ORIGINAL

Inactivation of Extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* by UVA-LED irradiation system

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Abstract : The prevalence of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* is increasing rapidly and spreading worldwide, particularly in Asia, compared to other regions. In the last ten years, in our hospital, in particular, there has been a < 30% increase. To prevent the spread of ESBL in hospitals and the community, the ultraviolet (UV) A-light-emitting diode (LED) irradiation device was used to inactivate ESBL-*E. coli* in human livestock and the environment. ESBL-*E. coli* and *E. coli* bacterial samples were collected from patients at Tokushima University Hospital (Tokushima City, Japan). The UVA-LED irradiation system had 365 nm single wavelength, and the current of the circuit was set to 0.23 or 0.50 A consistently. Results demonstrated that UVA-LED was useful for the inactivation of ESBL-*E. coli* and *E. coli*. The minimum energy dosage required to inactivate ESBL-*E. coli* and *E. coli* was 40.76 J/cm² (45 min) in the first type of UVA-LED and 38.85 J/cm² (5 min) in the second type. There were no significant differences between ESBL-*E. coli* and *E. coli*. The inactivation of ESBL-*E. coli* was dependent on energy. These findings suggest that UVA-LED with 365 nm single wavelength could be useful for surface decontamination in healthcare facilities. J. Med. Invest. 67 : 163-169, February, 2020

Keywords **:** *extended-spectrum β-lactamase (ESBL), ESBL-E. coli, E. coli, UVA-LED irradiation*

INTRODUCTION

Extended-spectrum β-lactamase (ESBL) is a β-lactamase that can hydrolyze penicillins, cephalosporins (1) and one or more oxyimino-β-lactams (cefotaxime, ceftazidime, and aztreonam) (2). For decades, resistance to β-lactams have been dramatically increasing, and a significant antimicrobial-resistant pathogen has emerged in the developed world. Centers for Disease Control and Prevention (CDC) trying to assess and understand why infections caused by ESBL-producing Enterobacteriaceae have increased since 2012 (3). In 2017, CDC estimated 197,400 cases of ESBL-producing Enterobacteriaceae among hospitalized patients, 9,100 people deaths, and 1.2 billion dollars to healthcare costs in the United States (3).

ESBL-producing *Escherichia coli* (ESBL-Ec) is considered a healthcare-associated problem, as outbreaks of infection by this organism have been occurring in hospitals or other healthcare facilities, such as nursing homes (1,4). The risk factor for the development of disease with ESBL-producing bacterial in the hospital are patients with prolonged hospitalization (3-5) and in whom invasive medical devices such as urinary catheters, endotracheal tubes, central venous lines for a prolonged duration (4-5). This infection can be spread from one person to another through contaminated hands, surfaces, and environments (3-5), such as ultrasonography coupling gel in particular intrapartum contamination (7) and the washing machine in the pediatric hospital ward in Germany (8). Stethoscopes and thermometers may also be risk factors in the nursing home (4-6).

Hospital environmental cleaning plays a vital role in the termination and reducing of healthcare-associated infections (9-12). The high-risk area of pathogen transmission should be cleaned and decontamination regularly (9). Traditional cleaning methods are currently inefficient for cleanup. New approaches have been proposed, including disinfectants, steam, automated dispersal systems, and antimicrobial surfaces (9). Each country has national standards for hospital environmental cleaning. The hospital has different standard specification in each area, such as operating theaters, outpatient sections, and non-clinical areas (10).

Thus, antibiotic-resistant bacteria have become a new challenge for disinfection. Besides the well-known shortcomings of chlorination, disinfection also has the potential to increase antibiotic-resistant gene transfer if adequate doses are not applied (13). As an innovative non-antibiotic approach, ultraviolet (UV) irradiation has been investigated as a potential decontaminant against environmental pathogens, including the disinfection of surfaces, instruments, and air (9). UV light-emitting diodes (LEDs) have emerged as the most promising new UV light source in the past decade, as they have a longer life and are less fragile and free of toxic components (14). UV-LED has three different bands depending on the wavelengths : UVC (< 280 nm), UVB (280–315 nm), and UVA (315–400 nm) (15). The mechanisms of the inactivation of microorganisms by UV are dependent on the irradiation wavelength, process conditions, and different organisms (14-16). Many studies have reported that UV-LEDs have been used for many years and at various wavelengths and developed as a new method for the inactivation

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of microorganisms (16).

