Bioluminescence Based Minimal Inhibitory Concentration (MIC) Determination for Biofilm Forming Bacteria

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ABSTRACT

Determination of antimicrobial susceptibilities of infectious agents is important in human and veterinary medicine. The aim of this study was to test minimal inhibitory concentrations (MIC) and bactericidal effects of antibiotics using light emission properties (bioluminescence) of live bacteria. In addition, this study tested the possibility to using the measurements of light emissions to evaluate the effects of antimicrobials on the bacteria in biofilm. Light emission requires presence of the *lux* operon that consists of *luxCDABE* genes of *Photorhabdus luminescens* which gives light only in living cells. These genes were inserted in pAT28 plasmid and used for transformation of *E. coli* DH10B and biofilm forming *E. coli* ADU40. Light output provided a sensitive method for real-time measurements of the effects of antibiotics. The results of our study showed that using light emission properties of live bacteria the MICs of antibiotics were in agreement with the results of the conventional microdilution method. Decrease of light emission of bacteria >10 fold was considered as a bactericidal effect. Results showed that addition of chloramphenicol, meropenem, amicasin, rifampisin gentamycin ciprofloxacin, cefazolin and cefoxitin to bacterial suspensions caused >10 fold decrease of light emission at MIC levels after 18 hours of incubation, in both biofilm producer and non-producer bacteria. It was concluded that Bioluminescence can be used for the determination of both MIC and bactericidal effects by antibiotics on bacteria. These methods have an important potential for use especially to determine the effects of candidate antimicrobials in veterinary and human medicine.

Keywords: Antimicrobial Effect; *lux* Operon; Cloning; Light Emission Measurements.

INTRODUCTION

The use of bioluminescence molecules in living cells has become widespread in different fields of science. Since the discovery of firefly luciferase, it has been widely used in various *in vitro* and *in vivo* systems to detect pathogenic bacteria and viruses, measure protein-protein and protein-ligand interactions, and test metabolites involved in cell communication and cell signaling (1). Bioluminescent bacteria are mainly found in marine habitats. All bioluminescent bacteria utilize the same unique mechanism for light emission, where photons are produced in a set of reactions requiring flavin mononucleotide (FMN), myristic aldehyde, oxygen and nicotinamide adenine dinucleotide (NADH) (1, 2). Bacterial luciferase found in *Vibrio harveyi* is a bioluminescence producing protein (3). For the synthesis of naturally occurring light from bioluminescent bacteria, five genes (*luxC*, *luxD*, *luxA*, *luxB*, *luxE*) are required within an operon (4). Luciferase reporter systems have been used for various purposes, such as measuring immunity to mycobacterial infection, testing the activity of new antimicrobial drugs, bacterial infections and environmental monitoring (5, 6).

Culture method is the most basic technique to determine whether microorganisms are viable and functional. In recent years, many different methods have been developed to de-

termine the viability of microorganisms. It is important to determine the viability of bacteria in biofilm structures that help them to grow and survive under unfavorable conditions. Biofilm develops on internal surfaces of living tissues, medical instruments and on dead tissue remains. Bacterial cells can stimulate antibody production due to their antigenic structures. However, antibodies cannot reach the bacteria in the biofilm. Even excellent cellular and humoral immune systems cannot help eliminate biofilm infections (7, 8, 9).

Determination of antimicrobial susceptibilities of agents causing infections is important to guide antibiotic selection for treatment and is one of the major subjects of microbiology. Disc diffusion testing is one of the most commonly used susceptibility test that has been used for more than 60 years (10). The Epsilometer test (E-test) developed in recent years, which also determines the minimum inhibitory concentration is also widely used (11). Automated antimicrobial susceptibility determination systems are based on MIC determination of a given species usually at one concentration, its breakpoint concentration of an antibiotic. All these tests are used to determine the antimicrobial susceptibilities of bacteria *in vitro*. However, the bacteria may be in biofilm or may cause intracellular infection. The effect of antibiotic in these conditions may not be reflected by a simple *in vitro* susceptibility testing.

One of the main purposes of this study was to develop an alternative time kill method and to find a technique that could measure MIC levels in different bacterial environments, especially in biofilms. For this purpose, MIC measurements were performed on bacteria containing *lux* genes, which can only give light through proteins that can be synthesized in living cells (12).

