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Research Article

USE OF *ESCHERICHIA COLI* K-12 STRAIN WITH GFP REPORTER GENE FOR SCREENING OF CEFUROXIME AND METRONIDAZOLE RESIDUES IN SURFACE WATER

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ABSTRACT

Antibiotic residues are constantly detected in environmental waters at relatively low concentrations. They can modulate the biological stability of ecosystems after entering the environment, therefore robust and reliable methods for determining their residues in environmental samples are required. In this work, *Escherichia coli* K-12 GFP-based bacterial biosensors strain was used to detect cyto- and genotoxic activity of cefuroxime and metronidazole at concentration of 500, 1000, 2000, 3000 and 5000 µg/ml. Experimental data indicated that metronidazole, at higher concentrations of 3000 and 5000 µg/ml showed stronger cyto- and genotoxix activity than cefuroxime to bacteria cells. Incubation of bacteria cells with surface water with both drugs modulated *gfp* gene expression. *E. coli* K-12 strain with genetic fusion of recA promoter and *gfp* reporter gene was a good model organism for screening cyto- and genotoxic effect of cefuroxime and metronidazole in applied in this experiment concentrations of these drugs.

Keywords: *recA::gfp* biosensor, cytotoxicity, genotoxicity, cefuroxime, metronidazole.

INTRODUCTION

Antibiotics are one of the pharmaceutical drug classes with high usage and consumption worldwide. They are widely used in both human and veterinary medicine mainly for treating bacterial infections. However, besides their therapeutic usage, they are also used as growth promoters in livestock animal production, as feed additives in fish farming and as a coccidiostatic drugs in the poultry industry [Kim and Aga, 2007; Grosa et. al., 2013].

Antibiotic residues are constantly detected in environmental waters at relatively low concentrations. Recent research investigations pointed out that some pharmaceuticals (including antibiotics) can exert adverse ecological and human health effects even at low concentrations found in the environment [Liu et. al., 2012; Mondragon et. al., 2011; Isidori et. al., 2009].

Cefuroxime is a second-generation β-lactam cephalosporin with a broad antimicrobial activity against both Gram-positive and Gram-negative bacteria. It is highly efficient and safe for the treatment of urinary and respiratory infections as well as many other types of infection. Clinical studies show that cefuroxime is effective in patients with infections of the lower respiratory tract, skin and skin structures, urinary tract or female reproductive system [Kessler et. al., 1985; Okamoto et. al., 1994; Brunton et. al., 2010].

Metronidazole (MNZ) belongs to the nitroimidazole group. It is employed in both human and veterinary medicine to treat diseases caused by anaerobic bacteria (*Bacteroides, Fusobacterium*, *Campylobacter*, *Clostridium*) and protozoa (*Trichomonas*, *Treponema*, *Histomonas*). However, this drug has been found to possess carcinogenic, mutagenic and toxic properties. In mammalian cells' DNA damage seems to be related to the production of reactive oxygen species. MNZ metabolites are also carcinogenic and mutagenic in some animal species, because the original nitroimidazole ring is retained [Wagil et. al., 2015].

As mentioned above, cefuroxime and metronidazole are used in human medicine and veterinary practice and also in aquaculture. After entering the environment, their biological properties may have significant and long-term effects on the stability of ecosystems, therefore robust and reliable methods for determining cefuroxime and MNZ residues in environmental samples are required.

Investigation concerning the level of drugs in the surface waters has been intense in recent years [Fatta-Kassinos et. al., 2011]. Antibiotics can accumulate in the aquatic environment, so these drugs can have a significant impact on non-target aquatic organisms and may lead to the formation of bacterial strains resistant to its effects. It has been reported that this phenomenon can occur not only in water organisms but also in sediments [Wagil et. al., 2015].

The US Food and Drug Administration requires environmental risk assessments to be performed for human and veterinary medicines on the effects on aquatic and terrestrial organisms before a product can be marketed [Fatta-Kassinos et. al., 2011].

For chemical and pharmaceutical risk assessment, microbial biosensors bio-tests are used. In such living cell systems, bacteria are especially attractive due to their rapid growth rate, low cost, and easy handling [Ptitsyn et al., 1997, Kostrzyńska et al., 2002, Zaslaver et al., 2004, Matejczyk 2010, Alhandrami and Paton 2013, Park et al., 2013].

