Determination of Minimum Inhibitory Concentrations using Thermal Ink-jet Printing

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Abstract

Ink-jet printing is a cost-effective technology that has had varied applications recently. The technology was applied here to an important microbiological procedure, minimum inhibitory concentration (MIC) determination.

MIC evaluations were conducted by printing varying concentrations of the antibiotics, ampicillin or tetracycline, onto agar-coated glass slides then printing an even film of known bacterial density of Lactobacillus acidophilus onto the antibiotic-imprinted slide. Results obtained were validated with a broth microdilution technique. The MICs for ampicillin and tetracycline obtained were two-fold higher than those determined by the conventional technique.

Introduction

Ink-jet printing (IJP) technology is well established for the reproduction of images and text. Recently, IJP has found increasing application in pharmaceutical research, such as the fabrication of personalised-dose medicines and to print biological materials; typically proteins and mammalian cells [1, 2]. However, very little work has been done with regards to microbiological applications.

Minimum inhibitory concentration (MIC) determination is an important microbiological technique for assessing the susceptibility of bacteria to an antibiotic [3]. Agar dilution and broth dilution methods are standard manual approaches to MIC determination. Agar dilution involves the incorporation of doubling concentrations of antimicrobial into nutrient agar then applying a known number of bacteria onto the agar plate whereas broth dilution is a technique in which containers holding identical volumes of broth with antimicrobial solution in geometrically increasing concentrations are inoculated with a known number of bacteria. Broth microdilution denotes performing broth dilution in microdilution plates with a capacity ≤ 500 µL [3, 4]. The difficulty and workload involved in obtaining varying drug concentrations is a major drawback with these techniques [5].

Automated procedures like the spiral gradient endpoint (SGE) technique are also available for high throughput determinations. MIC determinations via SGE involve a spiral plater. This is a syringe-driven dispensing pen that deposits samples onto a rotating agar plate. The pen moves from the near-center outward while the plate rotates as the syringe drives downward, depositing less of the sample as the pen moves outward. This results in a higher concentration of material at the center and a relatively lower concentration at the periphery of the plate. Hence, if an antibiotic solution is deposited during the lateral travel of the spiral plater dispensing pen across the surface of a rotating agar plate, a dilution gradient is created from the centre (high) to the periphery (low) of the agar [6].

The SGE technique is, however, an expensive technique due to the very high cost involved in the purchase of spiral plater [7].

In MIC determination, varying drug concentrations are applied to a constant number of bacterial population. A horizontal continuous gradient scale has been documented as a means of obtaining varied concentrations, however, an important factor like diffusion of drug was not factored in determinations [8]. Here, varying concentrations were obtained with an approach known as 'Y-value'. The term Y-value, is used because the only parameter varied for a series of rectangles (for ease of assay development rectangles were used) is the height and because changes in height occur vertically i.e., in the Y-plane. For a given series of rectangles, the width and colour (preferably 100% black) are maintained constant. An example is shown in figure 1, where all three rectangles have the same shade of black (100% black) and width of 0.5 cm but variations in height (0.5 cm, 1 cm and 1.5 cm respectively). Printing out these different 'Y-values' onto a fixed unit area results in linear increments in amount and this approach can be used to obtain different quantities of any material in solution which can be easily calculated. This was hence used as a means to obtain varying antibiotics concentrations.

Here we report on an inexpensive automated alternative for determination of MIC using an ink-jet printer.

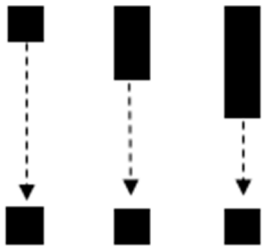

Figure 1- Illustration of y-value concept

Materials and Method

The thermal ink-jet printer used in this work was a Hewlett-Packard (HP) 5940 model (USA). This printer was chosen based on factors like cost, generation of model and cartridge properties.

