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作品名稱 Machine-learning assisted antibiotic detection and categorization using bacterial arrays

得獎獎項 大會獎：三等獎

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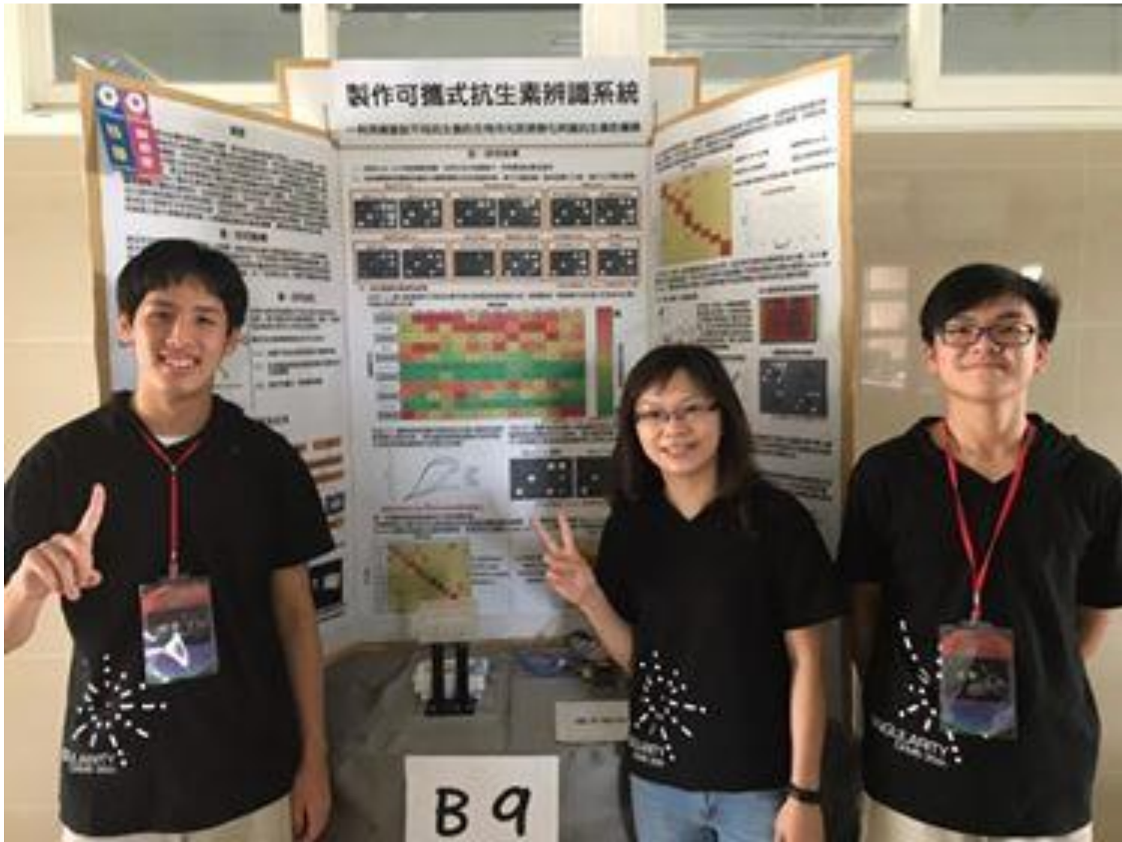


We both are students from Taipei Municipal Jianguo High School. We are interested in biology ,especially microbiology. Therefore, when we heard of a opportunity to do research in a laboratory, we didn’ t even have to think twice about taking it. Inside the lab, we are both young researchers with perseverance, ambition, and creativity. We both dedicated ourselves to the research. When most of our classmates were enjoying their high school lives, we still regarded doing research as our main goal. Besides, we are grateful to those who helped us, particularly our advisor, who gave us a lot of help. Also, we are thankful for the lab assistant’ s help and teacher advices. Without them, we couldn’ t have completed the research on our own.

Abstract

With the extensive use of antibiotics around the world, the problem of antibiotic residues have become severe. Therefore, a bacterial array solid-phase assay (BacSPA) method was established to detect and categorize antibiotics in samples. Suspension of 15 strains of *E. coli* genetically modified with *lux:CDABE* reporter genes were spotted on solidified agar individually mixed with 11 different antibiotics at 1.5 ppm concentration. The antibiotic stimulated the bacteria and the induced bioluminescence from different strains generated different patterns for different antibiotic classes. The luminescence pattern was monitored by time-lapse photography and the machine learning algorithm, Multiclass Decision Forest, was applied to train categorization models that either identify the compound or categorize the class of antibiotics using the indices. The best model got the best accuracy of 90% for class classification in three hours. The method was further tested for categorizing antibiotics at different concentrations. The final model could categorize the eight chosen antibiotics at a concentration range from 125 ppb to 1000 ppb with accuracies mostly higher than 70%. We hope to establish the database of the luminescence pattern for more kinds of antibiotics at different concentrations and with different combinations in the future. This method would become more powerful, and it could surely provide a good way for people to detect and categorize antibiotic residue in food or environment samples.

摘要

隨著抗生素的廣泛使用，抗生素殘留的問題日益嚴重。為此，本研究建立了一個稱作細菌陣列固相分析（BacSPA）的方法來檢測和分類樣品中的抗生素。本研究將 15 個在質體中植入生物冷光的大腸桿菌液，點在與 11 種不同的抗生素混合的固相培養基上，濃度為 1.5 ppm。不同的抗生素會刺激細菌，使其產生特定的生物冷光變化圖形。將圖形以延時攝影紀錄，並以機器學習演算法中的多重決策樹系訓練抗生素分類模型。得到的最佳模型能在三小時內，以 90％分類抗生素的類別。再對該方法進一步測試，以區分不同濃度的抗生素。最終模型能將濃度從 125 到 1000 ppb 的八種抗生素進行分類，其準確率大多高於 70％。在未來，我們希望能建立更多不同濃度、不同組合的抗生素發光圖形資料庫，以擴大此方法的應用範圍。這無疑為人們檢測與分類食品與環境樣品中的抗生素殘留，提供了一個很好的方法。

Introduction

With the extensive use of antibiotics around the world, the problem of antibiotic residues have become a severe issue. The antibiotics remaining in the environment or food will impose selection pressure on bacteria, which makes bacteria develop antibiotic resistance and turn into superbugs. Superbugs claim about five hundred thousand people's lives every year. Therefore, it is crucial to detect and categorize the antibiotics in samples more widely, such as food, water and so on. The method utilized now is Mass Spectrometry(MS) and ELISA. However, MS is quite expensive and takes long hours, so it is not suitable for preliminary use, and due to the chemical mechanism of ELISA, it cannot classify different antibiotics precisely. Thus, the biological method was utilized in the study. The 14 strains of genetically modified E. coli were used to detect and classify antibiotics in samples. Each strain of E. coli has a specific promoter on its plasmid, and each specific promoter was designed to be induced by a corresponding stress factor caused by a specific class of antibiotics. By using this biological method, the goal of quickness and convenience would be reached.

