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Short communication

FRET probe-based antibacterial susceptibility testing (F-AST) by detection of bacterial nucleases released by antibiotic-induced lysis

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ABSTRACT

This study demonstrates a novel and rapid antibacterial susceptibility testing (AST) method, called fluorescence resonance energy transfer (FRET) probe-based AST (F-AST), which relies on a nuclease-activated FRET probe that detects bacterial nucleases released by antibiotic-induced bacterial lysis. Three quality control (QC) strains and two additional clinically important strains were tested, and the minimum inhibitory concentration (MIC) values from both gold standard AST method (broth microdilution (BMD)) and the new F-AST method were compared. The resulting fluorescence signals from the F-AST method were obtained within 3–6 h and were consistent with MIC values obtained from the BMD method, which took more than 16 h. Thus, the F-AST method is a simple and rapid tool to detect antibacterial susceptibility, including MIC values, and provides a basis for rapid clinical treatments.

1. Introduction

The prevalence of infectious diseases caused by antibiotic-resistant pathogens is one of the most common causes of health complications and mortality worldwide (Sciarretta et al., 2016). Due to the widespread use of broad-spectrum antibiotics, multidrug-resistant pathogens are increasing significantly. To decrease the overuse and misuse of broad-spectrum antibiotics in preventing antibacterial resistance, antibacterial susceptibility testing (AST) is an essential step for the clinical treatment of infectious diseases (Barenfanger et al., 1999; Levy and Marshall, 2004).

The most widely used AST methods in clinical microbiology laboratories are based on observations of bacterial growth in the presence of the antibiotic being tested. Conventional culture-based methods, such as the disc diffusion test, E-test strips (bioMérieux, France), broth macrodilution, broth microdilution, and agar dilution susceptibility testing are time-consuming and labor-intensive. These traditional methods require 16–24 h to yield results (Jorgensen and Ferraro, 2009; Syal et al., 2017). To reduce hands-on and incubation times, various automated instrument systems have been developed, such as the VITEK2® (bioMérieux), MicroScan (Siemens, Germany) and BD Phoenix (BD Diagnostic Systems, USA) systems (Jorgensen and Ferraro, 2009). More recently, novel approaches to rapidly detect antibiotic resistance, such as PCR-based techniques, mass spectrometry, microarrays, microfluidics, and whole-genome sequencing, have been introduced in clinical microbiology laboratories. However, these techniques still require bulky, expensive equipment and/or skilled personnel (Choi et al., 2014; Dong and Zhao, 2015; Kaushik et al., 2017; Lee et al., 2017; Pulido et al., 2013).

Nucleases are enzymes that cleave the phosphodiester bonds of nucleic acids and may be endo- or exo-nucleases, DNases, RNases, restriction enzymes, or CRISPR/Cas9 systems (Bikard and Barrangou, 2017; Sato and Takenaka, 2014; Yang, 2011). Nucleases are involved in many biological processes, such as replication, DNA repair, recombination, and protection against viruses (Ceska and Sayers, 1998; Perona, 2002). Due to the biological and technological importance of nucleases and their useful applications in clinical research and in biotechnology, many different techniques have been developed to detect their activity (Dai and Kool, 2011; Su et al., 2013; Xu et al., 2015). In our previous study, simple and rapid detection of bacteria was