The aim of this study was to determine the effects of antibiotics on bacteria also in biofilms as well as to determine the killing effects of antibiotics on bacteria by bioluminescence measurement methods.

MATERIAL AND METHODS

Strains, growth conditions and plasmids

E. coli DH10B and *E. coli* ADU40 were used as recipient strains in the transformation experiments. *E. coli* DH10B strain is a reference strain from our laboratory collection. *E. coli* ADU40 is a biofilm producer clinical isolate from a patient in Aydın Adnan Menderes University Hospital. Bacterial strains were grown in Tryptic soy broth (TSB) or TS agar at 37 °C. The pAT28 was used as cloning vector. Recombinant plasmid was prepared to obtain bacteria that produced luciferase enzymes. The pAKlux1 plasmid containing the luciferase gene cluster (*luxCDABE*) was a gift from Attila Karsi (Addgene plasmid # 14073; http://n2t.net/addgene:14073; RRID: Addgene 14073) (13). Plasmids were extracted by plasmid miniprep kit (Thermo Fisher, Waltham, MA, USA) method.

Construction of recombinant plasmid

The *lux* genes were inserted in pAT28. Shortly, the pAKlux1 and pAT28 plasmids were digested with EcoRI (Fermentas, USA) and then the generated fragments were mixed, ligated, and transformed into electrocompetent *E. coli* DH10B. Transformants were selected on media with spectinomycin (*Oxoid*, Basingstoke, U.K.) 60 µg/ml. The recombinant plasmid that contained the *luxCDABE* gene cluster was confirmed by sequence analysis. The bioluminescent colonies were determined by using transilluminator in the dark mode. The presence of the recombinant plasmid was confirmed, and then this plasmid was designated as pAT28lux (Figure 1). pAT28lux plasmid was transferred to *E. coli* ADU40 for biofilm assay testing.

MIC measurement

Susceptibilities of *E. coli* DH10BΩpAT28lux transformants were tested for meropenem, cefazolin, ciprofloxacin, and chloramphenicol by broth microdilution method according to CLSI guidelines (14). The antibiotic concentrations used ranged from 0.06 to 128 µg/ml. Mueller Hinton broth without any added antimicrobial material was used as a negative control and only bacterial suspensions were used as a positive control. The MICs of *E. coli* ADU40 and *E. coli* DH10B for chloramphenicol (Applichem, Darmstadt, Germany), meropenem (Sigma-Aldrich, Munich, Germany), amikacin (Applichem, Darmstadt, Germany), rifampicin (Sigma-Aldrich, Munich, Germany), gentamicin (Sigma-Aldrich, Munich, Germany), ciprofloxacin (Biopharma, Istanbul, Turkey), cefazolin (Sigma-Aldrich, Munich, Germany), and cefoxitin (Sigma-Aldrich, Munich, Germany) was determined by conventional method.

Determination of luminometric MICs

Serial dilutions of antibiotics were prepared from 0.06 to 128 µg/ml in a 96-well plate. *E. coli* DH10B bacteria without

pAT28lux were used as the negative control group. The MICs of bacteria with *lux* genes were tested for meropenem, cefazolin, ciprofloxacin, and chloramphenicol antibiotics from 0.06 to 128 µg/ml. Antibiotic dilutions in liquid medium were prepared in 96 well plate and inocula of $5x10^5$ cfu/ml were added. For light measurements, the transilluminator (Multiskan FC Microplate Reader, Thermo, USA) was set to 37°C and programmed to take measurements every half hour during 18 hours of incubation. No filters were used for luminometric measurements. Thus, the effect of the antibiotics tested was determined by luminescence, which occurred only depending on the living cells because only living cells are able to give light (Table 1). The last concentrations before the first >5 fold light emission were defined as the MIC concentrations (Figure 2).

Conventional and bioluminescence MIC measurement of biofilm-forming bacteria

Biofilm formation in *E. coli* ADU40ΩpAT28lux was confirmed by the quantitative micro-dilution plate method. For this purpose, 2 mL of 0.25 % glucose containing TSB medium bacteria were incubated overnight at 37°C. The bacteria were diluted 1/40 with fresh medium with 0.25% glucose and were dispensed into 96-well U-based sterile micro plates and incubated at 37°C for 48 hours. After incubation, the plates were gently washed with 200 µl of phosphate buffer saline and dried at room temperature. Thereafter, 200 µl of 1% crystal violet was added to each well and the plate was incubated for 15 min. The dye was then removed and washed twice with distilled water. After drying at room temperature, ethanol-acetone (80/20) was added to dissolve any remaining stain. The absorbance was read with the Multiskan spectrophotometer (Multiskan FC Microplate Reader, Thermo, USA) at a wavelength of 595 nm and optical density > 1 was considered as indicator of biofilm formation (15).

