RAPID AND HIGH THROUGHPUT ANTIMICROBIAL SUSCEPTIBILITY TEST USING MORPHOLOGICAL ANALYSIS OF SINGLE CELLS WITH MICROFLUIDIC CHANNEL IN 96 WELL PLATFORM

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ABSTRACT

The rise in antimicrobial resistance has become a serious global health problem. For proper treatment of antimicrobial resistive bacteria and suppress the antimicrobial resistance, rapid antibiotic susceptibility test (AST) for clinical application is urgently needed. However, currently available AST platforms need 9 hours in average to obtain the susceptibility result. In this research, we develop a rapid AST based on the morphological determination in single cell resolution. For single cell tracking, we use the agarose as fixing agent in the microfluidic channel which is integrated with 96 well plate for user friendly and high throughput test for clinical application.

KEYWORDS: Antimicrobial susceptibility test, Single cell tracking, Morphological analysis, Microfluidic channel

INTRODUCTION

Antibiotic resistance increases after invention of the antibiotics and has become a upcoming problem[1]. Early initiation of correct therapy through rapid AST could reduce the emergence of the resistant bacteria[2]. The currently used AST platform in clinic rely on measuring the growth of bacterial population in the presence of antibiotic and, therefore, need more than 9 hours of incubation time for differentiate the susceptible and the resistive strains.[3] To reduce the time for AST, there are two kinds methods. The one group is using non-incubation method, such as : PCR-based techniques, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), DNA microarray. The other group is using incubation with imaging of single cell resolution in microfluidic chip. The limitations of these methods are that those methods are not validated for common strains for clinical application and has much difficulty to scale up to clinical application. Also, those methods are expensive and not user friendly. [4] Moreover, for image based application, such as real time imaging, they rely on measuring pixel number increased with bacterial growth. However, there are many cases that expansion of bacteria volume in the susceptible cases [5].

We previously demonstrated the rapid AST by tracking single cell growth in microfluidic channel[6]. This method is measuring the bacterial growth like currently used method, but, in single cell resolution. Therefore, the susceptibility of bacteria to the antibiotics could be determined in 3~4 hours. For fixation of motile bacteria, agarose was used for fixing agent and also for medium for antibiotic delivery by diffusion.

However, it was not useful for high throughput application due to the PDMS material and external pump and small number per chip. To solve this problem, we integrate with 96 well plate format in injection molding available material (PMMA). Moreover, we used the morphological analysis for determining the susceptibility of bacteria to the antibiotics. Measuring the area of bacteria can't be applied to the cases of beta-lactam antibiotics to Gram negative bacteria (*E.coli, K. pnuemonea, P. aeruginosa*). In the image, it prolongs or expands but actually it can't divide and susceptible to the antibiotics. We categorized such cases and established a specific determination standard for bacteria with antibiotics.

EXPERIMENTAL

The microfluidic chip contained the microfluidic channel and well for testing antibiotics were fabricated injection molding in the material of poly carbonate. The design of the microfluidic channel and the procedure of test is described in figure 1 and 2. The bacteria in preheated agarose were injected to the inlet of the channel and the bacterium was fixed after solidification of agarose. The antibiotics were applied to the each well and were diffused into the agarose. By measuring the growth of the bacterium under microscope in time lapse method (fig. 3), we could determine the MIC of this bacterium. For high throughput AST, 96 different antibiotics with different concentration could be tested in whole chip (fig. 4). Usually, the growth of bacteria is inhibited in sensitive case (fig 5.A) and the growth of resulted in formation of colony of bacteria (fig 5.B). However, in the case of Gram-negative with beta-lactam antibiotics, the bacteria formed filament which means sensitive to the antibiotics. Using morphological analysis of the images we could determine the susceptibility of bacteria in 3 hours.

RESULT AND DISCUSSION

For system validation, we tested 4 standard strains (*E. coli* ATCC 25922, *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853 and *E. faecalis* ATCC 29212) and checked that the values of minimal inhibitory concentration (MIC) obtained in 3 hours *(E. coli* and *P. aeruginosa*) and 4 hours (*S. aureus* and *E. faecalis*) from RAST are within the MIC range suggested from Clinical and Laboratory Standards Institute (CLSI). Further, the 39 clinical samples are tested

using RAST. As in table 1., the susceptibility data was obtained from RAST derives the result with 4.5% minor, 0.9% major and 0.7% very major discrepancies in comparison with gold standard method (broth-microdilution test). The recommendation from FDA for AST platform is 10% in minor error cases, 3% in major error cases and 1.5% in very major error cases. Therefore, this system satisfied the FDA recommendation. The morphological determination based RAST shows great potential for rapid susceptibility testing for clinical application.

The advantages of the proposed system is new method to overcome the fundamental limit of current method, rapid AST which can be applied to same day treatment, new insight of antibiotics effect to the bacteria and image checking available for error cases (Vitek2 is not available). However, there are also limitations that there is no bacterial identification procedure which is necessary for antibiotic prescribe. Therefore, this system should be related with other rapid bacterial identification system. Also, the system is not automated yet. For the further work, we are developing the automated imaging system and image analyzing program.

CONCLUSION

The presented system tracks the bacteria growth using agarose channel in 96 well format. The growth pattern and morphological analysis were used to determine the susceptibility of the antibiotics. By this analysis, the AST result was derived within 4 hours in the quality of FDA approval. This rapid AST system can be used in hospital for the full range of AST test.

