagent, beads and sample chamber, positive control reaction chamber, and negative control reaction chamber, respectively. The dimensions of the microfluidic chip were measured to be 65 mm $\times$ 88 mm $\times$ 7 mm. The detail information

FIG. 2. (a) Exploded view and (b) a photograph of the integrated microfluidic chip. The dimensions of the chip were measured to be 65 mm in width, 88 mm in length, and 7 mm in height. (c) Operating procedures of the microfluidic control and detection system including bio-sample and reagents loading, microfluidic chip loading, real-time fluorescence detection, and fluorescent signal analysis and diagnosis.

about the working principle and performance of the normally closed micro-valves and micro-pumps used in the study can be found in our previous works (Weng *et al.* (2011) and Chang *et al.* (2014)). Please note that entire detection procedures including bio-sample and reagents loading, microfluidic chip loading, real-time fluorescence detection, and fluorescent signal analysis and diagnosis can be completed automatically on the microfluidic control and detection system by just one single touch (Figure 2(c)).

C. Preparation of EMA

Ethanol was used to dissolve EMA (Molecular Probes™, Thermo Fisher Scientific, USA) to prepare a final concentration of 1 mg/ml and stored at -20°C before performing the diagnosis assay. Please note that EMA is a light-sensitive material. Therefore, it is important to keep it away from any light exposure.

D. Low temperature chemical lysis

The sample was first centrifuged at 12 000 rpm for 1 min. Then, the supernatant was discarded. Thirty-four μl of 50 mM ethylenediaminetetraacetic acid (AppliChem GmbH, German) was used to re-suspend the pellet. One μl of 1 $\mu\text{g}/\mu\text{l}$ lysostaphin (Sigma-Aldrich Corp., LLC, USA) was added to the reaction mixture with a final concentration of 10 ng/ μl .

E. Preparation of 16S rRNA nucleotide probe-coated magnetic beads

Two 5' amine group modified nucleotide probes, 16S rRNA F (5'ttttttttattagataccctggtagtccagcc) and 16S rRNA R (5'ttttttttgcgtatccccacctctcc) (Bergin *et al.*, 2010) were ordered from Sigma-Aldrich Co. LLC., USA and coated onto magnetic beads (Dynabeads® MyOne™ Carboxylic Acid, Invitrogen Corp., USA, 4×10^7 beads/ml) as per the following steps. Briefly, thirty μl of 100 μM 5' amine group modified nucleotide probes and 950 μl of magnetic beads were added into a 1.5-ml microcentrifuge tube. After mixing, 20 μl of 120 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Invitrogen Corp., USA) was added into the same 1.5-ml microcentrifuge tube and then incubated in the microcentrifuge tube at room temperature for 18 h. After washing with 0.02% Tween 20 (Sigma-Aldrich Corp., LLC, USA) and 0.1% sodium dodecyl sulfate (Sigma-Aldrich Corp., LLC, USA), 1 ml of 0.1 M of ethanol amine (Sigma-Aldrich Corp., LLC, USA) was used to block the magnetic bead surface where no nucleotide probes were occupied at room temperature for 1 h. Finally, 1 ml of distilled water was used to wash away the residual ethanol amine and suspended the nucleotide probes' magnetic beads. Note that the nucleotide probes' magnetic beads have to be stored at 4°C . The nucleotide probes' magnetic beads cannot be frozen at all times.

F. LAMP primers and LAMP reaction

The DNA sequence of *vanA* gene was obtained from the GenBank at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). LAMP primers specific to the *VanA* gene were designed by using Primer Explorer V4 software (<http://primerexplorer.jp/elamp4.0.0/index.html>) (Eiken Chemicals Co., Tokyo, Japan). The sequences of the *vanA* LAMP primers are as follows: F3 5'atactgtttgggggtgc, B3 5'ccgtgcatttttatccgg, FIP 5' ttccaatgtataacggctcgatttgag-gagcatgacgtatcg, BIP 5' cgaaatctggtgtatggaaatgtgagagtacagctgaatagcaa. The constituents of LAMP reagents are listed in Table 1 and the LAMP reaction was performed at 56°C for 1 h.