The aim of the present study was to validate the potential applications of reporter strains of *Escherichia coli* K-12 *recA*::*gfpmut2* for *in vitro* rapid screening of cytotoxic and genotoxic effects of cefuroxim and metronidazole. In the experiment more stable and fast folding mutant of *gfp* gene - *gfpmut2* with excitation and emission wavelengths of 485 and 507 nm was used [Zaslaver et al., 2004]. Earlier research confirmed the genotoxic sensitivity of *recA* promoter in fusion of *gfp*, in experiments, where cytotoxicity and genotoxicity of known genotoxins and drugs were assessed [Ptitsyn et al., 1997, Kostrzyńska et al., 2002, Zaslaver et al., 2004, Matejczyk 2010, Alhandrami and Paton 2013, Park et al., 2013].

MATERIALS AND METHODS

Bacteria strain and plasmid: In the experiment *Escherichia coli* K-12 MG1655 genetically modified stationary phase cells: *Escherichia coli* K-12 *recA*::*gfpmut2* and *Escherichia coli* K-12 *promoterless*::*gfpmut2*, were used. They contained a pUA66 plasmid-borne transcriptional fusion between DNA-damage, genotoxin-sensitive *recA* promoter involved in the SOS regulon response and fast folding GFP variant reporter gene-*gfpmut2* [Zaslaver et al., 2004].

Bacteria growth condition: *Escherichia coli* K-12 MG1655 strains: *Escherichia coli* K-12 *recA*::*gfpmut2* and *Escherichia coli* K-12 *promoterless*::*gfpmut2* were cultured overnight in LB agar medium (Merck, Germany) at 30 °C supplemented with 100 µg/ml of kanamycin (Sigma-Aldrich, Germany). Colonies were carried to LB broth medium (10 g NaCl, 10 g tryptone and 5 g yeast extract per 1000 ml of distilled water) with 100 µg/ml of kanamycin and incubated overnight at 30 °C. Followed that, cells were washed with PBS buffer (1,44 g Na_2HPO_4, 0,24 g KH_2PO_4, 0,2 g KCl, 8 g NaCl per 1000 ml of distilled water, pH = 7).

Monitoring of bacteria growth and bacteria concentration: At the start of the experiment the initial bacteria cells density was standardized to OD = 0,2 (Optical Density) value by use of spectrophotometer (Perkin Elmer Enspire 2300) at wavelength of 600 nm. The concentration of bacteria cells per ml of PBS was assessed by series dilutions system and expressed as Colony Forming Units per ml (CFU/ml) values. The growth dynamic of bacteria strains treated with cefuroxime and metronidazole (Bialystok pharmacy) was monitored with the use of standard spectrophotometer analysis of Optical Density values at wavelength of 600 nm. The values of bacteria growth inhibition (GI) during the treatment with the drugs were measured at the start of bacteria incubation with drugs – time 0 and after 3 and 24 hours, and were calculated according to the formula: GI (%) = OD_{CS} (%) – OD_{DS} (%), Where: OD_{CS} (%) – Optical Density of control sample = 100%, OD_{DS} (%) – Optical Density of bacteria samples treated with drugs.

Bacteria cells treatment with cefuroxime and metronidazole: 1 ml of stationary phase bacteria cells (1×10^7 CFU/ml; OD = 0,2) were suspended in 4 ml of PBS buffer and the following drugs were (was) used in testing: cefuroxime and metronidazole in five different concentrations: 500; 1000; 2000; 3000 and 5000 µg/ml. Drugs concentrations were selected experimentally. Bacteria strains with drugs were incubated for 1

hour at 20 °C. The bacteria incubation time with drugs was estimated for sensitivity monitoring of recA::gfp genetic construct for quick (1 hour) response. The control sample – Escherichia coli K-12 recA::gfpmut2 strain in PBS buffer was not treated with drugs. To verify the correct activity of recA promoter, Escherichia coli K-12 strain containing pUA66 plasmid without promoter - Escherichia coli K-12 promoterless::gfpmut2 was used as a control. Additionally, to assess genotoxic sensitivity of recA::gfpmut2 construct, 4% acetone was used as negative control and 50 μM methylnitronitrosoguanidine (MNNG, known genotoxin) as positive control [Ptitsyn et. al., 1997, Kostrzyńska et. al., 2002].