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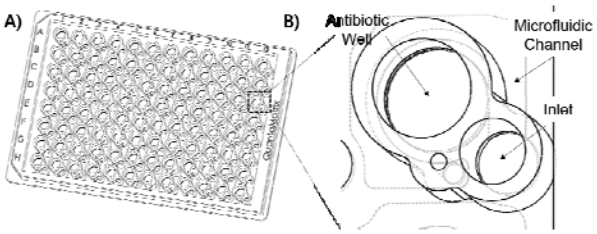

Figure 1: (A) 96 well plate format Microfluidic Agarose Channel (MAC) Chip (B) Detail of the single well

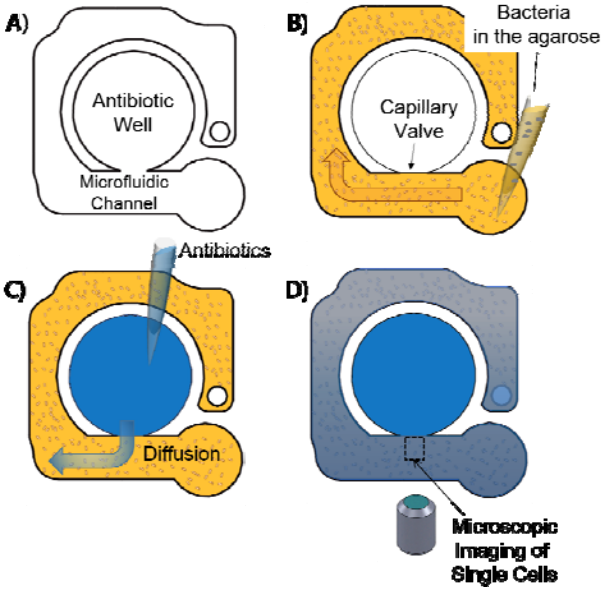

Figure 2. The process of the AST in the MAC chip.(A),(B) The bacterium is mixed with agarose and then injected into inlet. (C) Antibiotic solution is applied to the well.(D) The bacterial cell growth is tracked under microscope using time-lapse method.

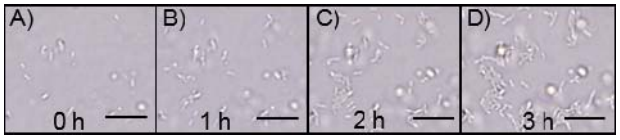

Figure 3. Time lapse image of single cell tracking method. The time duration is one hour. The scale bars represent 20 μm.

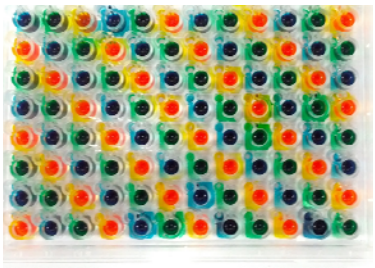

Figure 4. 96 antibiotics tests in one chip for high throughput test for clinical application.

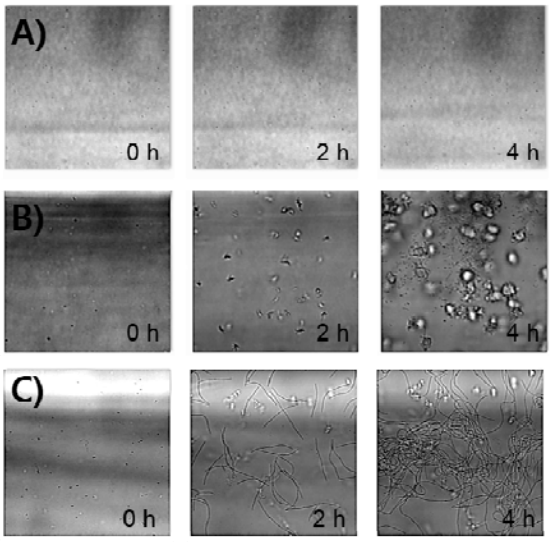

Figure 5. Morphological Determination of Susceptibility of antibiotics in case of P. aeruginosa (A) Sensitive case (B) Resistance case (C) Sensitive case of filament growth.

Table 1. Result of Clinical Sample Test

S. aureus	Error	cases	Percentage	Total cases	130
	MINOR	4	3.1%	Total S	75
	MAJOR	0	0.0%	Total I	0
	VERY MAJ	1	1.8%	Total R	55
P. aeruginosa	Error	cases	Percentage	Total cases	50
	MINOR	7	14.0%	Total S	31
	MAJOR	0	0.0%	Total I	7
	VERY MAJ	0	0.0%	Total R	12
E. coli	Error	cases	Percentage	Total cases	130
	MINOR	7	5.4%	Total S	63
	MAJOR	0	0.0%	Total I	6
	VERY MAJ	0	0.0%	Total R	61
E. faecalis	Error	cases	Percentage	Total cases	80
	MINOR	3	3.8%	Total S	58
	MAJOR	2	3.4%	Total I	3
	VERY MAJ	0	0.0%	Total R	19

Total cases	377	Error	cases	Percentage	FDA Standard
Total S	224	MINOR	17	**4.5%**	**≤10%**
Total I	14	MAJOR	2	**0.9%**	**≤3%**
Total R	139	VERY MAJ	1	**0.7%**	**≤1.5%**