MIC measurements of *E. coli* ADU40 with pAT28lux plasmid were also performed using the conventional MIC method as described previously (Table 2) (14). In order to obtain MIC values with biofilm formation, 0.25 % glucose was added to the broth medium and serial dilutions were

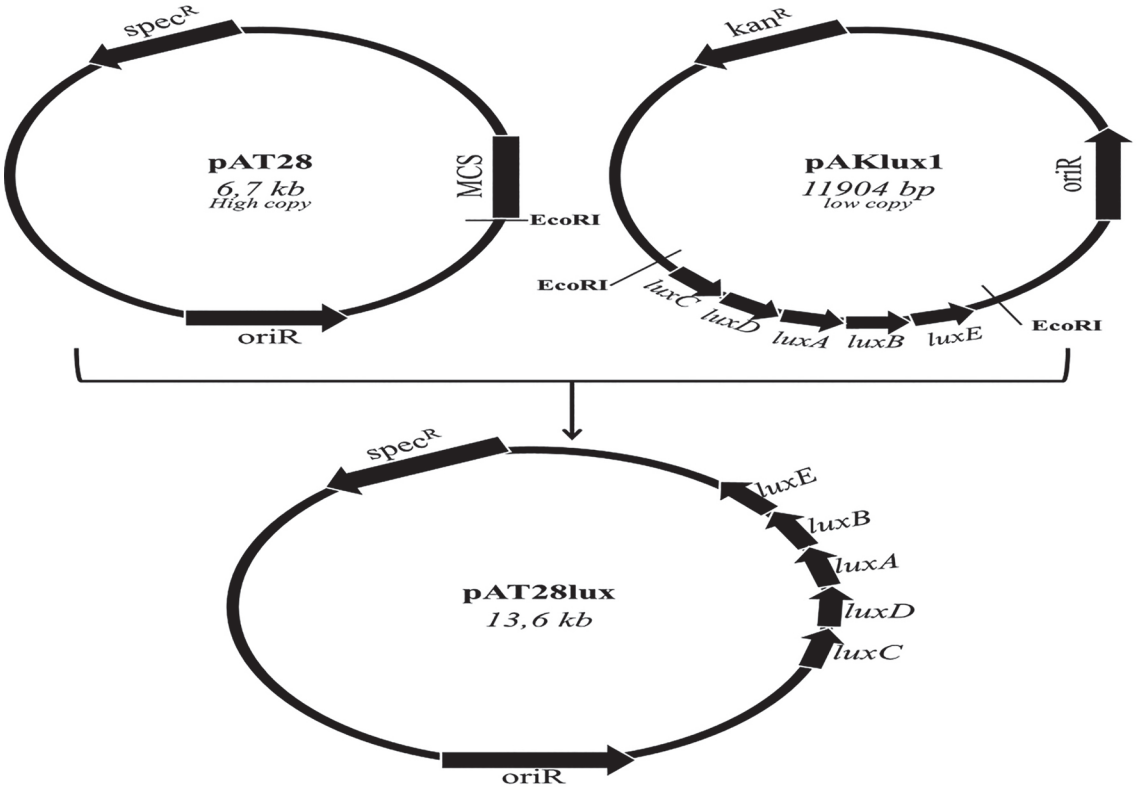

Figure 1: Constructed plasmid pAT28lux. The pAKlux1 plasmid was cut with EcoRI enzyme to obtain *luxCDABE* operon and cloned into the pAT28 plasmid cut with the same enzyme.

prepared and the luminometer (Multiskan FC Microplate Reader, Thermo, USA) values were read (Table 3). The last concentration before the first >5 fold light emission was defined as MIC concentration.

