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Resazurin reduction based colorimetric antibiogram in microfluidic plastic chip

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ABSTRACT

When drug-resistant bacteria pose a threat, a simple and inexpensive method for identifying their presence and quickly determining the right antibiotic for treatment, is critical. A simple optical detection technique combined with microfluidic technology will have great potential for configuring a portable antibiogram device. We report a colorimetric antibiogram method, based on viability-dependent resazurin dye reduction (intense blue colour changing to pink and leuco). The antibiogram protocol was developed initially in a 96-well plate format. This was tested using 20 clinical bacterial isolates against the minimum inhibitory concentration (MIC) of four antibiotics after 6 h of incubation. The antibiogram results obtained colorimetrically or visually were matched and confirmed with those of the liquid turbidity method (LTM). This antibiogram protocol was then transferred to a Poly(methyl methacrylate) (PMMA) plastic microfluidic chip containing 40 micro wells (8 rows and 5 columns, 20 μl volume per well). These microfluidic units can serve as disposable devices for portable instrumentation. The plastic microfluidic chip antibiogram showed 98% match with what was obtained in the 96-well plate method. The microfluidic chip offers a novel, compact and inexpensive platform technology for testing/screening of drug-resistant bacteria, and determining their antibiogram. This approach will lead the way for developing portable and low-cost point-of-care antibiotic susceptibility testing instrumentation for the future.

1. Introduction

The emergence of multidrug resistant pathogenic bacteria is of serious concern in the control of bacterial diseases. With the looming threat of the proliferation of multidrug-resistant bacteria, testing for antibiotic resistance is in considerable demand, but there is a lack of convenient and inexpensive rapid methods [1,2]. Normally, bacterial growth is measured by the turbidity as optical density (O.D.) of a culture at 600 nm [1], as in the case of determination of the minimum inhibitory concentration (MIC) of antibacterial compounds. Clinically, Kirby–Bueur disc diffusion method (DDM) is popular for testing antibiotic resistance, but prone to subjective errors, especially false-positives [3]. This could lead to unnecessary use of antibiotics. Furthermore, such methods are time-consuming and not adaptable for high throughput instrumentation, which is a necessity to handle a large number of dangerous clinical samples. The existing approaches in this direction are extremely expensive and are based on Raman spectroscopy and polymerase chain reaction [4,5].

A simple colorimetric method suited for automated instrumentation will be ideal for the antibiogram determination of a large number of samples, even in peripheral laboratories. Resazurin is a useful water-soluble dye in this regard. In the first stage, intensely blue coloured resazurin is converted to pink coloured resorufin by the loss of one oxygen atom and this reaction is not reversible by atmospheric oxygen. However, the pink coloured resorufin is further reduced to a colourless hydroresorufin in the second stage of reduction. This reaction is however reversible by atmospheric oxygen [6,7]. Resazurin is stable in an aqueous medium, but rapidly reduces in the presence of living cells. Resazurin has been used as an indicator for cellular growth, cell viability and toxicity [8]. In a recent study, resazurin based growth detection and antibiotic resistance testing methods have been developed for tuberculosis in a microtitre plate format and high-throughput instrumentation [8,9]. The necessity to develop easy-to-perform, miniature systems built with bio-degradable and disposable materials is necessary futuristic developments given the ever increasing incidence of multi-drug-resistance among a variety of microbes. This is our impetus to develop the methodology in microfluidic format.

Microfluidics is the science and technology of systems that manipulate or process small (in microlitres) amounts of fluids, utilizing channels with dimensions of tens to hundreds of micrometres [10]. The advantages of using microfluidics for fluid manipulation and processing include low sample/reagent volume,

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faster response times, low-sample wastage, cost efficacy (in terms of sample usage), and the possibility of developing disposable devices. Life sciences, analytical chemistry, biology, and the study of fluid transport are the main application fields of microfluidics [10]. The new application of microfluidics for antibiogram is particularly attractive because of the benefit of compactness and portability coupled with better throughput at a reduced cost.

Early microfluidic devices were mainly fabricated using silicon or glass, because the fabrication processes, photolithography and etching technologies, for these two materials were well established in the microelectronics industry [11–13]. Devices fabricated with silicon or glass require an expensive infrastructure. Consequently, silicon or glass based microfluidic systems can be expensive for many applications [14]. Therefore, the attention has shifted to plastics and polymers as the materials of choice. Plastics offer wider flexibility in processing techniques, and therefore, they render themselves to lower fabrication costs and reduced process complexity [14,15]. Plastic substrates, such as Poly(methyl methacrylate) (PMMA), provide a broad range of physical and chemical material properties for biochemical applications of microfluidics. PMMA is a thermoplastic material and has excellent optical transparency in visible light wavelengths. PMMA offers several advantages, such as low cost, ease of fabrication, and rapid prototyping [14].

In this study, the clinical isolates of pathogens were tested for their antibiotic resistance with various antibiotics in 96-well microtitre plates and a microfluidic PMMA chip respectively. The results, both visual and O.D., from the 96-well plate and microfluidic chip have been compared with the standard turbidity results to validate the utility of microfluidics in developing inexpensive and disposable antibiogram devices.