Materials and Methods

Antibiotics:

The 11 antibiotics used in this study were purchased from Sigma Aldrich and shown in Table 1. The "antibiotic compound" refers to the name of a specific antibiotic, while the "antibiotic class'' refers to the mode-of-action (MOA) class an antibiotic was in.

Table 1: The antibiotic compounds and their classes used in this study.

Antibiotic Class	**Antibiotic Compound Name**	**Catalog Number**
β-lactams	Amoxicillin	10039
	Ampicillin	A9393
	Ceftiofur	34001
	Cefapirin	SMB00605
Phenicols	Chloramphenicol	C0378
	Thiamphenicol	T0261
Tetracyclines	Doxycycline	D3447
	Tetracycline	31741
Rifampicin	Rifampicin	R3501

Quinolone	Ciprofloxacin	17850
Naphthyridine	Nalidixic acid	N8878

Bacteria strains:

The 14 strains of genetically modified *E. coli* used in this study were shown in Table 2. (Melamed, S., Lalush, C., Elad, T., Yagur-Kroll, S., Belkin, S., & Pedahzur, R. 2012) Each strain of E. coli has a specific promoter on its plasmid, and each specific promoter was designed to be induced by a corresponding stress factor (sensing element).

Without antibiotics, different strains of bacteria emit different intensities of natural bioluminescence, mainly caused by the intrinsic expression of the *luxCDABE* operon. However, when incubated with antibiotics, different antibiotics inhibited the growth of the bacteria by imposing different stress factors. They induced different extents of activation on different promoters, and eventually cause a variety of changes in luminescence intensity for different strains. This phenomenon allows the bacteria to be used to classify the antibiotics existing in their incubation environments.

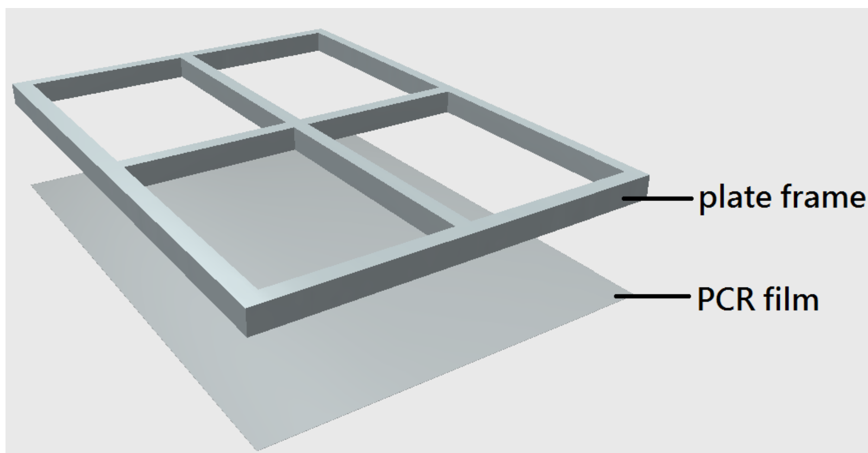
Table 2 The 14 strains of genetically modified *E. coli* used in this study. (Melamed, S., Lalush, C., Elad, T., Yagur-Kroll, S., Belkin, S., & Pedahzur, R. 2012)

Strain	Plasmid	Sensing element information
SM332	pBRlux-trp:*emrA::luxCDABE*	Cytoplasmatic membrane fusion protein
SM333	pBRlux-trp:*acrA::luxCDABE*	Periplasmic lipoprotein component of the AcrAB-TolC multidrug efflux pump
SM334	pBRlux-trp:*zwf::luxCDABE*	G6PDH, regulated by SoxS and MarA
SM335	pBRlux-trp:*soxS::luxCDABE*	Dual transcriptional activator, participates in the removal of antibiotics
SM337	pBRlux-trp:*tolC::luxCDABE*	Outer membrane porin involved in the efflux transport system
SM338	pBRlux-trp:*inaA::luxCDABE*	pH-inducible protein involved in stress response
SM340	pBRlux-trp:zntA*::luxCDABE*	Lead, cadmium, zinc and mercury transporting ATPase

SM341	pBRlux-trp:*marR::luxCDABE*	Multiple antibiotic resistance protein
SM342	pBRlux-trp:*recA::luxCDABE*	DNA recombination protein, induce the SOS response to DNA damage
SM343	pBRlux-trp:*micF::luxCDABE*	Antisense regulator of the translation of the OmpF porin, under SoxS regulation
SM344	pBRlux-trp:*katG::luxCDABE*	Bifunctional hydroperoxides I, having both catalase and peroxidase activity
SM345	pBRlux-trp:*sodA::luxCDABE*	Superoxide dismutase protein
SM346	pBRlux-trp:*rpoB::luxCDABE*	RNA polymerase, beta subunit
SM347	pBRlux-trp:*ompF::luxCDABE*	Outer membrane porin

Other materials:

25 g of LB powder(Difco LB Broth cat. no. 244620) was dissolved into 1 liter of water and sterilized to make LB Broth medium. 6 g of the agar powder (Bacto Agar) was added into LB Broth medium and sterilized to make agar. The formulation of the M9 medium had been mentioned in previous study. (Melamed, S., Lalush, C., Elad, T., Yagur-Kroll, S., Belkin, S., & Pedahzur, R. 2012)



(a)