Determination of antibiotic effects after 6 hours growth

To identify the effect of antibiotics after growth the bacteria were allowed to grow during 6 hours and then various concentrations of antibiotics were added. After antibiotic addition, cultures were incubated for 18 hours and light emissions were measured each 30 minutes. At this stage, the possible bactericidal effects of antibiotics were investigated by the luminometric measurement technique. Thereafter, a total of 10^5 cfu/ml bacteria were inoculated in microplates containing 150 µl of medium and incubated in the Multiskan luminometer at 37°C until the maximum time of the luminescence (6 hours). The microplates were then removed from the luminometer and the calculated antibiotic concentrations including 1/4MIC, 1/2MIC, MIC, 2MIC, 4MIC was added to the wells. After the addition of antibiotic, cultures were incubated at 37°C and the amount of luminescence in the culture was measured every half hour over an 18 hour period (Figure 3). A minimum >2 fold decrease in light emission was accepted as a bactericidal effect. The method also was used for biofilm forming bacteria so that the bactericidal effects of antibiotics were also determined in biofilm.

RESULTS

Construction of bioluminescent plasmid

In this study, *luxCDABE* was cloned in pAT28 as shown in Figure 1. Constructed plasmid pAT28lux was transferred to *E. coli* DH10B and *E. coli* ADU40 (biofilm producer) for MIC and time kill studies.