2. Materials and methods

2.1. Bacterial strains and growth condition

The bacterial strains used in this study were clinical isolates of diarrhoeagenic strains of *Escherichia coli*, *Shigella flexneri*, *Shigella boydii*, *Shigella sonnei* and *Uropathogenic E. coli* [3,16]. The bacterial strains were grown in Luria–Bertani broth in an incubator shaker at 180–200 rpm at 37 °C overnight (or for stipulated time spans).

2.2. Microfluidic plastic chip

The PMMA utilized in this research is OPTIX® (commercial medical-grade Acrylic) from Plaskolite. The PMMA, which normally comes in 12 in. × 12 in. (2 mm thick) sheets, was cut into 3 in. × 3 in. using a VersaLaser 3.60 CO_2 laser cutter/engraver [17]. (A laser cutter/engraver is a computer controlled cutting/engraving system using CO_2 laser as the cutting/engraving tool. By adjusting the laser power the instrument can be set to create a known depth of engraving or completely slice the sample. Typically these types of laser cutters are used in garment, footware and leather industry. In the recent times, VersaLaser is producing these instrumentations with dimensional and positional accuracy in the range of few tens of micrometres making it an ideal instrument to create microfluidic devices using plastic substrates [15].) The 3 in. × 3 in. substrates were directly patterned using the laser cutter, which ablates the PMMA to create the microfluidic channel pattern. The design consisted of reservoirs connected to each other via a microchannel. In this preliminary design, access holes were incorporated in each reservoir, so that the samples can be introduced individually into each reservoir. Fig. 1(a) shows the details of the design. A blank, 3 in. × 3 in. PMMA slide, is used as a backing plate for the laser-cut PMMA substrate, and these two units were bonded together to form the microfluidic chip. The exact bonding procedure is described in the following paragraph. The above design of the sample reservoir along with the connecting channels is used in our approach, in order to enable the flow of the medium and sample, in field trials where the reagents can be incorporated inside the chip, as dry powder for the longer shelf life of the functional chip. The VersaLaser 3.60 CO_2 laser cutter setting to produce the chip, is listed in Table 1.

Table 1
Laser cutter settings for patterning the PMMA.

Feature	Speed	Power	PPI
Channel	20%	20%	1000
Reservoir	38%	20%	1000
Access holes	80%	20%	1000

The patterned channels were (approximately) 215 μm wide at the top and 157 μm deep on an average, with a Gaussian channel shape. The Gaussian shape is typical of these types of patterning methods. Fig. 1(b) shows the Alphastep 500 profilometer (Tencor Instruments Inc.) measurements for the channel profile and dimensions. Furthermore, the reservoirs were measured to be 800 μm deep. Prior to bonding, the patterned PMMA samples were annealed in an oven at 80 °C for 1 h. This annealing step reduces the stress induced by laser during the laser-cutting process step. To clean the laser patterned PMMA samples, they were first rinsed with methanol and then placed in an Ultrasonic iso propyl alcohol (IPA) bath for 10 s. In this research, the optical transparency of the final microfluidic device is of paramount importance, which restricted the use of conventional thermal press for microfluidic bonding. Therefore, we developed a novel cost-effective bonding method using liquid PMMA and a transparency sheet to produce the enclosed channels. A thin layer of 950A11 PMMA (Microchem, USA) was spun on a clean over-head projector transparency sheet at 3500 rpm for 20 s. The transparency sheet (PMMA layer down) was then gently placed on the patterned PMMA samples. The samples were then kept, undisturbed for 1 h to ensure that the liquid PMMA sandwich layer dries and seals bottom side of the patterned grooves and produce the enclosed reservoir and connecting channels. To reinforce the bonding, a blank PMMA cover slide was glued to the transparency side of the sample. In this fabrication process, a transparency sheet was used as an intermediate layer for bonding because its flexibility enables the liquid PMMA layer to properly follow the topography of the patterned PMMA sample and seal the channels. Additionally, the transparency sheet is extremely lightweight, which prevents the liquid PMMA from getting into the channels. Fig. 1(c) illustrates the fabrication process flow for the plastic microfluidic chip. Once the glue dried, a coloured dye was passed through the channels to visually check for leaks. If the bonded microfluidic unit was found leak-free, de-ionized water was flushed through the channels to clear the coloured dye. Fig. 1(d) shows a typical ready-to-use microfluidic unit. In this photograph, in order to enhance the visibility of the details of the reservoir and the channels, a green dye was introduced inside the chip.

2.3. Preparation of a sterile microfluidic plastic chip for antibiogram

Microfluidic plastic chips with 8 rows and 5 columns of micro-wells (40 wells) were UV sterilized for 20–30 min. After sterilization, each well was filled with 20 μl of LB, using a micropipette, and allowed to dry for 90 min at 40–45 °C on a hot plate in a sterile environment. Except the controls (in each row), 2 μl of antibiotics at its MIC (Amikacin – 10 μg/ml, Gentamycin – 1 μg/ml, Kanamycin – 8 μg/ml and Ampicillin – 22 μg/ml) were added to the corresponding wells of the chip, and dried at room temperature in a