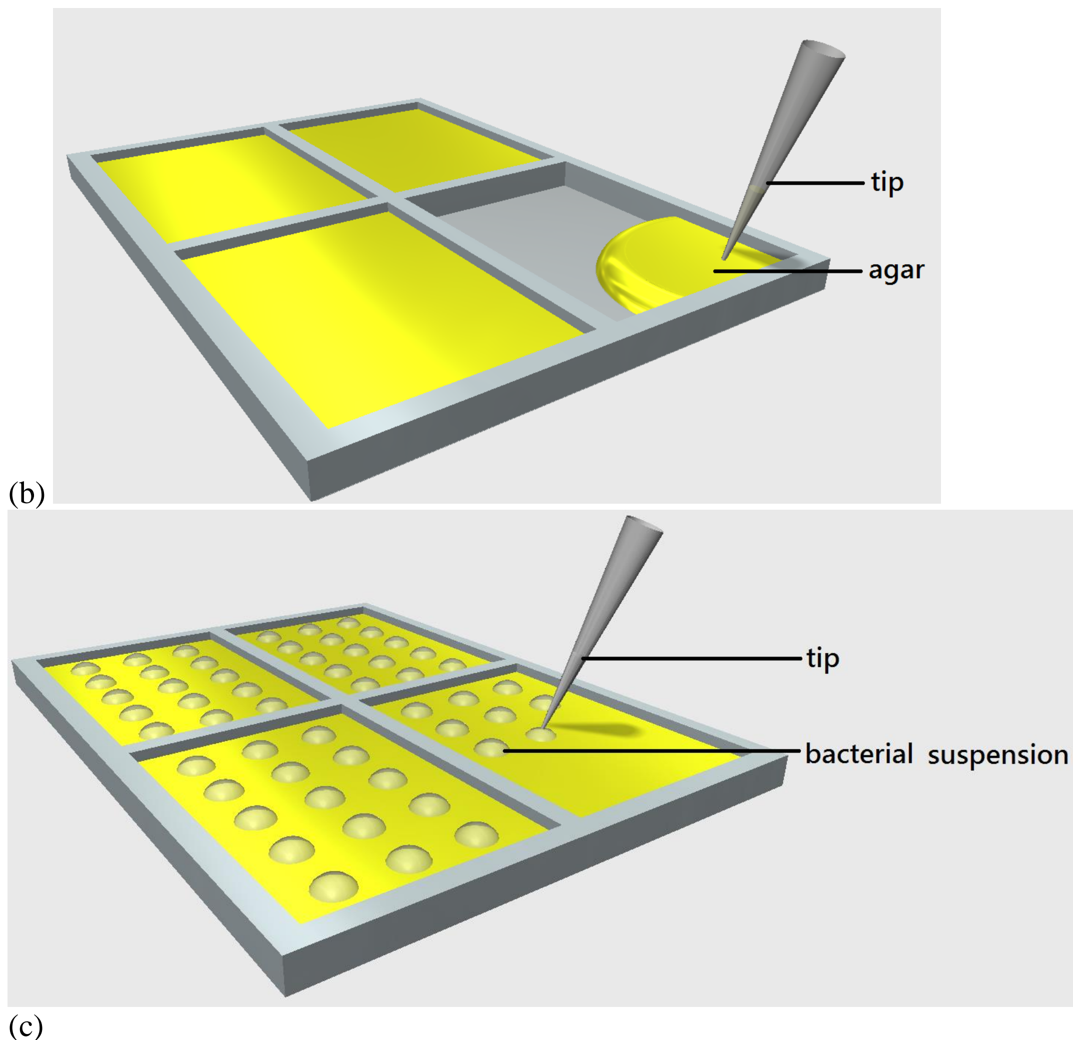


Figure 1. Procedure of the SPAA assay. (a) The structure of the four-well plate made of a PMMA frame and a layer of adhesive film. (b) Agar (yellow) mixed with the target antibiotic was pipetted into the wells and cooled to solidify. (c) The bacterial suspension was inoculated onto the agar surface, contacting the target antibiotic

Bacterial array solid-phase assay- BacSPA:

The BacSPA method allows the target antibiotics to stimulate the 14 bacteria strains simultaneously. Arrays of the bacteria were inoculated onto the surface of the agar containing the target antibiotics (Figure 1) for interaction. In comparison to the conventional liquid phase assay, the BacSPA method is simple and allows multiplex interactions to be monitored in a high-throughput manner. The detail of the BacSPA method is described below.

Solidified agar was held by a home-build four-well plate, which was composed of a plate frame (figure 3) and an underlying adhesive film. The plate frame was made of PMMA sheet (thickness 5 mm), cut by a CO_2 Laser scriber (Wavelength 10.6 micrometer, Company, V2000). Then, the adhesive film was adhered to the

bottom of the frame to make the four-well plate.

The agar containing the target antibiotics was prepared as follows. Antibiotic stocks were prepared by dissolving the target antibiotic powder in water or methanol to be 1,000X of the designated concentrations in the agar. Each stock was prepared with a volume of 10 ml and stored below 4 °C before mixed with agar to prevent degrading. For each experiment, the stock and the melted agar (60 °C) were mixed at a ratio of 1:1000 and vortexed for 1 minute. For the blank test, the melted agar was prepared with a mock solvent at the ratio identical to that of the experiment group. The melted agar was then poured into the four-well plate (Figure 1(b)) and allowed to cool at ambient temperature to solidify. The solidified agar was then ready for immobilizing bacterial suspension.

The bacterial suspension was prepared as follows. Bacterial stock (1.5 μL) stored at -40 °C was added into 15 mL culture tubes containing 1 mL of M9 salt medium and was grown overnight in an incubator (Dengyng, D9MD-DB45) at 37 °C. After the overnight growth, the bacterial suspension was diluted by 25 times with the LB medium and regrown in a new culture tube in the incubator at 37 °C for two hours. The culture tube was then stored in an ice bath to stop the bacterial growth. The suspension was then diluted to 0.2 O.D. (measured by a photometer Metertech, Model 6+ Mini) with LB medium. Finally, the bacterial suspension was centrifuged and then resuspend to a concentration of 2.0 to 3.0 O.D.

The concentrated bacteria suspension was then inoculated onto the surface of the solidified agar using a pipette, forming bacterial arrays (figure 1 (c)). The volume of each spot was 5 μL. The bacterial array was thus in contact with the target antibiotics, and the bioluminescence was then monitored by time-lapse photography.

Monitoring strain bioluminescence

The four-well plates containing the bacterial arrays were incubated at 37 °C and photographed using a camera (Diffraction Limited, STF-8300). Each experiment repeats consisted of four four-well plates mounting on a customized turntable made of PMMA cut by the CO_2 laser scriber. Each well in the four-well plate contains one condition (different compounds and concentrations), resulting in a total of 16 conditions in one experiment repeat. The turntable's rotation was integrated with the camera's exposure controlled by a MATLAB (https://www.mathworks.com/products/matlab.html) program to photograph each plate for 10 hours at an interval of 3 minutes. A total of 7 experiment repeats were conducted independently.

Luminescence of the bacterial arrays in the photographs was quantified using a program written in Python (https://www.python.org/downloads/) and its function library OpenCV (https://opencv.org/). The bacteria spots were first marked using plain circles in Photoshop manually, and a function called HoughCircle (https://docs.opencv.org/2.4/doc/tutorials/imgproc/imgtrans/hough_circle/hough_circle.html) in OpenCV circled out the edge of each spot. The photographs were compressed to 8-bit grayscale, and the color depths (i.e. intensity) of the pixels

within each spot were summed up into luminescence value. The background noise intensity was further subtracted from the luminescence values to obtain the raw bacterial luminescence intensity, shortened as "raw intensity, *Ln(t)*." below.