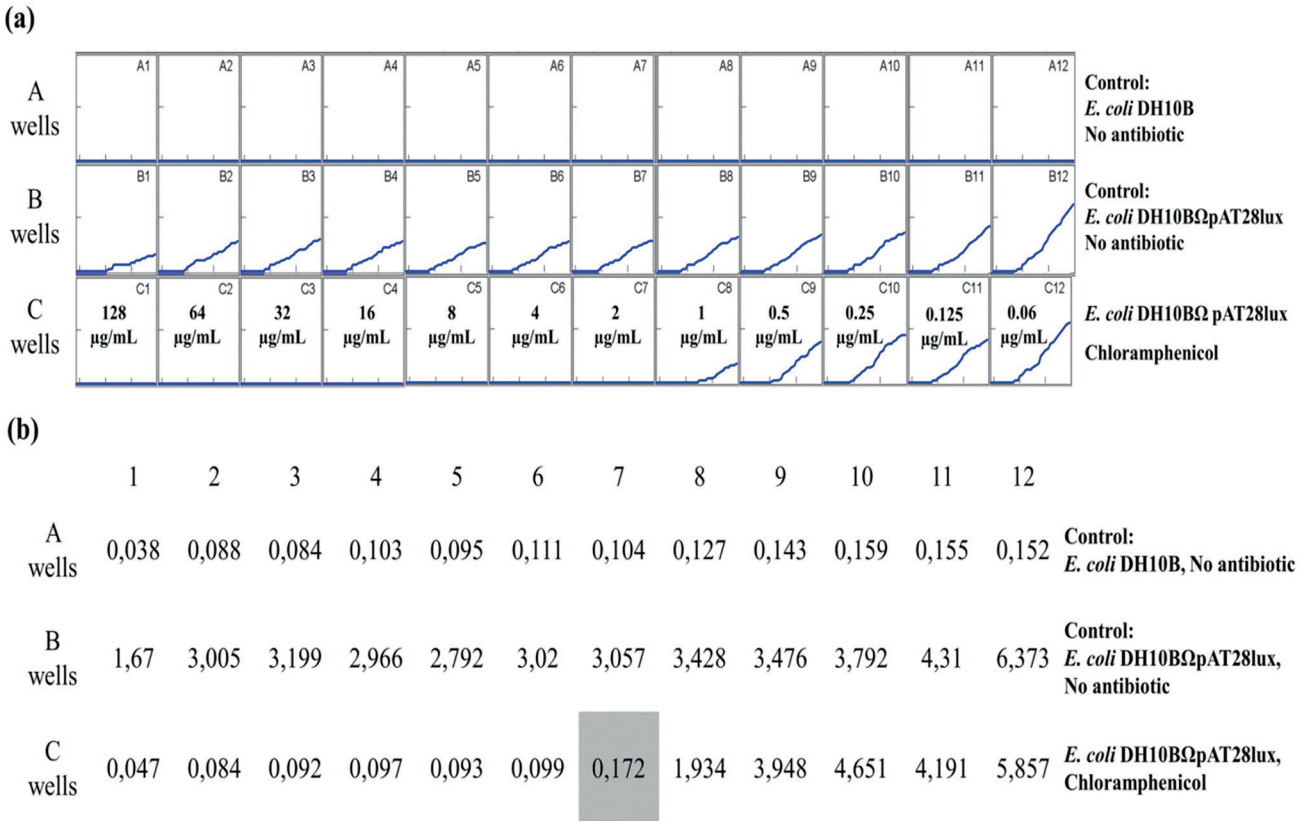

Figure 2: Luminometric measurements for the MIC determinations. (a): Light emission measurements of the plate. The light emission is measured for each well a total of 36 times during 18 hours. The A wells had only *E. coli* DH10B without lux genes and B wells had *E. coli* DH10BΩpAT28lux. Both A and B wells did not contain antibiotics and the concentration of antibiotic for C wells is presented. (b): Determination of MIC results was carried out by determining the well just prior to the first >5 fold increase in light emission. As can be seen the increase from C7 to C8 was > 10 fold.

Conventional MIC and luminometric MIC measurements

The MICs of meropenem, cefazolin, ciprofloxacin, and chloramphenicol for *E. coli* isolates with pAT28lux were determined by using the luminometric and conventional methods. Conventional and luminometric MIC values were found to be 0.06 and 0.03 µg/mL for Meropenem, 1 and 8 µg/mL for Cefazolin, <0.06 and <0.06 µg/mL for ciprofloxacin and 2 and 1 µg/mL for chloramphenicol, respectively, for *E. coli* DH10BΩpAT28lux (Table 1).

The MICs of *E. coli* ADU40 and *E. coli* DH10B for chloramphenicol, meropenem, amikacin, rifampicin, gentamicin, ciprofloxacin, cefazolin, cefoxitin and penicillin were determined by the conventional method (Table 2), The results were found to be 2 µg/mL for chloramphenicol, 2 µg/mL for meropenem, 4 µg/mL for amikacin, 16 µg/mL for rifampicin, 1 µg/mL for gentamicin, <0.06 µg/mL for ciprofloxacin, 2 µg/mL for cefazolin, 4 µg/mL for cefoxitin and 16 µg/mL for penicillin, for *E. coli* DH10B. The MIC values for *E. coli* ADU40 were found as 8 µg/mL for chloramphenicol, 1 µg/mL for meropenem, 4 µg/mL for amikacin, 16 µg/mL for rifampicin, 128 µg/mL for gentamicin, 128 µg/mL for ciprofloxacin, 32 µg/mL for cefazolin, 32 µg/mL for cefoxitin and >128 µg/mL for penicillin.

Conventional and luminometric MIC of biofilm forming *E. coli* ADU40

According to the results of conventional and luminometric MIC measurements in biofilm-forming *E. coli* ADU40ΩpAT28lux strain were found as 8 µg/mL for both