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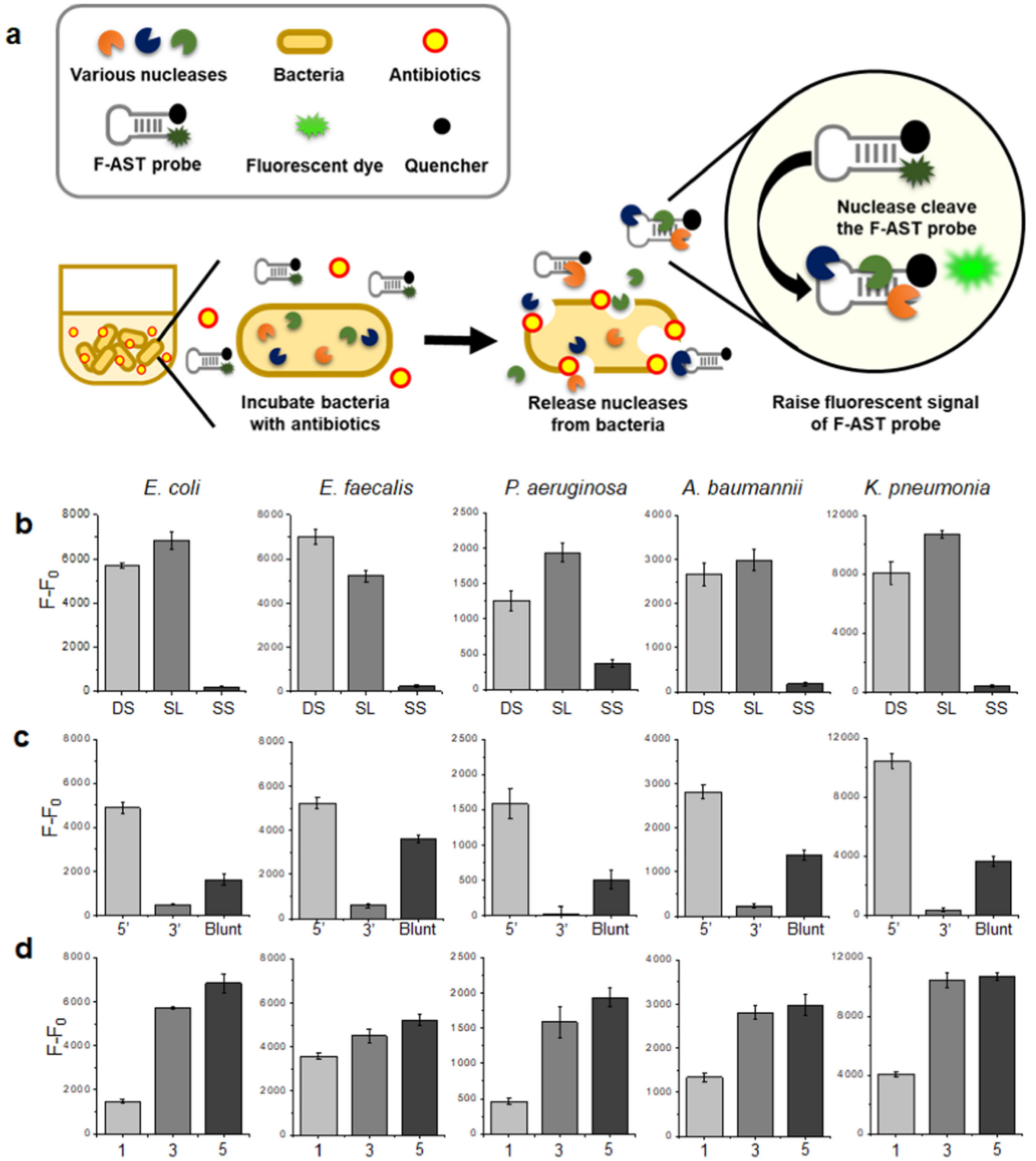

Fig. 1. (a) Schematic illustration of the F-AST method using a FRET probe to detect nuclease activity released by antibiotic lysis. (b-d) Optimization tests of the FRET probe for five different bacteria strains, including *E. coli*, *E. faecalis, P. aeruginosa, A. baumannii,* and *K. pneumonia* under various conditions, such as (b) probe shapes (double-stranded (DS), stem-loop (SL), and single-stranded (SS)), (c) overhang types (5′, 3′, and blunt) of SL, and (d) 5′-overhang length (1, 3, or 5 bases) of SL-5′. All experiments were conducted in triplicate.

performed by determining nuclease activity in a quantitative manner using a nuclease-responsive DNA probe via fluorescence resonance energy transfer (FRET) (Lee et al., 2018). In particular, we developed a well-designed DNA probe, which can be efficiently cleaved by various nucleases from either gram-negative or gram-positive bacteria within several minutes.