Intensity data processing:

The human error occurred when condensing or inoculating bacteria might cause errors in the amounts of bacteria spotted on the agar surface. The amount of plasmids in each bacteria cell also varies. These errors manifest in the luminescence intensity emitted from the cells, causing the fluctuation of the raw bioluminescence intensity.

The raw intensity data were further processed using different mathematical ways to increase the accuracy by machine learning. For a specific antibiotic, the raw luminescence intensity was calculated according to the following four methods, generating four sets of indices.

Method 1: Ratio to blank, $R_n(t)$

The ratio of a strain's raw luminescence intensity, *Ln(t)*, in an antibiotic to the corresponding one in the blank experiment was calculated to $R_n(t)$ using the following equation. The index $R_n(t)$ could diminish the error caused by unstable intrinsic bioluminescence emission by designating each intrinsic emission as the baseline.

$$R_n(t) = \frac{L_n(t)}{B_n(t)} \text{ eq.(1)}$$

, where n is the strain number, $L_n(t)$ is the raw intensity of a strain in an antibiotic emitted at time t. $B_n(t)$ is the raw intensity of the corresponding strain emitted in the blank experiment at time t.

Method 2: Time normalization, $T_n(t)$

The raw intensities of a strain in an antibiotic at time t were normalized to $T_n(t)$ by all the corresponding time points using the following equation. The index T_n is aiming for reducing the effect from the luminescence fluctuation over time between different experiment repeats.

$$T_n(t) = \frac{L_n(t) - avg(L_n(0) \ to \ Ln(t))}{std(L_n(0) \ to \ Ln(t))} \text{ eq.(2)}$$

, where n is the strain number. $L_n(t)$ is the raw intensity of a strain in an antibiotic emitted at time t. $L_n(0) \ to \ Ln(t)$ refer to the raw intensity of the corresponding strain in the corresponding antibiotic at the time points from time 0 to time t. The function avg refers to the average and std the corrected standard deviation.

Method 3: Strain normalization $S_n(t)$

The raw intensities of a strain in an antibiotic at time t were normalized by all those from all the 14 strains to $S_n(t)$ using the following equation. The index aims to diminish the error caused by overall environmental instability among different experiment repeats that affects all the bacterial strains in forms of linear transformations. (Craig A. Mertler., 2007)

$$S_n(t) = \frac{L_n(t) - \text{avg}(L_{1\sim14}(t))}{\text{std}(L_{1\sim14}(t))} \quad \text{eq.(3)}$$

, where n is the strain number. $S_n(t)$ is the raw intensity of a strain at time t. $(L_{1\sim14}(t)$was all the bioluminescence intensity of all 14 strains in the corresponding antibiotic to $L_n(t)$

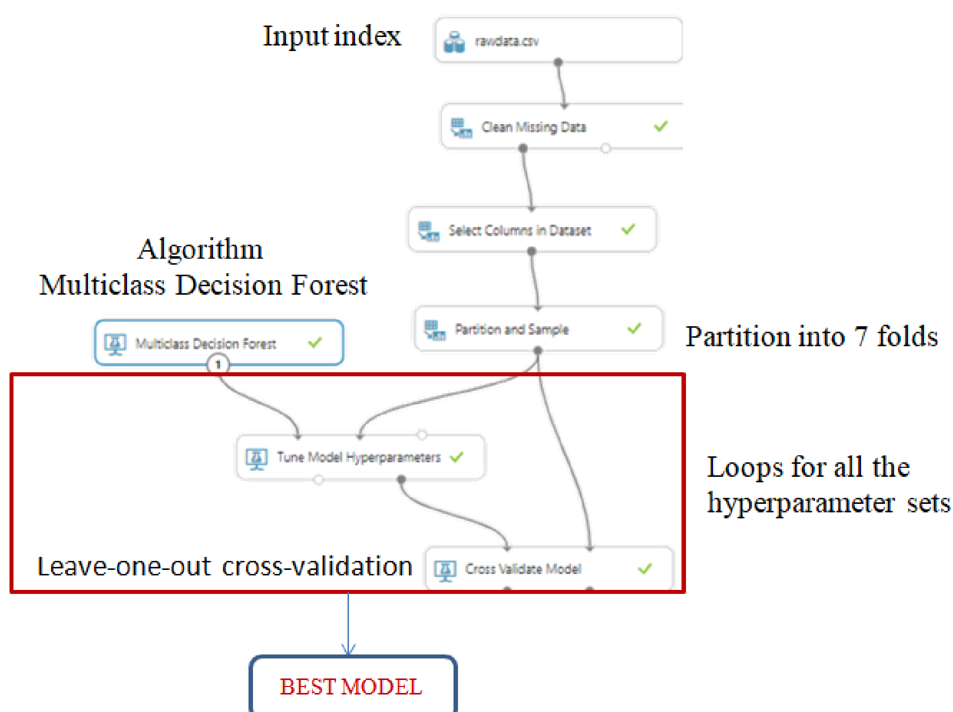
Method 4: Omnibus index $A_n(t)$

The three indices obtained using the three methods above were combined into $A_n(t)$as shown below. The index $A_n(t)$ was a set of data tuples that contained the information of all the three indices.

$A_n(t)$=($R_n(t)$, $T_n(t)$, $S_n(t)$), where n is the strain number.

Categorization Model Training and Testing :

Microsoft Azure machine learning Studio (https://studio.azureml.net/) was used for constructing the machine learning models used for identifying and categorizing the antibiotics objectively (Scheme 1). The algorithm, Multiclass Decision Forest (MDF), was applied to train categorization models that categorized the antibiotic in the sample using the bioluminescence intensity data of the bacteria stimulated by the antibiotics.

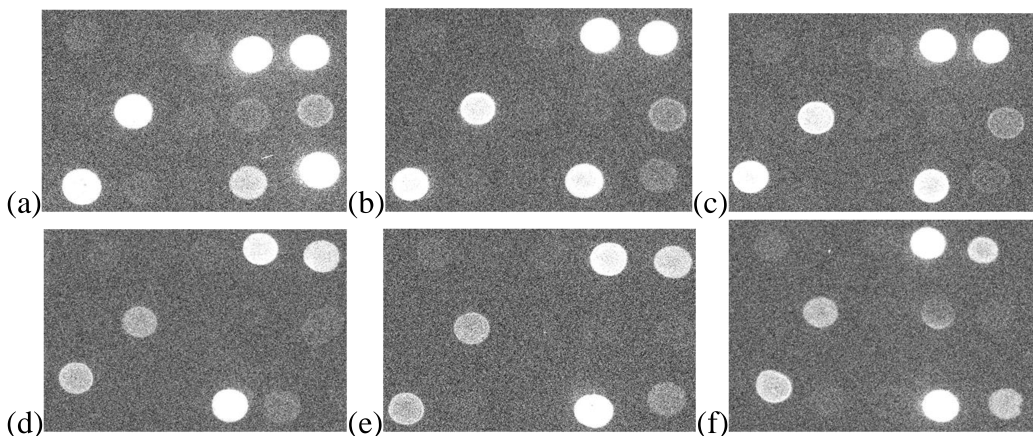


Scheme 1. The flowchart for training and validation of the machine learning models.