G. Electrophoresis

LAMP products were first analyzed by conventional slab-gel electrophoresis after the entire process to confirm consistency between the electrophoresis results and fluorescence signals. Two grams of agarose (MB755-0100, GeneDireX, USA) was dissolved in 100 ml Tris/borate/ethylenediaminetetraacetic acid buffer (Sigma-Aldrich Corp., LLC, USA) to form 2% agarose gel for slab-gel electrophoresis. After loading 5 μl of LAMP products with 1 μl of DNA loading

dye (Promega Corporation, USA) into the slab-gel, 100 V was applied to separate the DNA fragments for 40 min. Five-hundred ml of 50 mg/ml Ethidium bromide with a concentration of 50 mg/ml (Sigma-Aldrich Corp., LLC, USA) was then used to stain the LAMP products. After exposure under 302-nm ultraviolet light (UVTM transilluminator, UVP, Canada) for 2 s, the LAMP products can be observed on the slab-gel clearly. Note that one hundred base-pair (bp) DNA ladder (108 ng/ μ l, 5 μ l) (DM003-R500, GeneDireX, USA) was used to compare the size of the DNA fragments.

H. Clinical samples

The clinical samples used in this study were under the approval of Chang Gung Memorial Hospital Institutional Review Board (104-7148C, 103-6831B, and 104-2206C).

I. Microfluidic antibiotics-resistant gene detection system

A new microfluidic control and detection system was developed by Jabil Circuit Inc., Ltd., Taichung, Taiwan and shown in Fig. 3, in which all the above-mentioned experimental procedures could be carried out automatically. After loading LAMP reagents, bio-samples, wash buffer, and mineral oil into corresponding chambers on the LAMP LabChip (as indicated in the frame, Fig. 3(a)), the microfluidic chip was placed into the integrated control system and the diagnostic process was launched by just touching one button (Fig. 3(b)). The fluorescence signals were collected during the thermal reaction process and could be shown in a real-time format (Fig. 3(c)). The analyzed results (infection or non-infection) were displayed after the whole reaction was completed (Fig. 3(d)).

III. RESULTS AND DISCUSSION

A. Optimization of low temperature chemical lysis of live VRE diagnostic assay

The optimal condition for low temperature chemical lysis of live VRE was first explored in this study. 10^6 CFU of VRE bacteria were treated with different concentrations of lysostaphin at a volume of 34 μ l. Then, the temperature was elevated to the optimal working

FIG. 3. (a) Optimal working concentration for low temperature chemical lysis. 10^6 CFU of VRE bacteria were treated with lysostaphin with different concentrations at a volume of 34 μ l. Then, the temperature was elevated to the optimal working temperature for lysostaphin, which was 37 °C for 10 min and the samples were performed with all live VRE diagnostic process. The results showed that 10 ng/ μ l of lysostaphin is enough for low temperature chemical lysis. M: 100-bp ladder and N: negative control (distilled water). (b) Optimal working time for low temperature chemical lysis. 10^6 CFU of *E. faecalis* were treated with 1 μ g/ μ l lysostaphin to make a final concentration of 10 ng/ μ l. The temperature was elevated to 37 °C for different times, and the samples underwent the all on-chip process to determine the optimal working time for low temperature chemical lysis. The results demonstrated that as short as 2.5 min is sufficient for low temperature chemical lysis. M: 100-bp ladder and N: negative control (distilled water).

temperature for lysostaphin, which was 37 °C for 10 min and the samples underwent the all live VRE diagnostic process. As shown in Figure 3(a), a final concentration of 10 ng/ μ l in the reaction mixture was enough to carry out the low temperature chemical lysis. Therefore, 10 ng/ μ l was chosen as the optimal concentration for lysostaphin in this developed system.

Next, 10^6 CFU of *E. faecalis* were treated with 1 μ g/ μ l lysostaphin to achieve a final concentration of 10 ng/ μ l. The temperature was elevated to 37 °C for different time periods and the samples underwent the all on-chip process to determine the optimal working time for low temperature chemical lysis. The electrophoresis slab gel of these samples is shown in Figure 3(b). The results indicated that an operating period as short as 2.5 min is sufficient for low temperature chemical lysis. Compared to the high temperature lysis method (Chang *et al.* (2013)), this method further saved 2.5 min (5 min vs 2.5 min). Most importantly, the low temperature chemical lysis did not denature proteins in the clinical samples to cause clots because the working temperature is the same as that of human body.