Based on our previous results, in the present study, we developed a FRET probe-based AST (F-AST) for rapid detection of antibiotic

Table 1
Comparison of MIC values and acceptable MIC ranges for QC strains given by the CLSI (2017) guidelines for six antibiotics determined by BMD and F-AST.

QC strains	Antibiotics	Test/report group	Testing range (μg/mL)	CLSI-approved QC range (μg/mL)	MIC by BMD (μg/mL)	MIC by F-AST (μg/mL)
E. coli	Ampicillin	A	0, 1 – 32	2–8	8	8
	Meropenem	B	0, 0.002 – 0.06	0.008–0.06	0.015	0.008
E. faecalis	Ampicillin	A	0, 0.25 – 8	0.5 – 2	2	1
	Penicillin G	A	0, 0.25 – 8	1 – 4	2	1
	Vancomycin	B	0, 0.25 – 8	1 – 4	2	2
P. aeruginosa	Colistin	O	0, 0.25 – 8	0.5 – 4	2	2
	Meropenem	B	0, 0.06 – 2	0.25 – 1	0.12	0.12
	Piperacillin	O	0, 0.5 – 16	1 – 8	2	2
A. baumannii	Colistin	O	0, 0.25 – 8	–	2	2
	Meropenem	A	0, 0.12 – 4	–	1	1
K. pneumoniae	Colistin	–	0, 0.25 – 8	–	1	1
	Meropenem	–	0, 0.004 – 0.12	–	0.03	0.03

susceptibility. To the best of our knowledge, this is first study to report minimum inhibitory concentration (MIC) values of clinically important strains using a FRET probe with comparable accuracy to the broth microdilution (BMD) method, which is the gold standard for AST. Moreover, the resulting time to obtain MIC values using the F-AST method was dramatically reduced from that of the BMD method. Therefore, the F-AST method can be a powerful tool for rapid detection of antibacterial susceptibility in short clinical treatments.

2. Results and discussion

2.1. Optimizing the FRET probes for F-AST

Fig. 1a illustrates the F-AST detection strategy, which was designed using an optimal DNA probe to detect the activity of nucleases released from bacterial lysates caused by antibiotic action. The FRET probe basically consists of a nuclease substrate with a 6-carboxyfluorescein (FAM) at its 5′ end 5 a black hole quencher 1 (BHQ-1) moiety at its 3′ end between the oligomers. The DNA sequence and structure were modified from our previous probe for the optimization of AST. The sequences and structures of the probes are shown in Table S1.

We first compared three different probes (double-stranded, stem-loop, and single-stranded probes indicated as DS, SL, and SS, respectively) to identify the optimal AST structure. For the test, both the DS and SL probes were hybridized to achieve stable structures, while the SS probe was denatured to prevent double-stranded formations. To compare the results of the probes in different bacteria, five important clinical strains (*E. coli, E. faecalis, P. aeruginosa, A. baumannii,* and *K. pneumonia*) were used. To measure the fluorescence signal of the FRET probes, the bacteria were incubated with antibiotics and the different FRET probes for 5 h at 35 °C. Ampicillin was tested at 16 and 4 μg/mL for *E. coli* and *E. faecalis*, respectively. In the cases of *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*, colistin was tested at 4, 4, and 2 μg/mL, respectively. The antibiotic concentrations were two times higher than the MIC value evaluated by the BMD method. After 5 h of incubation, the fluorescence signal was detected by a microplate reader. F-F_0 represents the fluorescence intensity difference, and F_0 is the fluorescence intensity of the growth control well (drug-free control: 0 μg/mL). As shown in Fig. 1b, the fluorescence signals, in order of highest to lowest, were in the following order: SL > DS > SS probe for all strains except *E. faecalis* for which the signal from the DS probe was a little higher than that of the SL probe. Thus, we chose the SL probe as an optimal DNA structure and tested further modified SL probes with different ends, such as a 5′- or 3′-overhang or blunt ended. As shown in Fig. 1c, the probes with 5′-overhangs revealed higher fluorescence signals than the other end-modified probes, and fluorescence was hardly exhibited in the case of the 3′-overhang probe. Subsequently, we also tested SL probes having 5′-overhangs of various lengths (e.g., 1, 3, and 5 bases) in the same manner. Fig. 1d shows that the fluorescence signal gradually increased with the length of the 5′-overhang. On the basis of these results, a stem-loop structure with a 5′-overhang of 5 bases was chosen as the optimal F-AST probe for the different bacterial strains.

