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A microfluidic device for antimicrobial susceptibility testing based on a broth dilution method ☆

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ABSTRACT

Bacterial resistance to antimicrobial compounds is increasing at a faster rate than the development of new antibiotics; this represents a critical challenge for clinicians worldwide. Normally, the minimum inhibitory concentration of an antibiotic, the dosage at which bacterial growth is thwarted, provides an effective quantitative measure for antimicrobial susceptibility testing, and determination of minimum inhibitory concentration is conventionally performed by either a serial broth dilution method or with the commercially available Etest® (Biomerieux, France) kit. However, these techniques are relatively labor-intensive and require a significant amount of training. In order to reduce human error and increase operation simplicity, a simple microfluidic device that can perform antimicrobial susceptibility testing automatically via a broth dilution method to accurately determine the minimum inhibitory concentration was developed herein. As a proof of concept, wild-type (ATCC 29212) and vancomycin-resistant *Enterococcus* cells were incubated at five different vancomycin concentrations on-chip, and the sample injection, transport, and mixing processes occurred within five reaction chambers and three reagent chambers via the chip's automatic dispensation and dilution functions within nine minutes. The minimum inhibitory concentration values measured after 24 h of antibiotic incubation were similar to those calculated using Etest®. With its high flexibility, reliability, and portability, the developed microfluidic device provides a simple method for antimicrobial susceptibility testing in an automated format that could be implemented for clinical and point-of-care applications.

1. Introduction

Antibiotics have been routinely used to fight bacterial infections for around 70 years. In the first several decades of their use, a relatively small percentage of bacterial species acquired antibiotic resistance via random mutation. However, indiscriminate utilization of antibiotics in human medicine and animal husbandry in recent years has accelerated the selection process, leading to a rise in the number of antibiotic-resistant bacteria. The consequent increase in difficulty in fighting bacterial infections has resulted in longer hospitalization periods and worse prognoses, both of which are associated with increases in medical expenses (El-Tahawy, 2004; Levy and Marshall 2004). Indeed, the World Health Organization (WHO) highlighted antibiotic resistance as a global concern (Center for Disease Dynamics, 2015; Mendelson and Matsoso, 2015). In the WHO action plan, optimization of antibiotic administration was promoted as one means of slowing the increase in the number of antibiotic-resistant bacteria. It will therefore be a key to rapidly and accurately determine the presence and concentration of such antimicrobial resistant microbes.

The current protocol for antibiotic susceptibility testing (AST), which is provided by the Clinical and Laboratory Standards Institute (CLSI, 2013), involves first identifying the bacterial flora present by culturing bacteria isolated from patient specimens in liquid broth via broth microdilution (BMD) or via disk diffusion on agar plates (for the commercially available Etest®) (Brown and Brown, 1991) to measure the minimum dosage of antibiotic that inhibits bacteria growth (i.e., the minimal inhibitory concentration, MIC) (Jorgensen

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Nomenclature

AST	Antimicrobial susceptibility testing
BHI	Brain heart infusion
BMD	Broth microdilution
CCD	Charge-coupled device
CFU	Colony-forming unit
CLSI	Clinical and Laboratory Standards Institutes
CNC	Computer numerical control
DNA	Deoxyribonucleic acid
EMVs	Electro-magnetic valves
HAI	Hospital-acquired infection
MALDI-TOF MS	Matrix-assisted laser desorption ionization/time-of-flight mass spectrometry
MCA	Melting curve analysis
MIC	Minimum inhibitory concentration
OD	Optical density
PCR	Polymerase chain reaction
PDMS	Polydimethylsiloxane
PI	Propidium iodide
PMMA	Poly-methyl methacrylate
VRE	Vancomycin-resistant *Enterococcus*
WHO	World Health Organization
dsDNA	Double-stranded DNA

and Ferraro, 2009). These standardized methods are relatively labor-intensive, time-consuming (20–72 h), and insensitive (with a limit of detection of 10^7 CFU/mL) (Dalgaard et al., 1994). Currently, several automated instruments, including VITEK®2 (BioMérieux, USA), BD Phoenix (BD, USA), and MicroScan WalkAway® (Beckman Coulter, USA), can carry out AST in under 16 h on multiple samples using highly sensitive optical detection systems (Jorgensen and Ferraro, 2009; Kobayashi et al., 2004). However, they are relatively expensive and bulky. Alternatively, matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS) and multiplex polymerase chain reaction (PCR) detection (Clark et al., 2013; Patel et al., 1997; Strommenger et al., 2003) can rapidly identify bacteria and document the presence of several antibiotic-resistant genes in 1–5 h. However, they do not directly provide MIC data. Therefore, a quick and accurate bacterial identification method featuring a MIC test is of great need and could lead to proper patient antibiotic treatment (van Belkum and Dunne, 2013).