A process termed as "model hyperparameter tuning" attempted different training hyperparameters (Scott Hartshorn., 2016) during training. The hyperparameters include the numbers of decision trees, the maximum depths of the decision trees, the number of random splits per node, and the minimum number of samples per leaf node. The evaluation method "Leave-one-out cross-validation (Scott Hartshorn., 2016) ", was used to score the accuracy of the models with different hyperparameters, which chooses the best model hyperparameters with the highest accuracy and prevents overfitting at the same time. The detailed training steps are described below.

The data (the L, R, T, S, and A indices) were partitioned into seven-folds, each fold containing the indices from one experiment repeat. The cross-validation process worked in a round-robin way. The tuning module first designated a set of training hyperparameters of the decision tree. One data fold was first left out (i.e., excluded from the model training), and it was used to score the categorization model trained using the remaining six data folds by the decision forest with the designated hyperparameters. After that, the left-out data fold was swapped with another data fold, scoring the second categorization model trained using the new combination of six remaining data folds with the same sets of hyperparameters. After all the seven data folds have been left out respectively with a set of seven models trained, the accuracy obtained using the set of hyperparameters was defined as the average accuracy of the seven models. After all the seven combinations of data have been used for training the MDF model, a set of optimal hyperparameters was determined. By changing and tuning the training hyperparameters, the final accuracy is defined as the accuracy of the optimal (having the highest average accuracy) set of hyperparameters, and the final categorization model was considered trained with the resultant hyperparameter set.

Results and Discussions



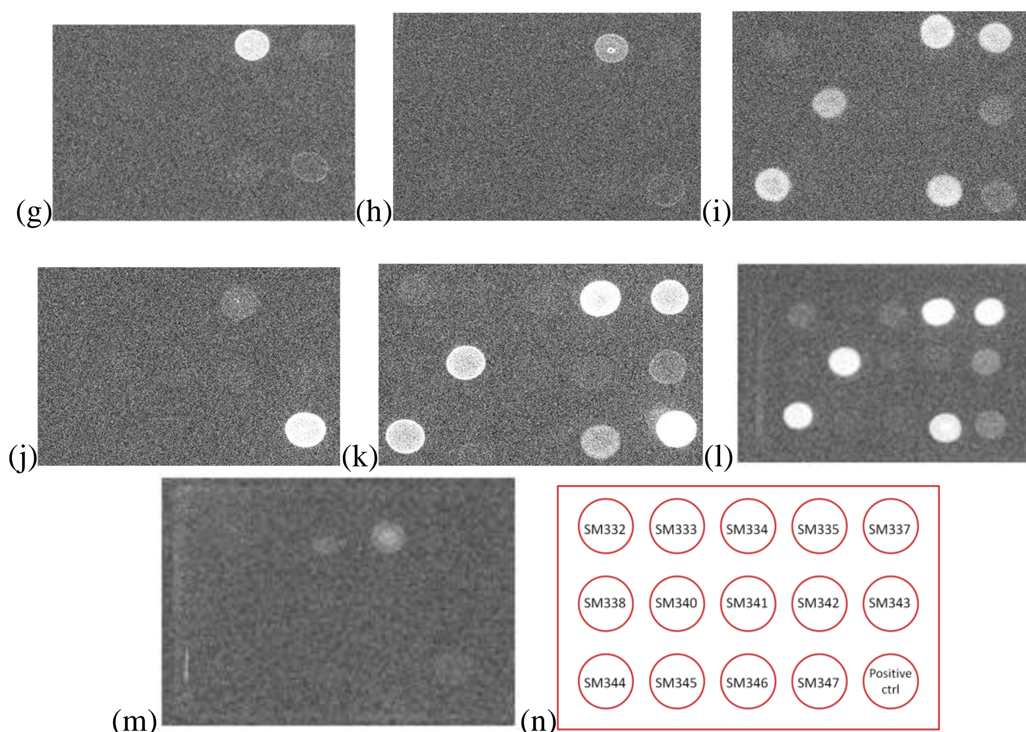


Figure 2. Photographs of luminescence from the 15 strains of bacteria stimulated by different antibiotics in the agar at concentration 1.5 ppm for 3 hours. (a) Amoxicillin. (b) Ampicillin. (c) Ceftiofur. (d) Cefapirin. (e) Chloramphenicol. (f) Thiamphenicol. (g) Doxycycline. (h) Tetracycline. (i) Rifampicin. (j) Ciprofloxacin. (k) Nalidixic Acid. (l) Blank. (m) Nalidixic Acid at concentration 6 ppm. (n) The layout of the 15 strains on the agar surface.

Luminescence from stimulated bacteria:

Figure 2 shows the photographs of the luminescence from the 15 strains of bacteria when stimulated by the antibiotics spiked in the agar. With a moderate antibiotic concentration, different antibiotics caused different extents of activations on different promoters. As a result, each antibiotic generated a specific luminescence pattern, which could be used to identify and classify the MOA of the antibiotic.

For antibiotics in the same class, the luminescence from the strains generated similar patterns, mainly because a similar mode of action should take place. For example, doxycycline (figure 2(g)) and tetracycline (figure 2(h)) yielded a similar pattern because they both belong to the tetracycline class and inhibit the work of 30S subunits in ribosomes (Chopra, Roberts M., 2001.).

In contrast, amoxicillin (figure 2(a)) acts on bacteria through the weakening of cell walls (Pharmacol Ther.,1985) and generated a different luminescence pattern from that by doxycycline (figure 2(g)). In a blank experiment with no antibiotics added (figure 2(l)), some strains emitted observable bioluminescence because of the intrinsic expression of the *LuxCDABE* operons. On the other hand, for

antibiotics in a concentration high enough to inhibit the growth of most strains intensely (figure 2(j)), almost all strains exhibited only weak bioluminescence. To better compare the differences of the luminescence pattern stimulated by different antibiotics, the raw intensities were normalized to three indices using the methods described in the Materials and Methods section.