2.2. Reference MIC values determined by BMD

Standard testing using QC strains is essential in clinical microbiology to guarantee AST results (Marsik, 2011; Karatuna, 2012). The BMD method was chosen as the reference method for this study and was performed according to CLSI guidelines (Marsik, 2011; Karatuna, 2012; Ericsson and Sherris, 1971). Published guidelines also suggest testing precision using three separate strains, as characterized by the control strains (Njoroge and Nichols, 2014). Therefore, *E. coli, E. faecalis*, and *P. aeruginosa*, which are the most commonly used QC strains for AST, were selected and tested to provide reliable antibiotic susceptibility results. More details about antibiotic selection criteria are described in Supporting information. In addition, we also tested two more clinically significant strains, *A. baumannii* and *K. pneumonia*.

To obtain reference MIC values for the BMD method, individual antibiotics were prepared in six, two-fold dilutions (e.g., 1, 2, 4, 8, 16, and 32 μg/mL) in a 96-well plate, including two negative controls, drug-free (0 μg/mL), and sterility (uninoculated) controls; and bacterial suspensions were also inoculated. Following an overnight incubation at 35 °C, bacterial growth was measured by turbidity using a microplate reader, and the MIC value was defined as the lowest antibiotic concentration that inhibited bacterial growth. The acceptable MIC ranges from the CLSI guidelines for the three QC strains for various antibiotics are presented in Table 1. In the absence of an acceptable MIC range according to CLSI guidelines, such as for *A. baumannii* and *K. pneumonia*, we measured their MICs by BMD to compare with F-AST results.

Table 1 summarizes the results of MICs and proposed testing ranges determined by the standard BMD. The MIC values of *E. coli* were 8 μg/mL for ampicillin and 0.015 μg/mL for meropenem. We also obtained MICs for ampicillin, penicillin G, and vancomycin against *E. faecalis* in the same manner, and the MICs were 2 μg/mL for ampicillin, 2 μg/mL for penicillin G, and 2 μg/mL for vancomycin. The MIC values for *P. aeruginosa* were detected at 2 μg/mL for colistin, 0.12 μg/mL for meropenem, and 2 μg/mL for piperacillin. The MIC values for *A. baumannii* were 2 μg/mL for colistin and 1 μg/mL for meropenem. The MIC values for *K. pneumoniae* were 1 μg/mL for colistin and 0.03 μg/mL for meropenem. These results indicate that the three QC strains evaluated using the standard BMD method provided MIC values that were all within the acceptable CLSI QC ranges for the particular antibiotics. These data suggest that the QC strains and antibiotics were suitable to apply for the test.

2.3. MIC values determined by F-AST

To validate the accuracy of the F-AST method, we performed F-AST with the five strains that were previously tested and verified by the BMD method. The F-AST method was designed to follow the BMD