Recently, microfluidic technology has successfully miniaturized a number of biomedical devices, allowing for 1) the need for only small sample and reagent volumes, 2) more precise liquid handling, 3) affordable costs, 4) automation, 5) high throughput, and 6) improved detection. Owing to these favorable properties, microfluidics could be an ideal platform for advanced bacterial diagnostics. Indeed, previous studies have reported several microfluidic droplet-based microfluidic system (Baraban et al., 2011; Kang et al., 2014; Liu et al., 2016), an inertial microfluidics-controlled chip (Hou et al., 2015), a single-cell chip (Choi et al., 2014), an electrochemical sensing chip (Besant et al., 2015). Some of the chip designs were based on the broth dilution method, including a dilution/gradient generation chip (Mohan et al., 2015; Kim et al., 2015; Derzsi, et al. 2016), an antimicrobials pre-coated microchannels (Matsumoto et al., 2016), and a colorimetric-based MIC chip (Cira et al., 2012). Most of these microfluidic devices overcome the aforementioned limitations of conventional AST. However, the dissemination of these diagnostic platforms might be limited since they all require expensive instrumentation or complicated imaging software. For instance, high-resolution, charge-coupled device (CCD), which is generally coupled with microfluidic droplet systems or a gradient chip for bacterial detection, was inevitable. Also, the accuracy of imaging analysis is still limited due to the fact that various bacteria with different sizes, shapes, motilities and growth rates may exist during AST. Furthermore, the chips for AST/MIC analysis or single-cell chips for analysis of single bacteria are relatively difficult to fabricate. Finally, most of chips/devices still require several steps of manual operation and could not be classified as fully automated MIC/AST systems. Thus, there are still challenges to overcome and improvements to be made for MIC-calculating microfluidic devices to see widespread clinical usage.

To overcome these hurdles, a simple microfluidic device was designed herein to automatically determine antibiotic MIC values. In this system a pneumatically-driven micro-pump and normally-closed micro-valves transported reagents and samples within the microchambers, and five different concentrations of antibiotics could be transported to the reaction chambers in a proportionally- or serially-diluted manner. Strains of vancomycin-resistant *Enterococcus* (VRE), a type of bacteria that has caused a large number of severe, hospital-acquired infections (HAI) in the United States and Asia (Handwerger et al., 1993; Lu et al., 2012; Murray, 2000; Willems et al., 2005), were used as the biological material for the proof of concept, and reproducible MIC data across four VRE genotypes from 14 clinical strains were obtained. This chip-based technology has several notable advantages over the current gold standard for AST, the Etest®, and could greatly benefit clinicians and researchers seeking to rapidly determine the presence of antibiotic-resistant bacterial strains. It is the first time that AST was automated on an integrated microfluidic system by utilizing the broth dilution method provided in CLSI guidelines (CLSI, 2013). Not only does the design and fabrication of this microfluidic chip is relatively simple and easy for batch production, but the device also reduces complicated manual operation and errors. In this device, robust normally-closed micro-valves effectively blocked the contamination and interference of samples while bacterial growth, as well as improved the accuracy of liquid injection. The circular micro-pump in the center and circular arrangement of chambers could minimize the dead volume and chip size of the microfluidic device. Compared with clinical methods of AST, the developed device increases the accuracy and precision of MIC that provides effective treatment portfolios to clinicians. This microfluidic device with high flexibility and reliability could provide a simple and user-friendly platform for the AST and could be implemented for clinical and point-of-care applications.

2. Materials and methods

2.1. Antibiotic and biological material preparation

The vancomycin stock solution was prepared according to CLSI guidelines. Briefly, vancomycin (Sigma-Aldrich, USA) was dissolved in deionized water at a final concentration of 10 mg/mL and stored at −20 °C prior to usage. Next, the stock solution was diluted in pH-dependent colorimetric broth to the target concentration used in the reactions. It is one of the unique features of this study as the interval of diluted concentration is changeable by switching two operation modes (proportional and serial dilution modes) as long as preparing the suitable initial concentration of antibiotic solution. Vancomycin-susceptible *Enterococcus faecalis* strain 29212 was purchased from ATCC (Manassas, VA, USA). According to their resistance to vancomycin, *Enterococcus* can be