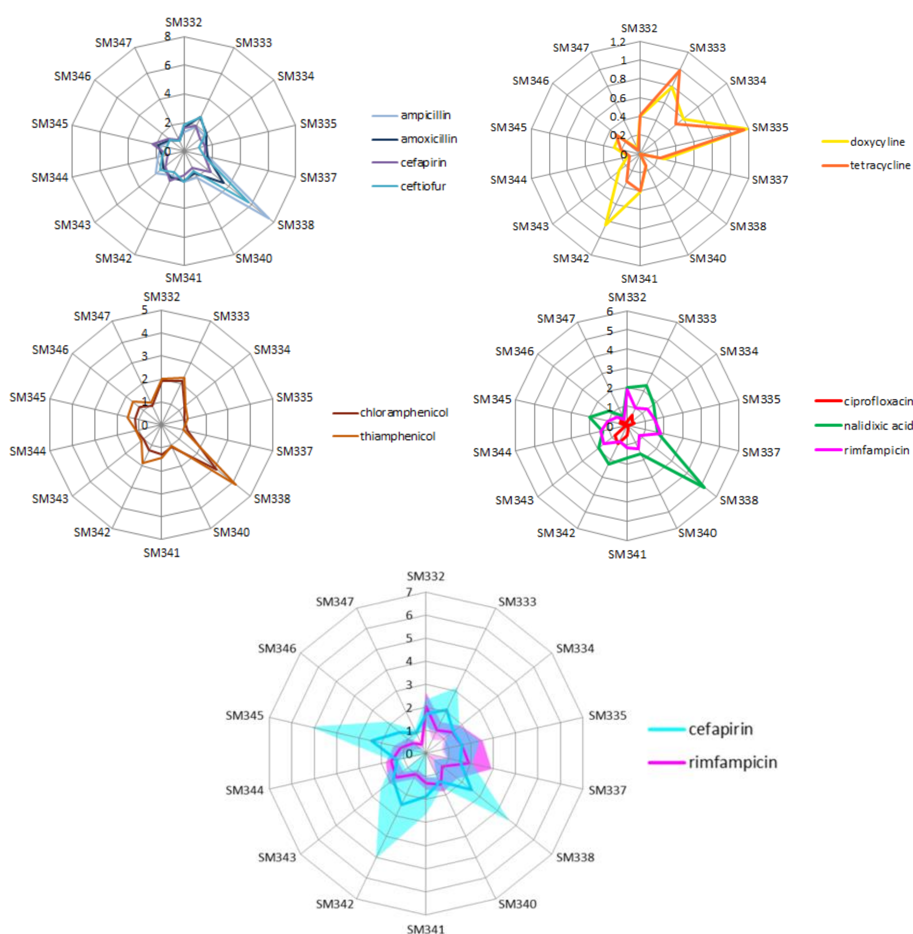


Figure 3. Radar charts of the luminescence indices, *Rn(3hr)*, for different antibiotic classes. (a) β-lactams. (b) Tetracyclines. (c) Phenicols. (d) Other three classes, including Naphthyridines, Quinolones, and Rifampicins. (e) 95% confidence intervals for Cefapirin and Rifampicin.

Radar charts were drawn using the $R_n(3hr)$ index of antibiotics at 1.5 ppm to demonstrate the relationship of luminescence patterns between antibiotic classes, as shown in Figure 3. The radial axes represent the 14 bacterial strains, while the radii indicate the $R_n(3hr)$ value of the strains.

Figure 3(a) shows the charts of the four β-lactam antibiotics (Amoxicillin, Ampicillin, Ceftiofur, and Cephapirin). This radar chart reveals the significant-high spikes at strain SM338 and the minor spikes at strains SM333, SM343, and SM345 for most of these antibiotics.

Figure 3(b) shows the chart of the two Tetracycline class antibiotics (Tetracycline and Doxycycline). In this chart, all the $R_n(3hr)$alues are smaller than 1.5, and both antibiotics showed a small pike in strains SM333 and SM335.

Contrary to the charts for the beta-lactam class (figure 3(a)), a visible dent in strain SM338 was observed for the Tetracycline class.

Figure 3(c) shows the charts of the two Phenicol class antibiotics (Chloramphenicol and Thiamphenicol). This chart seems to be similar to the β-lactam antibiotics chart, as they both showed a prominent spike in strain SM338. However, in the Phenicols chart, $R_n(3hr)$or strains SM344 and SM346 are smaller than those for the beta-lactam class. As a result, the shapes of the two radar charts appear different.

Figure 3(d) represents the remained three classes of antibiotics, including the Naphthyridine class (Nalidixic Acid), the Quinolone class (Ciprofloxacin), and the Rifampicin class (Rifampicin). For Nalidixic Acid, it shows a prominent spike in strain SM338, similar to the Tetracyclines and β-lactams. Nevertheless, the curve around the other strains is much smoother in the Naphthyridine chart, making it differentiable with the other two classes. For Rifampicin, the chart reveals a relatively round curve for $R_n(3hr)$ values around 1.5, and two minor spikes in strains SM332 and SM344. For Ciprofloxacin, all the $R_n(3hr)$values are smaller than 1.0. Compare to the tetracycline class, the threshold of $R_n(3hr)$is even smaller, and no prominent spikes are found in the Ciprofloxacin chart.

Figure 3(e) shows that the difference between classes may not be statistically different for all the strains. It is difficult to select the representative strains to distinguish the difference between the antibiotic classes. Therefore, Machine learning was utilized to classify the antibiotics. In machine learning, the Decision Forest algorithm was known for its capability to dig out the differentiable features concealed in a bulge of noised data, and multiple biological tasks, including protein and DNA arrays classification (Chen, X., & Ishwaran, H., 2012), had been successfully attempted. The categorization models trained using this algorithm tried to tell the class of an antibiotic from the response of its bioluminescence pattern.

Table 3. Accuracy of categorization models trained using different indices W to L

Data Forms (t=3hr)	$L_n(t)$	$R_n(t)$	$T_n(t)$	$s_n(t)$	$A_n(t)$
Compound	53%	56%	59%	52%	65%
Class	63%	81%	76%	77%	90%

Accuracy of machine learning categorization models:

Preliminarily, the concentrations of the antibiotics were confined to 1.5ppm to simplify the training procedure.

Ten categorization models were trained using the method mentioned in "Categorization Model Training and Testing." The bioluminescence-pattern data were given in five forms, including the raw intensity data ($L_n(t)$, $R_n(t)$, $T_n(t)$, $S_n(t)$, and $\boldsymbol{A_n(t)}$). Two different models were trained using each pattern-data form that identified either the compound name (such as Thiamphenicol, or Ampicillin) or classified the class (such as Phenicols, or β-lactams) of the antibiotic.