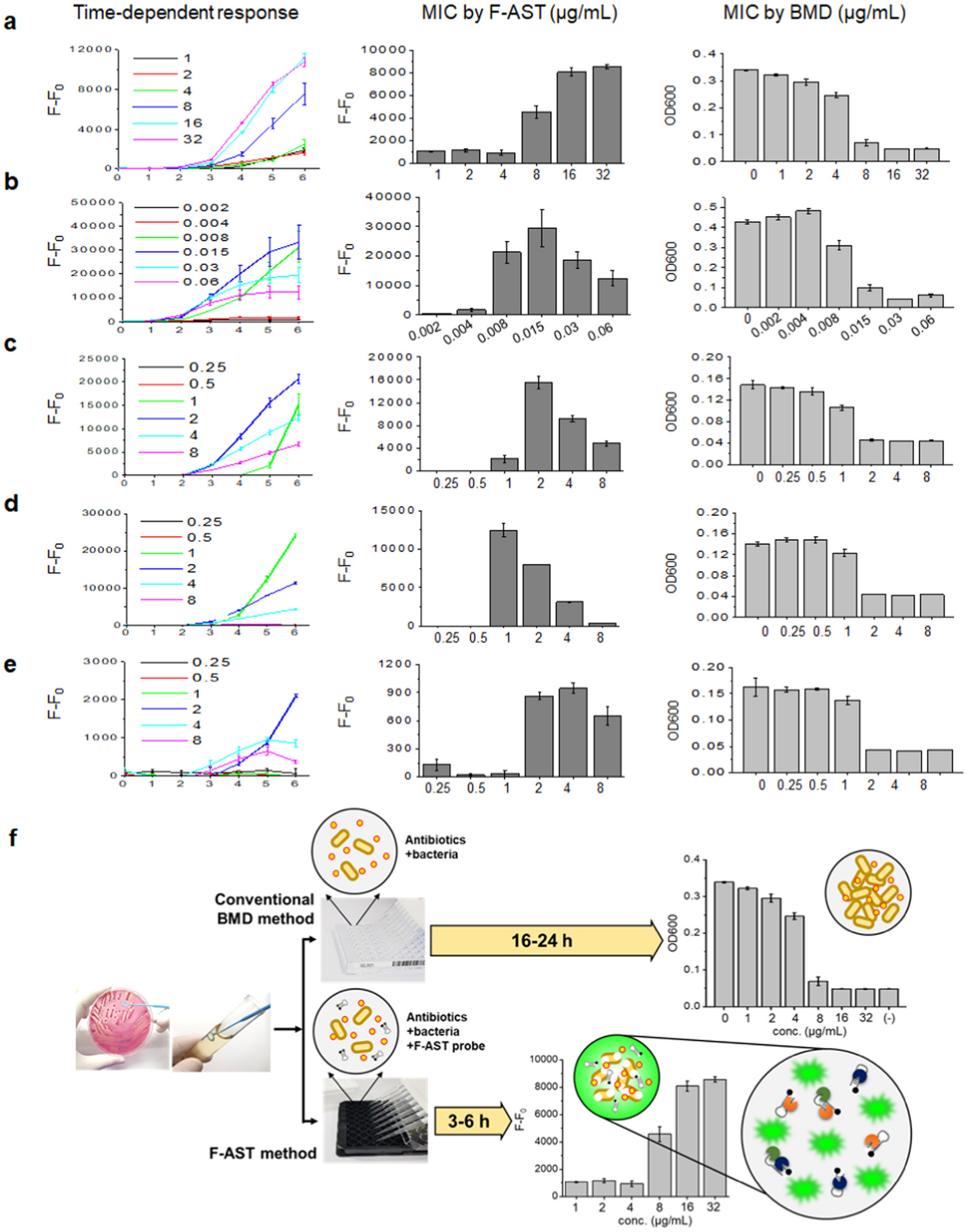

Fig. 2. (a-e) The MIC values of *E. coli and E. faecalis* with various antibiotics determined by measuring the fluorescence signals from the antibiotic treatments using the F-AST method and turbidity using the BMD method. *E. coli* was tested with (a) ampicillin and (b) meropenem and *E. faecalis* was tested with (c) ampicillin, (d) penicillin G, and (e) vancomycin. All experiments were done in triplicate. (f) A comparison of the F-AST and conventional BMD, as a gold standard AST method.

protocol with some modifications. To obtain the optimized reaction buffer, various reaction buffers were tested, including restriction enzyme buffers and nuclease reaction buffers. Finally, the restriction enzyme buffer (known as CutSmart buffer) was used for *E. coli* and *E. faecalis*, and the DNase I reaction buffer was used for *P. aeruginosa, A. baumannii*, and *K. pneumonia* (Fig. S1). A bacterial suspension was grown in 96-well plates with antibiotics and the FRET probe at 35 °C. The results were obtained by measuring fluorescence intensities every hour during the incubation.