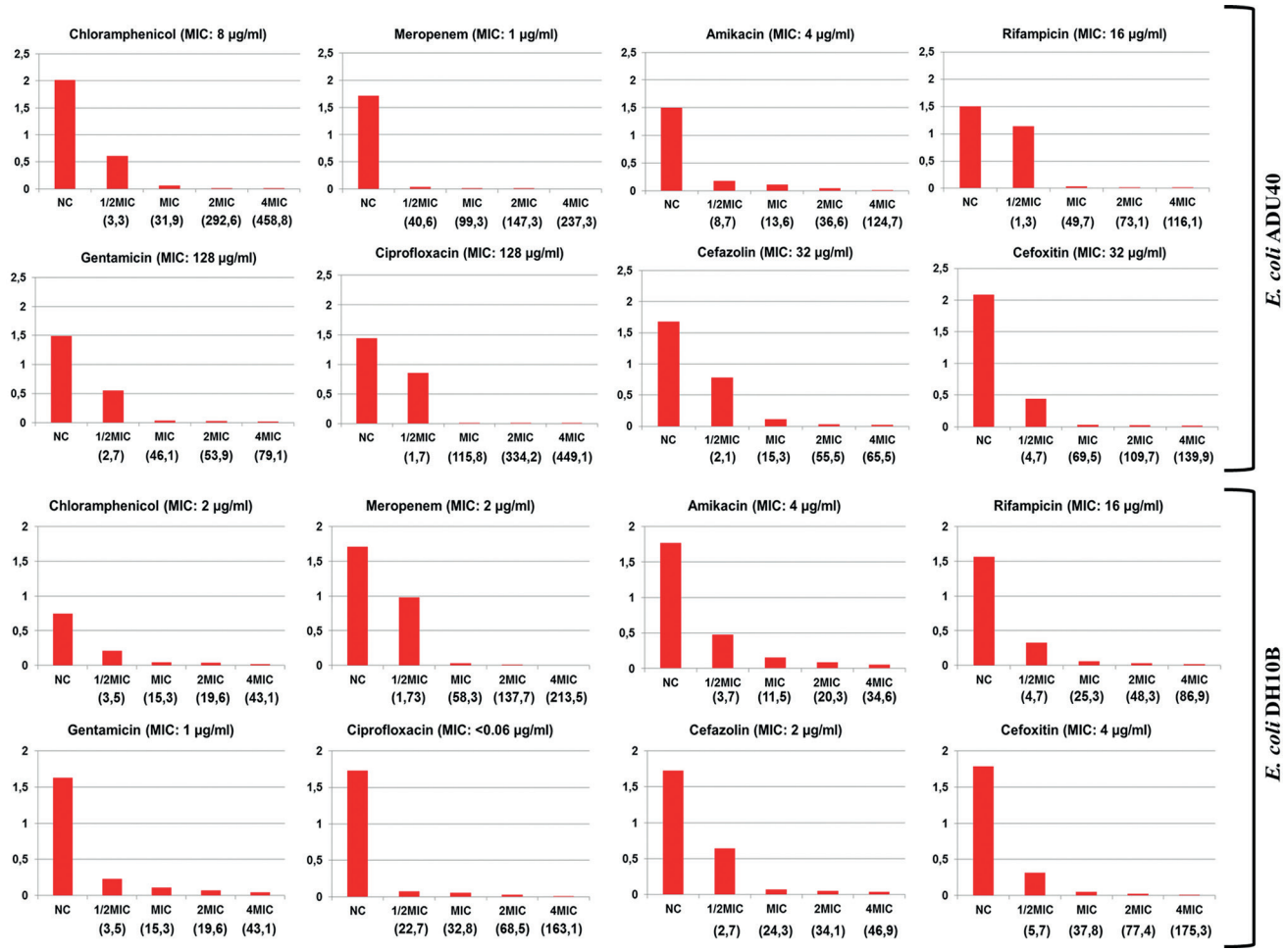

Figure 3: Testing of bactericidal effects of antimicrobials. As the MICs of the antibiotics were different for each tested bacteria different antibiotics were used. Chloramphenicol, meropenem, amikacin, rifampicin, gentamicin, ciprofloxacin, cefazolin and cefoxitin were tested for biofilm forming *E. coli* ADU40 and non-producer *E. coli* DH10B.

techniques for chloramphenicol; 0.06 µg/mL for both techniques for meropenem; >128 µg/mL for both techniques for ciprofloxacin and 32 µg/mL for the conventional MIC method and 16 µg/mL by the luminometric technique for cefazolin (Table 3).

Determination of bactericidal effect

Chloramphenicol, meropenem, amikacin, rifampicin, gentamicin, ciprofloxacin, cefazolin and cefoxitin antibiotics were used for biofilm forming *E. coli* ADU40 and non-biofilm forming *E. coli* DH10B. The first sharp decrease in light emission (> 10 fold decrease) was considered as a bactericidal effect. The fold change was calculated by division of arbitrary light emission value of the control strain by the light emission value obtained for a given concentration of each antibiotic. For example, for chloramphenicol testing the value of no antibiotic control light emission for E. coli ADU40 was 2.019 and emission value at MIC concentration was 0.063. When 2.019 is divided by 0.063 the result (32.04 fold) showing the fold decrease in light emission. As this value is greater than 10, this concentration chloramphenicol is bactericidal for *E. coli* ADU40.