Bacteria cells treatment with cefuroxime and metronidazole in surface water: Surface water samples were collected in sterile flasks from Białka river. Samples were sterilized by filtration. 1 ml of stationary phase bacteria cells (1×10^6 CFU/ml) was suspended in 4 ml of surface water contaminated with the drugs used in genotoxicity testing. Drugs concentrations (5000 μg/ml) were selected to the highest inhibition of *gfp* gene expression in PBS buffer The conditions of bacteria incubations and the control protocols were the same as above.

Analytical method for the intensity of *gfp* gene fluorescence (FI) analysis: After exposition of bacteria cultures to the tested drugs, the strains were washed with PBS buffer and the intensity of fluorescence of *gfp* gene in the volume of 1 ml of bacteria cells suspension (1×10^4 CFU/ml) in PBS buffer was measured with spectrofluorometer (Perkin Elmer Enspire 2300). The measurements were at excitation and emission wavelengths of 485 and 507 nm.

Assessment of SFI values: The specific fluorescence intensity (SFI) value, which is defined as the fluorescence intensity (FI) divided by the optical density (OD) measured at each time point at 600 nm, was calculated according to the following formula to detect the level of genotoxic activity of drugs:

$$SFI = \frac{F}{D},$$

where: SFI – Specific Fluorescence Intensity;
FI – the Fluorescence Intensity of the strains at excitation and emission wavelengths of 485 and 507 nm;
OD – Optical Density at 600 nm of the strains.

Detection of $I_{gfpexp.}$ values: For each concentration of tested drugs the levels of inhibition ($I_{gfpexp.}$) were calculated, according to the formulas for the SFI values with a decrease with the level of *gfp* expression in comparison to the control sample:

$$I_{gfpexp.}(\%) = SFI_{CS}(\%) - SFI_{DS}(\%),$$

where: SFI_{CS} (%) – SFI for control sample =100%;
SFI_{DS} (%) – SFI values for tested drugs sample.

Detection of GI values: The dynamics of the growth of bacteria strains treated with drugs was monitored by the use of standard spectrophotometer analysis of OD values at the wavelength of 600 nm. The values of bacteria growth inhibition (GI) during the treatment with drugs at the start of bacteria incubation with drugs (time 0 and after 1 h) was calculated according to the formula:

$$GI(\%) = OD_{CS}(\%) - D_{ODTS}(\%),$$

where: OD_{CS}(%) - OD of control sample = 100%;
D_{ODTS}(%) - the decrease in the value of OD of bacteria samples treated with drugs.

Statistical analysis: Experiments were conducted in three independent series. Statistics data obtained in this study are expressed as mean ± standard deviation (SD) for n = 8. The data were analyzed with the use of one-way Student`s test for multiple comparisons to determine the significance between different groups. The values of $P<0.05$ were considered as significant.

RESULTS

Stationary phase *Escherichia coli* K-12 MG1655 *recA*::*gfpmut2* strain treatment with cefuroxime and metronidazole showed that both drugs caused a significant dose- and time-dependent decreased ($P<0,05$) in SFI value and increased the inhibition of *recA* promoter activity and intensified $I_{gfpexp.}$ value compared to non-treated cells in PBS buffer (control sample) (Table 1). A sustained decrease in SFI values was observed for the both drugs after 1 hour incubation of bacteria cells, especially at concentrations of 3000 and 5000 μg/ml.

Progressive inhibition of SFI values and *gfp* expression were obtained for the whole applied concentration of drugs, during 1 h incubation comparing to control sample. The maximum point for *recA* promoter inhibition was observed for the higher concentration of the both drugs (5000 μg/

Table. 1. SFI values for stationary phase *E. coli* K-12 *recA::gfp mut2* treated with cefuroxime and metronidazole in PBS buffer at concentration of 500; 1000; 2000; 3000 and 5000 µg/ml in comparison with the control sample

C (µg/ml)	t (h)	Control SFI±SD	Cefuroxime SFI±SD	$I_{gfpexp.}$ (%)	Metronidazole SFI±SD	$I_{gfpexp.}$(%)
500	1	31.50±2.91	29.57±1.44	6.13	28.51±1.48	9.5
1000	1	31.50±2.91	30.08±2.13	4.51	27.06±1.04	14.1
2000	1	31.50±2.91	29.53±2.44	6.26	$25.27±1.22^{abA}$	19.78
3000	1	31.50±2.91	$25.55±0.82^{bcd}$	18.90	$21.80±1.26^{abcB}$	30.8
5000	1	31.50±2.91	$18.88±2.29^{abcde}$	40.07	$14.71±1.23^{abcdeC}$	53.3

t – time of bacteria strain incubation with drugs, $I_{gfpexp.}$ (%) – the percent of inhibition of *gfp* expression after treatment of bacteria cells with drugs in comparison with the control sample (100%).