According to the time-dependent responses shown in Fig. 2a-e and Fig. S2, the fluorescence signals increased at antibiotic concentrations in which bacteria died, and fluorescence intensity increased over time. Meanwhile, the fluorescent signal increased very little at lower antibiotic concentrations. The MIC value from the F-AST method was defined as the lowest antibiotic concentration at which the fluorescence signal increased. We also found that the fluorescence signal did not always increase with increasing antibiotic concentration, presumably due to high doses of antibiotics killing bacteria initially, resulting in lower bacteria growth and weak fluorescence signal detection.

Mostly, the fluorescence intensity differences between live and dead bacteria began to increase at 3 h of incubation and was clearly distinguishable at 5 h, except for colistin and piperacillin. The fluorescence signal for colistin started to increase at 2 h and decreased at 4 h, while the signal from piperacillin was detectable at 5 h (Fig. S2). The difference in reaction times may be caused by the mechanisms of the antibiotics. For example, colistin shows extremely rapid initial killing against *A. baumannii, K. pneumoniae,* and *P. aeruginosa* (Bergen et al., 2010; Owen et al., 2007; Poudyal et al., 2008) compared to other antibiotics. Therefore, we selected different fluorescence measurement values, at 3 h for colistin to avoid the decrease in total signal, while the values for ampicillin and meropenem were 5 h and 6 h for piperacillin, values which represent the concentration-dependent curves of F-AST in a similar manner to those from the BMD method.

Consequently, the fluorescence signals against *E. coli* increased at concentrations > 8 μg/mL for ampicillin and > 0.008 μg/mL for meropenem (Fig. 2a-b). We also obtained the signals for ampicillin, penicillin G, and vancomycin with *E. faecalis* in the same manner, and the results were > 1 μg/mL, 1 μg/mL, and 2 μg/mL for ampicillin, penicillin G, and vancomycin, respectively (Fig. 2c-e). The MIC values for *P. aeruginosa* were detected at > 2 μg/mL for colistin, 0.12 μg/mL for meropenem, and 2 μg/mL for piperacillin. For *A. baumannii*, the MIC values for colistin and meropenem were 2 μg/mL and 1 μg/mL, respectively. The MIC values for *K. pneumoniae* were 1 μg/mL for colistin and 0.03 μg/mL for meropenem (Fig. S2). The MIC values obtained by F-AST were compared with those of the BMD method and were similar or two-fold lower than the references, indicating that the accuracy is enough to substitute for the BMD method with reduced times (Fig. 2a-e, Fig. S2, and Table 1). The illustration in Fig. 2f clearly shows the difference between F-AST and the conventional BMD method by comparing the procedures. The entire F-AST procedure was designed based on the BMD method, one of the most widely used AST methods in the clinical field. Both started from a concentration-adjusted bacterial suspension after an overnight bacterial culture in an agar plate. Then, the F-AST method results were obtained within 3–6 h of incubation, while the conventional culture-based AST system required an overnight incubation time (16–24 h) for accurate AST results. The F-AST method also provides quantitative MIC values similar to the BMD method.

3. Conclusions

In summary, we demonstrated the F-AST method, which relies on detecting the activity of bacterial nucleases released from antibiotic-induced bacterial lysates. Compared to the traditional culture-based AST, BMD, which takes 16–24 h, our F-AST method provides results, including MIC values, within 3–6 h. This study indicates that F-AST can rapidly differentiate resistant and susceptible strains in agreement with a standard AST and provide a basis for novel rapid AST methods. As a consequence, the F-AST system is rapid (assay can be completed within 3–6 h), cost-effective (needs only a FRET probe), and easy to operate (requires a common fluorescence microplate reader). Thus, we believe that this simple, facile, and rapid AST system based on a FRET probe can reduce inappropriate antibiotic use, decrease healthcare costs, and reduce the prevalence of antibacterial resistance.

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Declaration of interests

None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2019.01.033.

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