Modification of Printer and cartridge

The printer was modified such that rather than the substrate (paper in the unmodified printer) passing through the printer's rollers during operation, printing would be done onto a stage underneath the cartridge print head without the printer detecting the absence of paper. The key point with the printer modification was to identify and make accessible the printer's sensors to enable manual activation of these when needed. Accessibility to printer's sensors was ensured by removal of paper feeder trays and printer lid as well as the paper guide structures. Caution was taken to preserve the cartridge cradle and sensor cablings from unnecessary stress. Once sensors were accessible, the working stage was fixed well below the print head level of cartridge to prevent damaging the print head or the driving cradle during printer operation.

HP black ink cartridges number 337 were modified and used. Modification was by carefully cutting the cartridge top from the body, removing the foam and inner membrane then rinsing out ink several times until clear with deionized water.

The functionality of the modified printer was confirmed after modification. The effect of the printing procedure on bacterial population was assessed quantitatively by enumerating pipetted bacterial solution (control) and ink-jet printed bacterial solution. This was performed before the printer was used for analytical work.

Model for MIC determination using Ink-jet printing

A model was designed in which varying amounts of antibiotics could be applied to a standard bacterial population such that the lowest amount of antibiotic that inhibited the visible growth of a microorganism after incubation could be easily identified. Varying concentrations were achieved with the Y-value concept. The linear relationship that exists between different Y-values and the quantity printed implies the MIC can in principle be determined from the Y-value at which no growth is first observed.

Prior to MIC determination, cartridges were standardized to know the volume of "ink" the cartridges jet out for each Y-value. 1 mg/mL Fast Green dye solution was prepared and used. Y-values with height of 0.5 cm, 1 cm, 2 cm, and 3 cm and a constant width of 0.2 cm were printed individually onto clean acetate sheets using 1 mg/mL dye in the modified cartridges. The acetate sheets were carefully cut into bijou containers and 1 mL of deionized water added to dissolve the dye. The bijou containers were vortexed accordingly to ensure appropriate dissolution of the dye. 1 mL of the resulting solutions was then transferred into vials for analysis using High Performance Liquid Chromatography. A gradient system was adopted and the composition of mobile phases detailed in table 1.

A flow rate of 1 mL/min was maintained for 10 minutes with a sample injection volume of 10 µL. A wavelength of 600 nm was used to detect Fast Green dye and a column temperature set at 30 ºC.

Table 1- Gradient system used in HPLC

Mobile phase	Time (minutes)		
	0	**5**	**6**
Acetonitrile	15%	60%	15%
55 mM Acetate buffer	85%	40%	85%

The protocol for MIC determination was as follows. 1 mL of known antibiotic concentration was put into the ink compartment of a sterilized black cartridge. This was used to print the Y-value set (0.5 cm and 1 cm), (1.5 cm and 2 cm) and (2.5 cm and 3 cm) onto de Man, Rogosa and Sharpe (MRS) agar-coated slides on a stage 2 mm from the cartridge head. An interval of 2.5 cm (this was to cater for any potential diffusion arising from the different concentrations) was allowed between the different Y-values (figure 2). Each set was duplicated. The cartridge was then removed and the main parts of the printer that came into contact with the cartridge were wiped with 70% ethanol and allowed to dry. 1 mL of *Lactobacillus acidophilus* (10^5 CFU/mL) was put into another sterile cartridge and used to print an even film (1 cm x 5 cm rectangle) of bacteria across all the slides. A control was also set up which had just bacterial film with no drug. The experiment was repeated with varying antibiotic concentrations. The slides were then incubated under anaerobic conditions for 48 hours. Broth microdilution was simultaneously performed using 96 well plate and results used to validate the IJP results.

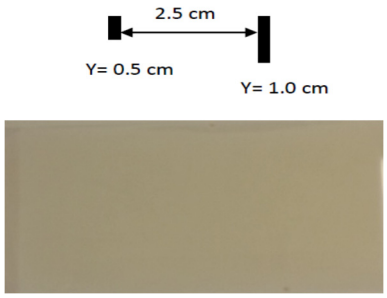

Figure 2- Illustration of MIC determination template for printing antibiotic

Results and Discussion

An evaluation of the impact of IJP on bacterial viability showed that the printing procedure did not have any significant effect on bacterial numbers. The number of cells after ink-jet printing was very similar to that of control (pipetted), approximately 1 x 10^7 colony forming units (CFUs) per mL (figure 3). Further confirming that the assumed temperature rise of about 200 ºC that accompanies bubble formation during printing does not have a significant impact on cells [2].