Data points represent mean values ± SD; n=8; a - significantly different from control ($p<0.05$); b - significantly different from 500 µg/ml group ($p<0.05$); c - significantly different from 1000 µg/ml group ($p<0.05$); d - significantly different from 2000 µg/ml group ($p<0.05$); e - significantly different from 3000 µg/ml group ($p<0.05$); A - significant difference between cefuroxime and metronidazole groups at 2000 µg/ml ($p<0.05$); B - significant difference between cefuroxime and metronidazole groups at 3000 µg/ml ($p<0.05$); C - significant difference between cefuroxime and metronidazole groups at 5000 µg/ml ($p<0.05$);

ml). Metronidazole at all applied concentrations showed more intensive effect of *gfp* expression inhibition than cefuroxime.

Metronidazole in relation to cefuroxime caused statistically significant increase in the *gfp* expression inhibition at concentrations of 2000; 3000 and 5000 µg/ml.

The monitoring of bacteria cultures growth (OD) at the start of bacteria incubation (time 0) and after 1 h with drugs, indicated 35% significant increase in GI (growth inhibition) values for metronidazole at concentration of 5000 µg/ml in comparison to the control sample. In the case of cefuroxime there were no statistically important differences for OD values (Figure 1)**.**

Metronidazole in relation to cefuroxime caused statistically significant stronger increase in the cytotoxic and genotoxic activity, especially at higher concentration of metronidazole. Bacteria incubated with PBS buffer (control sample) without any drugs, resulted in no statistical differences in OD value for 1 h continuous cultivation.

Treatment of *E. coli* K-12 *recA::gfpmut2* biosensor bacteria strain in surface water (n = 8) weakened the reactivity of *recA::gfpmut2* genotoxic system and decreased the inhibition of *gfp* expression and SFI value in comparison to incubation in PBS buffer (Table 2).

To assess genotoxic sensitivity of *recA::gfp* genetic biosensing system 4% acetone was tested as negative control. For this chemical F_I values did not increase during 3 h and 24 h of incubation. Methylnitronitrosoguanidine (MNNG) at concentration of 50 µM was used as positive control. For this chemical F_I (induction factor) = 8.4 during 24 h incubation and F_I = 2.8 during 3 h were obtained (data not shown). These results showed stronger sensitivity of *recA::gfp* biosensing system for

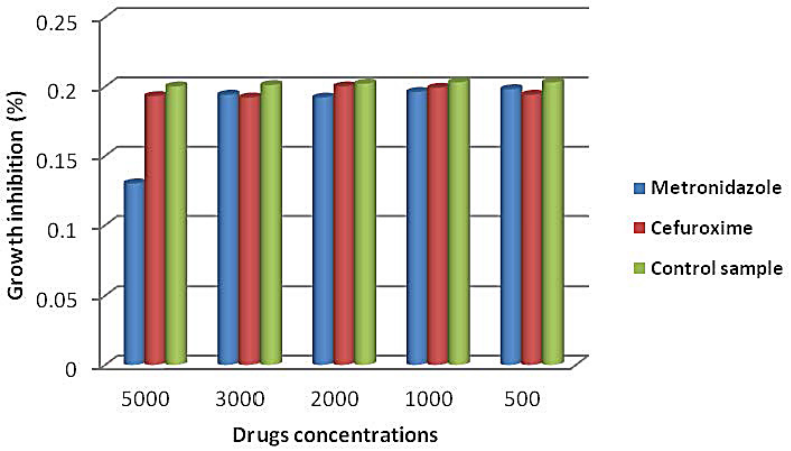

Figure 1. Growth inhibition GI (%) values for 1 h incubation of *E. coli* K-12 *recA::gfpmut2* with cefuroxime and metronidazole

Table 2. SFI values for stationary phase *E. coli* K-12 *recA::gfp mut2* treated with cefuroxime and metronidazole in surface water at concentration of 5000 μg/ml in comparison with the control sample

C (μg/ml)	t (h)	Control SFI±SD	Cefuroxime SFI±SD	$I_{gfpexp.}$ (%)	Metronidazole SFI±SD	$I_{gfpexp.}$ (%)
5000	1	29.32±1.95	20.27±2.68^{a*}	30.87	18.48±1.57^{a*}	36.98

t – time of bacteria strain incubation with drugs, $I_{gfpexp.}$ (%) – the percent of inhibition of *gfp* expression after treatment of bacteria cells with drugs in comparison with the control sample (100%).