As shown in Table 4 and Figure 3, decrease in light emissions of *E. coli* ADU40 were found for 1/2MIC, MIC, 2MIC and 4MIC concentrations were 3.3, 32.04, 288.4 and 504.8 fold for chloramphenicol; 40.6, 100.5, 155.3 and 244.1 fold for meropenem; 8.3, 13.6, 36.6 and 124.7 fold for amikacin; 1.3, 49.9, 74.9 and 115.2 fold for rifampicin; 2.7, 46.1, 53.1 and 78.3 fold for gentamicin; 1.7, 115.8, 359.2 and 379.0 fold for ciprofloxacin; 2.1, 15.2, 55.5 and 65.5 fold for cefazolin; 4.7, 69.5, 109.7 and 139.9 fold for cefoxitin, respectively.

Decrease in light emissions of *E. coli* DH10B were found for 1/2MIC, MIC, 2MIC and 4MIC concentrations to be 3.5, 15.2, 19.6, and 43.8 fold for chloramphenicol; 1.73, 56.9, 142.3 and 213.5 fold for meropenem; 3.7, 11.5, 20.3 and 34.6 fold for amikacin; 4.7, 25.3, 48.9 and 86.9 fold for rifampicin; 7.1, 15.3, 23.9 and 37.9 fold for gentamicin; 22.7, 33.2, 69.1 and 172.8 fold for ciprofloxacin; 2.7, 24.3, 34.1 and 46.9 fold for cefazolin; and 5.7, 38.04, 77.4 and 175.3 fold for cefoxitin, respectively.

Table 1: Comparison of conventional MIC and bioluminescence MIC.

	Luciferase	
	Conventional MIC	**Luminometric MIC**
Antibiotics	*E. coli* DH10BΩpAT28lux (µg/ml)	*E. coli* DH10BΩpAT28lux (µg/ml)
Meropenem	0.06	0.03
Cefazolin	1	2
Ciprofloxacin	<0.06	<0.06
Chloramphenicol	2	1

Table 2: The MICs of *E. coli* ADU40 and *E. coli* DH10B by conventional MIC

	Conventional MIC	
Antibiotics	*E. coli* DH10B (µg/ml)	*E. coli* ADU40 (µg/ml)
Chloramphenicol	2	8
Meropenem	2	1
Amikacin	4	4
Rifampicin	16	16
Gentamicin	1	128
Ciprofloxacin	<0.06	128
Cefazolin	2	32
Cefoxitin	4	32
Penicillin	16	>128

Table 3: Determination of MIC values after biofilm forming *E. coli* ADU40ΩpAT28lux strain

	Conventional MIC	**Luminometric MIC**
Antibiotics	*E. coli* ADU40ΩpAT28lux (µg/ml)	*E. coli* ADU40ΩpAT28lux (µg/ml)
Chloramphenicol	8	8
Meropenem	0.06	0.06
Ciprofloxacin	>128	>128
Cefazolin	32	16

DISCUSSION

In our study, MIC was measured with *E. coli* bacteria transformed with *luxCDABE* genes. Similar results were obtained with bioluminescence-based MIC method compared to conventional methods, conventional and luminometric MICs were found to be almost the same at maximum 1 dilution difference. It was considered very important to determine the ability of antibiotics to affect the bacterial growth in biofilm.

The firefly bioluminescent ATP assay releases light through the luciferin-luciferase reaction and in the presence of ATP. The light produced is proportional to the amount of bacterial ATP present in the sample and is therefore proportional to the bacterial cells (12). Therefore, this assay allows to indirectly measure the number of bacteria. For this purpose, in a previous study, it was aimed to apply a fast method using

firefly bioluminescent ATP test in which bacteria were tested against antibiotics used in clinical practice. According to the findings obtained in the study, a rapid test method, which can measure with similar sensitivity to other commercial methods and yield antibiotic sensitivity within 2 hours, was developed (12).

In a similar study, the researchers tried to determine the MIC values in positive blood cultures with a bioluminescence-based test. In that study, a fast ATP bioluminescence-based method was used to determine antibiotic resistance and only rapid detection of levofoxine resistance was performed. Researchers used only one concentration and one inoculum size for determination of antimicrobial susceptibility and commented that the effect of different inocula concentrations and antibiotics on MIC values should be tested (16).

In another recent study, a fluorescence-based MIC measurement method was used to monitor the growth of biofilm-forming *P. aeruginosa*. In this way, a real-time fluorimetric MIC analysis was performed with doubling concentrations of antimicrobials (9). In our study we have also tested susceptibilities with bacteria containing green fluorescent protein (GFP) gene however the fluorescence emission lasts even after the bacteria dies so that the bactericidal effect of antibiotics with GFP could not be determined (Data not shown). Therefore, we continued our study based on the determination of MIC values according to light emission only with bioluminescence bacteria, while the bacteria were alive.