The accuracies of the models trained using the data from time equals the 3rd hour were shown in Table 3. It showed that most of the accuracies increased prominently with data that had been preprocessed into indices. Different indices excelled at classifying different antibiotics, while the omnibus indices tended to integrate the advantages of them and resulted in the best accuracy of 65% for compound identification and 90% for class classification.

The significant 25% increase in the accuracy for the class categorization compared to that for the compound identification demonstrated that the bacterial array is advantageous for categorizing antibiotics based on the mode of action (MOA). As mentioned, the same class of antibiotics generated similar luminescence patterns, resulting in similar patterns that lowered the accuracies for discriminating antibiotics in the same class. For class categorization, however, antibiotics in the same class share the same categorization label. In other words, the MDF algorithm did not have to tell the same-class antibiotics apart, which share similar pattern features, and thus significantly increased the categorization accuracy.

Table 4. Confusion matrix of the class classification model trained using a combination of all three indices. (The boxes in white equals 0.00.)

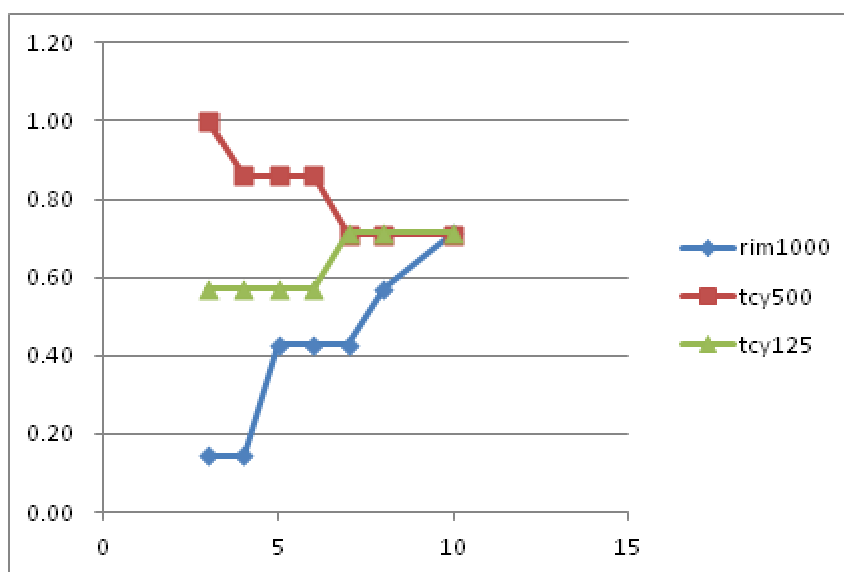
	blank	*phenicols*	*β-lactams*	*Quinolone*	*naphthyridone*	*rimfampicin*	*tetracyclines*
blank	0.86	0.14					
chloramphenicol		1.00					
thiamphenicol		1.00					
amxoxicillin		0.28	0.57		0.14		
ampicillin			1.0				
cefapirin	0.14		0.86				
ceftiofur		0.14	0.86				
ciprofloxacin				1.00			
nalidixic acid		0.14	0.14		0.71		
rimfampicin						1.00	
doxycycline							1.00
tetracycline							1.00

The scoring results of the class categorization model trained with $A_n(t)$ are visualized using a table called Confusion Matrix shown in Table 4. The vertical axis represents the antibiotic compounds that were used in the sample, while the horizontal axis represents the class-categorization results by the MDF model. The numbers in the grids represent the ratio of the corresponding sample being classified into the correct class. The black-boxed grids represent the ones that were accurately classified.

Most of the 11 antibiotics were accurately categorized, with nine accuracies higher than 80 %. It is more crucial to detect the existence of antibiotic in a sample than identifying the compound, meaning that this model shouldn't have high false-negative and false-positive rates for Blank. In Table 4, samples with antibiotics could be detected except for Cefapirin, which has a rate of 0.14 to be categorized as Blank. In other words, all antibiotics except Cefapirin show no false-negative rate. For the blank samples, there is only a rate of 0.14 to be categorized as phenicols, showing that this model has a low false-positive rate for Blank.

For categorization, there were low false-categorization rates for Cefeparin (14%), Ceftiofur(14%), Nalidixic Acid (28%) and Amoxicillin (40%) and no false-categorization results for the other antibiotics. Compared to what was observed in the radar chart in Figure 3(e), in which Cefapirin (β-lactam class) and Rifampicin (rifampicin class) could not be distinguished statistically, the MDF model successfully told apart from the two classes. The result demonstrated the superiority of using ML for categorization.

Categorization models for different concentrations of antibiotics:



(a)

Concentration (ppb)	Thl	Amx	Amp	Cef	Cip	Rim	Dcy	Tcy
1000	0.86	1.00	1.00	1.00	1.00	0.71	1.00	0.86
500	0.57	0.71	1.00	1.00	0.86	0.57	0.86	0.86
250	0.86	0.86	0.86	1.00	0.71	0.71	0.71	0.71
125	0.86	0.57	0.86	1.00	0.86	0.29	0.71	0.71

Reaction time: 4hr / 10hr

Figure 5. (a) line chart of the classification accuracy change of Rifampicin at 1000 ppb, Tetracycline at 500 ppb, and tetracycline at 125 ppb through time. (b) Scoring result of the combination method classification. The letters in red were the accuracies obtained in the fourth hour while the letters in blue were those obtained in the tenth hour. (Thl= Thiamphenicol, Amx= Amoxicillin, Amp= Ampicillin, Cef= Ceftiofur, Cip= Ciprofloxacin, Rim= Rifampicin, Dcy= Doxycycline, Tcy= Tetracycline.)

We further tested whether the MDF categorization could be useful for antibiotics at different concentrations. In this research, four lower concentrations (1000 ppb, 500 ppb, 250 ppb, and 125 ppb) of eight chosen antibiotics were used

to demonstrate the ability for the MDF categorization method to distinguish antibiotics at different concentrations.

A classification model was trained with the $\boldsymbol{A_n(t)}$ indices and the concentration value of the 32 kinds of antibiotic samples. The time *3*hr ≦ t ≦ *10*hr were used fo$\boldsymbol{A_n(t)}$. It was found that the same compound of antibiotics with different concentrations in the experimental range didn't emit bioluminescence with significant differences. Therefore, the classification model was designated to output the class but not the concentration of the antibiotic in a sample.

From the scoring results, the antibiotic samples could be partitioned into two groups, depending on their classified accuracy through time. For antibiotics in higher concentrations (≥ 500 ppb), they were classified accurately in the fourth hour but dropped their accuracy through time. However, for antibiotics in lower concentrations (≤ 250 ppb) and the antibiotic Rifampicin, which imposed weaker stress, they were classified poorly in the fourth hour, but more accurately in the 10th hours. Three examples were shown in Figure 5(a).