Data points represent mean values ±SD; n=8; a - significantly different from control ($p \leq 0.05$); * - significant difference between PBS buffer and surface water groups at 5000 μg/ml of cefuroxime and metronidazole ($p < 0.05$).

MNNG than acetone stressor. According to references Ptitsyn et al., [1997] and Kostrzyńska et al., [2002], a chemical was identified as a genotoxin if its induction factor was two or more ($F_I \geq 2$).

DISCUSSION

Genetically engineered microorganisms biosensors based on fusing of the *gfp* gene reporter to an inducible gene *recA* promoter have been widely applied to assay cytotoxicity, genotoxicity and bioavailability of known genotoxins and drugs [Ptitsyn et al., 1997, Kostrzyńska et al., 2002, Zaslaver et al., 2004, Matejczyk 2010, Alhandrami and Paton 2013, Park et al., 2013].

According to the results obtained in our experiment cefuroxime and metronidazole modulated and decreased the *gfp* expression in transcriptional fusion of this reporter with *recA* promoter in *E. coli* K-12 living cells.

Faure et. al. [2015] reported the clinical findings and retinal function of a patient who presented retinal toxicity signs after cefuroxime use for a phacoemulsification surgery. Earlier studies indicated dose-dependent cefuroxime toxicity on cultured human corneal endothelial cells started at a concentration above 2750 μg/ml. Transient ocular hypertony was also noticed. Macular edema (ME) and even macular infarction had been noted with excess cefuroxime dose over 40 mg in all the cases [Faure et. al. 2015]. The results of the above experiment confirm possible influence of cefuroxime on living cells, because in 20% of cases, there were significant differences (comparable to the control sample) in the level of *gfp* expression after stationary phase bacteria treatment, especially with the higher concentration of this antibiotic (5000 μg/ml).

Our results indicated that bacteria exposure to metronidazole resulted in a progressive intensification of the inhibition of *gfp* expression in comparison to cefuroxime samples. The strongest inhibition of *recA* promoter and *gfp* expression was noticed after incubation of the higher (2000; 3000 and 5000 μg/ml) than for lower metronidazole concentration.

Metronidazole was found to possess carcinogenic, mutagenic and toxic properties [Wagil et. al., 2015]. The higher (2000; 3000 and 5000 μg/ml) investigated concentrations of this drug inhibited SFI values of bacteria culture and OD value (at concentration of 5000 μg/ml) in the stationary phase. It can be suggested the possible direct influence of metronidazole on DNA activity (genotoxic effect). Our results showed that metronidazole treatment at concentration of 5000 μg/ml significantly inhibited *E. coli* K-12 bacteria cells growth (cytotoxic effect).

The incubation of *E. coli* K-12 *recA::gfpmut2* strain with 5000 μg/ml cefuroxime and metronidazole in surface water decreased *gfp* gene expression and reactivity of *recA::gfpmut2* genetic construct. The influence of components of surface water on *gfp* expression in bacteria cells could be a consequence of the presence of different organic or inorganic chemicals (e.g. compounds from hospitals) in surface water used in our experiment. These unknown water compounds could modulate *gfp* expression. Therefore, the all river's water (specially in the hospital's surroundings) should be check for the presence of drugs from these groups of chemicals.

In above experiment we applied *E. coli* K-12 bacteria cells as a model organism for genotoxic studies. The obtained data are generally in agreement with other results which were previously obtained in *in vivo* and *in vitro* tests of higher organisms, including human cells.

CONCLUSIONS

Genetically engineered *E. coli* K-12 strain with *gfp* reporter gene was a proper model organism for screening cyto- and genotoxic effect of

cefuroxime and metronidazole in applied in this experiment concentrations of drugs.

1. Metronidazole, especially at the higher concentrations, indicated stronger cyto- and genotoxix effect than cefuroxime to investigated bacteria cells.
2. Incubation of bacteria cells with surface water with both drugs modulated *gfp* expression.
3. Next steps in research will include more experimental analysis to validate *recA::gfpmut2* genetic system in *E. coli* K-12 on different drugs.

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