In a recent study, researchers used *luxCDABE* operon of *Photorhabdus luminescens* for rapid high-throughput screening of compounds targeting *Mycobacterium tuberculosis*. Accordingly, they report that the Lux system is a convenient tool for easy, fast, reliable, real-time high-throughput screening of antibacterial compounds. In addition, there is considerable savings in terms of plate pouring efforts, plating efforts, plates, and medium requirements (17).

In this study the effects of antibiotics on bacteria in the

Table 4: Light emissions with and without antibiotics for biofilm forming *E. coli* ADU40ΩpAT28lux and non-biofilm forming *E. coli* DH10B ΩpAT28lux. More than 10 fold decrease was accepted as a bactericidal effect.

	***E. coli* ADU40ΩpAT28lux**					***E. coli* DH10BΩpAT28lux**				
Antibiotics	NA[1]	1/2MIC	MIC	2MIC	4MIC	NA	1/2MIC	MIC	2MIC	4MIC
	Ems[2]	Ems (fold)	Ems (fold)	Ems (fold)	Ems (fold)	Ems	Ems (fold)	Ems (fold)	Ems (fold)	Ems (fold)
CHL[3]	2.019	0.608	0.063	0.007	0.004	0.746	0.214	0.049	0.038	0.017
		3.3	**(32.04)**[4]	288.4	504.8		3.5	**(15.2)**	19.6	43.8
MER	1.709	0.042	0.017	0.011	0.007	1.708	0.983	0.030	0.012	0.008
		(40.6)	100.5	155.3	244.1		1.73	**(56.9)**	142.3	213.5
AMK	1.497	0.179	0.110	0.041	0.012	1.769	0.476	0.154	0.087	0.051
		8.3	**(13.6)**	36.6	124.7		3.7	**(11.5)**	20.3	34.6
RIF	1.498	1.140	0.030	0.020	0.013	1.565	0.330	0.062	0.032	0.018
		1.3	**(49.9)**	74.9	115.2		4.7	**(25.2)**	48.9	86.9
GEN	1.488	0.551	0.032	0.028	0.019	1.630	0.231	0.106	0.068	0.043
		2.7	**(46.5)**	53.1	78.3		7.1	**(15.3)**	23.9	37.9
CIP	1.437	0.849	0.012	0.004	0.003	1.728	0.076	0.052	0.025	0.010
		1.7	**(119.7)**	359.2	479.0		**(22.7)**	33.2	69.1	172.8
CEF	1.678	0.783	0.110	0.030	0.025	1.724	0.642	0.071	0.050	0.037
		2.1	**(15.2)**	55.5	65.5		2.7	**(24.3)**	34.1	46.9
CFX	2.085	0.442	0.030	0.019	0.015	1.788	0.311	0.047	0.023	0.010
		4.7	**(69.5)**	109.7	139.9		5.7	**(38.04)**	77.4	175.3

[1]NA: No antibiotic, [2]Ems: Light emission arbitrary value [3]CHL: Chloramphenicol, MER: Meropenem, AMK: Amikacin, RIF: Rifampicin, GEN: Gentamicin, CIP: Ciprofloxacin, CEF: Cefazolin, CFX: Cefoxitin, [4]Bactericidal concentrations are shown in bold.

biofilm were also tested. The results were consistent with MIC values without biofilm formation. Using GFP fluorescence emission results of a recent study that measured MICs of antibiotics for bacteria in biofilm also showed that in or out of biofilm, the MICs of antibiotics for *P. aeruginosa* were not dissimilar (9). Also in our study there was no significant difference in MIC of antibiotics tested.

The results of present study showed that light emission can be used for determination of the effects of the antimicrobials. These methods may be used as a rapid screening test for MIC and bactericidal effect determination of a candidate molecule, a future antimicrobial compound. The only limitation of this method is the necessity to use bacteria transformed with *lux* genes. Other than antibiotics these methods can be used to test also the effects of extracts from animals, microorganisms and plants on bacteria in a quick and easy manner. Each method that will contribute to the development of new antibiotics will also contribute to human health with increasing treatment success.

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