A combination of two models was applied to enhance the accuracies for low concentration and Rifampicin classification. The scoring results of this combination method were shown in Figure 5(b) The final model was trained with the data in the fourth hour (letters in red) and modified using the model trained with the data in the tenth hour (letters in blue). This model categorized the eight antibiotics at a concentration range from 125 ppb to 1000 ppb with accuracies mostly higher than 70%. Except for Rifampicin at 125 ppb, which may be too weak for the present bioreporters to detect.

Table 6. Confusion matrix of the classification model with three unknown antibiotics

	blank	*phenicols*	*β-lactams*	*quinones*	*naphthyridones*	*rifampicins*	*tetracycliness*
blank	0.71	0.29					
chloramphenicol	0.29	0.71					
amoxicillin		0.14	0.71		0.14		
cefapirin		0.14	0.86				
ceftiofur	1.00		0.86				
ciprofloxacin				1.00			
nalidixic acid		0.14	0.14		0.71		
rifampicin			0.14			0.86	
tetracycline							1.00
thiamphenicol	0.14	0.71	0.14				
ampicillin			1.00				
doxycycline							1.00

As mentioned, the categorization method classified antibiotics based on the similar patterns caused by similar stress factors. This mechanism enabled the categorization method to categorize an unknown compound of antibiotic if an antibiotic from the same class had been included in the training database.

The training dataset of the classification model in Table 4 was modified to demonstrate the ability of the method to classify unknown antibiotics. Three compounds of antibiotics (Ampicillin, Thiamphenicol, Doxycycline) were excluded from the training database, representing unknown antibiotics, with at least one same-class antibiotic included in the database. A new model was trained using this database and scored using the data of the three removed ones. The confusion matrix of the model is shown in Table 6. The overall accuracy also dropped from 90% to 85 %, which occurred in both the retained and excluded antibiotics. The accuracies of the three excluded antibiotics were all greater than 70%, showing that they were well classified even if they weren't in the database.

The ability to detect and categorize unknown antibiotics reveals another advantage of this method. For most of the existing antibiotic detection methods, a database for all the antibiotic compounds needs to be established in order for those methods to work. However, for our method, only the representative antibiotics for each MOA class need to be in the luminescence pattern database, and the remained ones could be successfully detected and categorized. Not only could it

save lots of time in database establishing, but also newly created antibiotics with no detailed information could be tested to see which MOA class might them belonged to. More detailed comparisons with existing antibiotic detection methods would be listed in the next paragraph.

Comparisons with existing antibiotic detection and categorization methods:

Two most commonly used existing antibiotics detection methods are High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) (Lúcia Santos, Fernando Ramos. , 2016) and Enzyme-Linked Immunosorbent Assay (ELISA) (Mahgoub, Osman & Kadim, Isam & Mothershaw, Ann & Zadjali, S.A. & Annamalai, K.. 2006.).

In HPLC-MS, the HPLC separates samples into different components and the MS identified the components by their mass-to-charge ratio (m/z). This method could detect and identify the antibiotic residues in samples with significantly high accuracy and could even tell the amounts or concentration of the residues. Due to its high precision, HPLC-MS serves as the government regulations for many countries. However, at the cost of its precision, the MS instruments are relatively expensive, and the amount of the instruments is rather small, meaning that it's not practical for many samples to be tested by this method. In comparison, the bacterial array method could detect the presence of antibiotics with almost no false-negative rates, and categorize the antibiotic residues in an accuracy of about 90%. The accuracy may not be as high as HPLC-MS, however, the only instruments for the bacterial array method are just cameras and incubators, which are much more affordable and convenient than MS. Therefore, the method could serve as a widely screening process that could be implemented on large numbers of samples, and if necessary, the detected samples could be further tested with HPLC-MS to tell the concentration of the residues.

ELISA is a screening method that is widely used in different detection fields. The antibody in the test kit binds to the specific antigen in the sample and the enzyme on the immobilized antibody emits visible signals that could be recognized. The process of this screening method could be done in a short time, however with relatively low accuracy, and high false-negative rates. Also, one ELISA antibodies could only detect one compound of antibiotic, meaning that for an unknown sample, one may need to utilized many times of different ELISA antibodies to meet most of the possibilities of the compounds.The bacterial array method, in comparison, could give its results in four to ten hours with much higher accuracies, and by doing only one time of test, all the antibiotic classes in the database could be detected and categorized. Moreover, the establishment of the luminescence database could be done in just seven times of E.coli incubation and monitoring, while the creation of an antibody with specific selectivity is rather complicated. These reasons clearly show the importance of the bacterial array method.

Conclusion

A bacterial array solid-phase assay (BacSPA) method was established to detect and categorize antibiotics in samples. Suspension of 15 strains of *E. coli* genetically modified with *lux:CDABE* reporter genes were spotted on solidified agar individually mixed with 11 different antibiotics from 7 classes at 1.5 ppm concentration. The antibiotic stimulated the bacteria and the induced bioluminescence from different strains generated different patterns for different antibiotic classes. The luminescence pattern was monitored by time-lapse photography and the data were processed into four kinds of indices for antibiotic categorization. The machine learning algorithm, Multiclass Decision Forest, was applied to train categorization models that either identify the compound or categorize the class of antibiotics using the indices.

The omnibus indices tended to integrate the advantages of the other three indices and resulted in the best accuracy of 65% for compound identification and 90% for class classification in three hours. The method was further tested for categorizing antibiotics at different concentrations. A combination of two models from the fourth and tenth hour could categorize the eight chosen antibiotics at a concentration range from 125 ppb to 1000 ppb with accuracies mostly higher than 70%. Also, three antibiotics were excluded from the training database and successfully categorized. It demonstrated the ability of the categorization method to categorize an unknown compound of antibiotics if an antibiotic from the same class had been included in the training database.

The bacterial array method is fully extendable, with more luminescence data monitored, the detection range of this method would be wider and wider. We hope to establish the database of the luminescence pattern for more kinds of antibiotics at different concentrations and with different combinations in the future. The method would become more powerful, and it could surely provide a good way for people to detect and categorize antibiotic residue in food or environment samples.

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抗生素濫用及耐藥性細菌的存在實為本世紀重要的課題，故本文研究動機及想法很有創意，建構螢光偵測系統利用已知濃度的抗生素及菌種做機器學習，希望作為未來抗生素檢測的方法。然而實驗測試中何以不同的抗生素會刺激細菌，使其產生特定的生物冷光圖形有不同的變化，作者未詳述其原理，是否所檢測到的讀值具有實質意義，相關對照組應詳列，且機器學習需要大量的數據，本研究學習的資料不多，是否可以有顯著性地反應測試的結果有待進一步驗證。

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