B. Detection of live VRE using 16S rRNA nucleotide probe coated magnetic beads

The nucleotide probes tested in this section are universal probes for over 80% commonly found bacteria clinically because their sequences are complementary to the highly conserved region of the 16S rRNA genome (Bergin *et al.* (2010)). The limit of detection (LOD) of two nucleotide probes (R probe and F probe) was investigated in this study. 10^5 – 10^0 CFU of *E. faecalis* were spiked into un-infected joint fluid samples and used as the target of the proposed microfluidic system. The LOD results of two nucleotide probes are shown in Figures 4(a) and 4(b). It indicated that the LOD of the 16S rRNA R probe is better than that of the 16S rRNA F probe (10^1 CFU/reaction vs. 10^2 CFU/reaction). The 16S rRNA R probe (20 bps) is shorter than the 16S rRNA F probe (25 bps) for 5 nucleotides. Higher complementary pairing requirement might compromise the sensitivity so that the sensitivity of the 16S rRNA R probe is one order higher than that of the 16S rRNA F probe. The LOD of the 16S rRNA R probe (10^1 CFU/reaction) is better than that from previous studies (Chang *et al.* (2014), (2015)). The results indicated that the performance of live VRE detection assay with LAMP reaction is sufficient for prosthetic joint infection (PJI) diagnosis and can be used for surgery decision.

C. Specificity of vanA LAMP reaction

As mentioned previously, amongst the different kinds of VRE genes, *vanA*, *vanB*, and *vanC* are clinically common strains and *vanA* is the most widely spread one, which should be monitored closely due to its high vancomycin-resistant property and potential danger to cause vancomycin-resistant *Staphylococcus aureus*. Therefore, the proposed microfluidic system aims to detect *vanA* gene specifically. The specificity of the developed assay was explored by using different VRE clinical strains carrying *vanA*, *vanB*, and *vanC* and the results are shown in Figure 4(c). Samples containing thirty-four μ l of VRE clinical strains carrying *vanA*, *vanB*, and *vanC* bacteria of 10^6 CFU were tested following the experimental process mentioned in Section II A and analyzed by slab-gel electrophoresis for comparison. The results indicated that only VRE strains carrying *vanA* show LAMP signals but not VRE strains carrying *vanB* and *vanC*. The proposed assay is demonstrated to be highly specific to *vanA* gene detection.

D. Optimization of optical detection system on the integrated control system

After exploring the optimal conditions of the proposed assay, genomic DNA from VRE carrying *vanA* gene was used to verify the optical detection system of the integrated control system. As shown in Figure 5(a), the ratio between the signals from the positive control and the negative control (fluorescent signals) is over 3 when using 1 μ l of SybrGreen dye (Please see detail information in Table I). One μ l of Sybr Green dye was therefore chosen for the

FIG. 4. The limit of detection of the developed microfluidic system by using different nucleotide probes was determined. The results showed that the detection of 16S rRNA F was 10^2 CFU/reaction (a) and that of 16S rRNA R was 10^1 CFU/reaction (b). M: 100-bp ladder, N: negative control (distilled water), and others: 10-fold serial dilutions of VRE with an initial concentration of 10^5 CFU. (c) The specificity results of the proposed system showing that the probe was highly specific to VRE carrying *vanA* gene. N: negative control (distilled water), M: 100-bp ladder, 1: clinical strain 102 (*vanA* type), 2: clinical strain 107 (*vanA* type), 3: clinical strain M072300105 (*vanA* type), 4: standard strain 51299 (*vanB* type), 5: clinical strain M071300151 (*vanC* type).

proposed assay due to its good performance (signal-to-noise ratio is over 3) and relatively low cost (around 1 USD (United States dollar)/reaction). Figure 5(b) demonstrates the fluorescent signals collected when performing the whole process on the microfluidic chip with VRE (10^6 CFU). The ratio between the fluorescent signals of the sample to the negative control is over 3 (0.67 ± 0.03 V vs. 0.21 ± 0.1 V). This indicated that the whole process can be performed on the proposed system and the slab gel electrophoresis data further confirmed this result (Figure 5(c)).