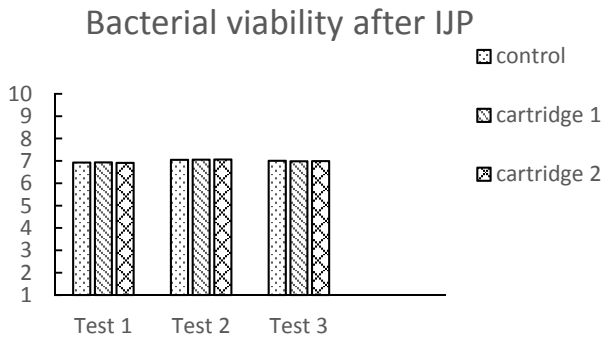

Figure 3- Effect of IJP on bacterial viability (y-axis, cell number expressed as log cfu/mL; x-axis, triplicate sampling of two independent cartridges; control represents the original cell number before printing).

A calibration curve (figure 4) for Fast Green dye was obtained with a good linearity (r^2 = 0.9999) between 0.0625 µg/mL to 2 µg/mL. The graph of Y-value against area under curve (AUC) (figure 5) confirmed the linearity (r^2 = 0.9992) of the Y-value concept. The graph representing Y-value against characteristic ratio (figure 6) was obtained by combining figures 4 and 5. This graph informs about the ratio of cartridge concentration printed per Y-value. A characteristic ratio (Eqn. 1) was obtained by expressing the concentration of Fast Green dye per Y-value as a fraction of the cartridge concentration used in printing Y-values. This ratio is a constant per Y-value. A plot of the characteristic ratio against the Y-value gave a linear graph (r^2 = 1).

$$\text{Characteristic ratio} = \frac{\text{equivalent concentration}}{\text{cartridge concentration}} \quad (1)$$

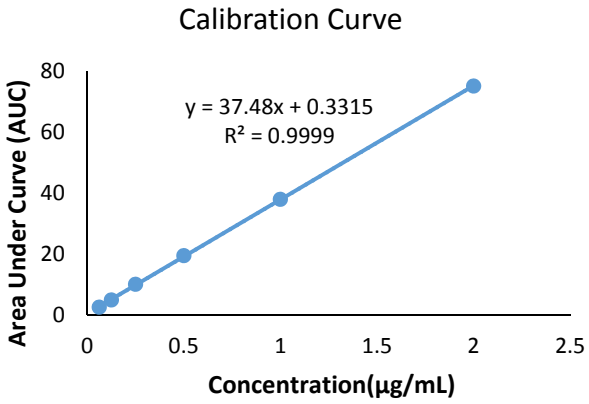

Figure 4 - Calibration curve

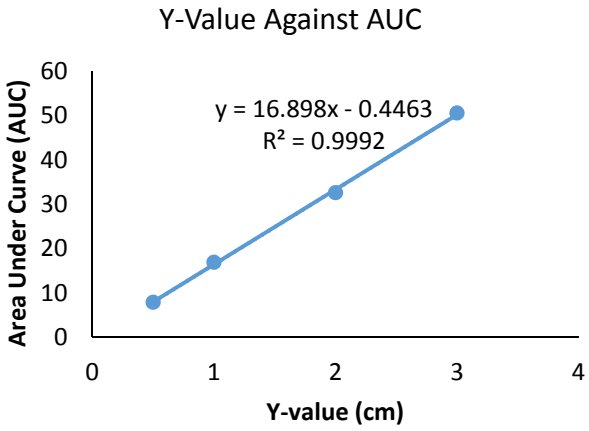

Figure 5- Y-value against AUC

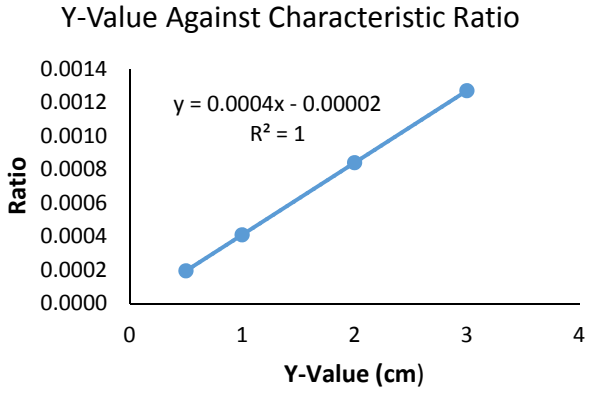

Figure 6- Y-value against characteristic ratio

Table 2- Ampicillin MIC for *L. acidophilus* determined via IJP

Cartridge concentration (mg/mL)	Y-value (cm)					
	0.5	1.0	1.5	2.0	2.5	3.0
0.10	+	+	+	+	+	-
0.20	+	+	-	-	-	-
0.25	+	+	-	-	-	-

n=3

+ : growth - : no growth

Ampicillin MIC determine by broth microdilution was 0.5 µg/mL

Table 3 - Tetracycline MIC for *L. Acidophilus* determined via IJP

Cartridge concentration (mg/mL)	Y-value (cm)					
	0.5	1.0	1.5	2.0	2.5	3.0
0.1	+	+	+	+	+	+
0.2	+	+	+	+	+	+
0.3	+	+	+	+	+	+
0.6	+	+	+	+	-	-
0.7	+	+	+	-	-	-

n = 3

+: growth - : no growth

Tetracycline MIC determined by broth microdilution was 4 µg/mL

Using ampicillin data to illustrate MIC calculation.

Corresponding drug concentrations for ampicillin sodium
Using 0.1 mg/mL
MIC at 3 cm = 0.1 x (characteristic ratio at 3 cm)
From graph, 3 cm = 0.00118
MIC = 0.1 x 0.00118 = 1.18 x 10^{-4} mg/mL (0.12 µg/mL)

Table 4 - Comparison of MIC values determined by IJP and broth microdilution

Antibiotic	Cartridge concentr-ation (mg/mL)	MIC by ink-jet printing (µg/mL)	MIC by broth microdilution (µg/mL)
Ampicillin	0.10	0.12	0.50
	0.20	0.12	0.50
	0.25	0.15	0.50
Tetracycline	0.60	0.59	4.00
	0.70	0.55	4.00

The MIC obtained by the broth microdilution method was recorded in a standard manner i.e., if growth was observed at 0.5 µg/mL and no growth was observed at 1 µg/mL, the MIC was documented as 1 µg/mL. However, with the ink-jet printing, because concentration terms were in linear rather than in doubling increments, MICs were rounded off to the next incremental doubling dilution to enable direct comparisons with the broth microdilution technique. A similar concept is usually adopted when comparing spiral gradient endpoints (SGE) with standard techniques [9]; since the IJP model design is similar to the SGE procedure, the same approach of comparison was adopted.

The MICs obtained for ampicillin and tetracycline using IJP were 0.12 µg/mL and 0.55 µg/mL respectively (Tables 2, 3 and 4). The corrected MIC values were 0.125 µg/mL for ampicillin (0.12 µg/mL) and 1 µg/mL for tetracycline (0.55 µg/mL). The resulting MIC value for both ampicillin and tetracycline were within two doubling dilutions, but outside the acceptable range (i.e. one doubling dilution). One possible reason for this is the fact that the inoculum density used in this instance was 10^5 CFU/mL as compared to 10^8 CFU/mL and 10^9 CFU/mL used with spiral plating experiments [10, 11].

However, being a new model, further optimisation and experiments are needed to confirm the validity of the procedure. Also, other antibiotics with different modes of action as well as a variety of test organisms will be used. These will aid in elucidating whether variations from standards follow a constant trend which can be taken into account when interpreting the MIC results. Notwithstanding, the aim is to obtain results without ambiguity in comparison to standard techniques.

This technique could potentially aid the accurate determination of MICs since the range between growth and growth inhibition can easily be narrowed down with small linear increments after initial MIC determination.

Conclusion

An inexpensive automated alternative for the determination of MIC using an ink-jet printer has been demonstrated here. The model was used to determine the MICs of ampicillin and tetracycline against *Lactobacillus acidophilus*. The MIC values obtained for ampicillin and tetracycline were found to be one doubling dilution lower than the acceptable range.

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