E. Detection of live VRE on the proposed integrated microfluidic system from spiked samples

After exploring the feasibility of the whole process for detecting live VRE with the proposed system, a non-infected joint fluid sample spiked with VRE was further used to test the capability of the integrated microfluidic system in the clinical samples. The results of detecting bacteria from the spiked joint fluid samples are shown in Figure 5(d). The slab gel electrophoresis is shown in Figure 5(e) for confirmation. It showed that VRE in joint fluid samples could be detected by the proposed system successfully. This indicated that not only is the proposed system capable of performing live VRE detection successfully, but it also confirms its usage under the micro-environment of the joint fluid samples.

FIG. 5. (a) Optimization of SybrGreen usage for the proposed assay by genomic DNA from VRE. (b) Feasibility of the proposed live VRE detection system by using overnight VRE culture of 10⁶ CFU/reaction: fluorescent signals of LAMP products; (c) The feasibility results from gel electrophoresis. M: 100-bp marker; 1: negative control using ddH₂O; 2: Products of 10⁶ CFU/reaction overnight VRE culture. (d) Real-time fluorescent signals of LAMP products from spiked sample on the integrated control system. Red line: spiked sample, black line: positive control, and blue line: negative control (distilled water). (e) Electrophoresis results used to confirm fluorescent signals obtained from the integrated control system. M: 100-bp marker; N: negative control (distilled water); P: positive control; and S: spiked sample.

IV. CONCLUSIONS

Antibiotic-resistant bacteria have become a big threat globally. Some bacteria have resistance toward multiple types or classes of antibiotics. The lack of effective antibiotics results in undermining our ability to fight infectious diseases and manage the infectious complications. It is estimated that at least 2×10^6 people are infected with bacteria that are resistant to one or more of the antibiotics and at least 23 000 people die from these antibiotics-resistant infections in the United States each year (CDC (2013)). VRE is one of the notorious antibiotics-resistant bacteria. Not only does it prolong hospitalization periods, but it also easily transmits its antibiotics-resistant gene to create super bacteria. Among all subtypes of VRE, *vanA* is of crucial attention because of its potential to create super bacteria which have no drug to treat. It has been demonstrated that *vanA* can be transferred to *Staphylococcus aureus* in laboratory tests (Noble *et al.* (1992)) and indeed some wild-type of methicillin-resistant *Staphylococcus aureus* also having vancomycin-resistance has been reported (Hiramatsu (2001)). Therefore, timely and accurate detection of *vanA* gene for monitoring and controlling purpose is of crucial need.

Although the gold standard of identifying antibiotics-resistant bacteria is AST, the lengthy operation and detection time (at least 72 h after sampling) makes timely monitoring impossible. Some molecular diagnostic assays such as MALDI-TOF, PCR, and LAMP have been developed to detect *vanA* gene faster. However, none of these assays can distinguish live bacteria from dead bacteria and may generate false-positive results. To solve the above-mentioned issues, an integrated microfluidic system capable of detecting *vanA* gene from live VRE in clinical samples was presented in this study. This system includes EMA for live/dead bacteria distinction, nucleic acid specific probes for target DNA isolation, low temperature chemical lysis for

bacteria lysis without denaturing proteins in protein-rich samples, and LAMP for nucleic acid amplification and detection. The experimental results showed that the whole diagnosis assay could be performed within 1 h after sampling with a LOD of 10 CFU, which makes timely and sensitive detection possible. Furthermore, the estimated prices of the proposed system are 5000 USD for one microfluidic control and detection system and 5 USD for one disposable chip and reagents. When compared with existing molecular diagnosis products currently in the market, the estimated price is very competitive. Therefore, the proposed system might serve as a promising tool for immediate clinical decision making and choice of antibiotics in hospital routine procedures in the near future.

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