Our previous studies have reported that an originally developed UVA-LED with a 365 nm irradiation system could inactivate bacteria in the environment, in particular, water disinfection systems. The UVA-LED irradiation system was able to inactivate bacteria (17-20,28), such as *V. parahaemolyticus*, enteropathogenic *E. coli*, *S. aureus*, and *E. coli* DH5∝ at minimum energy 315 J/cm^2 in 75 min. Inactivation of *S. enterica* serovar *enteritidis* required energy 672 J/cm^2 in 160 min by UVA-LED (18). The UVA-LED was reported to induce cellular membrane damage and delay growth (21-23). The mechanisms indirectly by increasing the levels of reactivating oxygen species, including superoxide anion radicals, hydroxyl radicals, hydrogen peroxide, and singlet oxygen (18). UVA light exposure resulted in modest reductions of vegetative microorganisms and reduced recovery of pathogenic bacteria from in-use medical equipment (24).

Here, we explored the effectiveness of UVA-LED irradiation to inactivate ESBL-Ec and *E. coli* from patients at Tokushima University Hospital. We investigated how UVA-LED inactivated ESBL-Ec and *E. coli* with different irradiation times and various energies. Further, we compared the different effects of UVA-LED radiation on the inactivation of *E. coli* and ESBL-Ec.

MATERIALS AND METHODS

Screening and confirmation of ESBL production

Bacterial samples obtained from two compartments were clinical isolate strains and food isolate strains. Six *E. coli* and 12 ESBL-Ec clinical isolates from patients from December 2017 to June 2018 at Tokushima University Hospital were measured using MALDI Biotyper (Bruker) MALDI-TOF mass spectrometer. A confirmation test for ESBL-Ec based on the microliquid method of the Clinical and Laboratory Standards Institute (CLSI ; M100-S22) was performed. Dry plate E-EP01 for Gram-negative bacilli was used together with a microbial susceptibility analyzer (DPS192iX).

ESBL-Ec food isolates collected from chicken meats were purchased from three supermarkets in Tokushima City, Tokushima, Japan, from May to June 2018. Two grams of each sample was stomached in 18 ml Enterobacteriaceae enrichment mannitol broth (Merck, Germany). After incubation at 37°C for 16 ± 2 h, a loopful of enriched culture was streaked onto MacConkey agar (Difco™ Mac Conkey, Becton, Dickinson and Co., USA) containing 1 mg/l CTX (Nihon Becton Dickinson, Tokyo, Japan) and incubated at 37°C for 24 h.

Detection of ESBL-Ec genes

The isolation of ESBL-Ec DNA and DNA sequence was done using DNeasy Blood and Tissue Kit (Qiagen). Polymerase chain reaction (PCR) was performed using two kinds of multiplex PCR using Cica Geneus ESBL Genotype Detection Kit (Kanto Chemical Co., Inc.) to identify *ESBL* genes, including *bla*TEM, *bla*SHV, *bla*CTX-M-1 group, *bla*CTX-M-2 group, *bla*CTX-M-8 group, and *bla*CTX-M-9 group.

Antimicrobial susceptibility testing

All samples of clinical isolates and food isolates were sent to the Department of Laboratory Medicine, Tokushima University Hospital, for antimicrobial susceptibility testing. A total of 13 antibiotics were tested : ampicillin, piperacillin-tazobactam, cefazolin, cefmetazole, ceftriaxone, flomoxef, aztreonam, imipenem, meropenem, cefoperazone/sulbactam, ciprofloxacin, gentamicin, and selfamethoxazole-trimethoprim. The minimum inhibitory concentration was measured based on the guidelines of the CLSI. The disc diffusion method was used, in which CPDX inhibited clavulanic acid with AmpC/ESBL disc (Kanto Chemical).

UVA-LED irradiation procedure

The UVA-LED (Nichia Corp., Japan) with 365 nm wavelength was used as the sterilization device. In this experiment, two types of 365 nm UVA-LED were used. The first type [NC4U133B (T)] was equipped with eight 365 nm LEDs (Fig. 1a) (17-19), whereas the second type [NVSU233A (T)-D1)] was equipped with three 365 nm LEDs with the lens (Fig. 1c). We connected the UVA-LED device with a direct-current power supply (Fig. 1b). The current of the circuit was set to 0.23 or 0.50

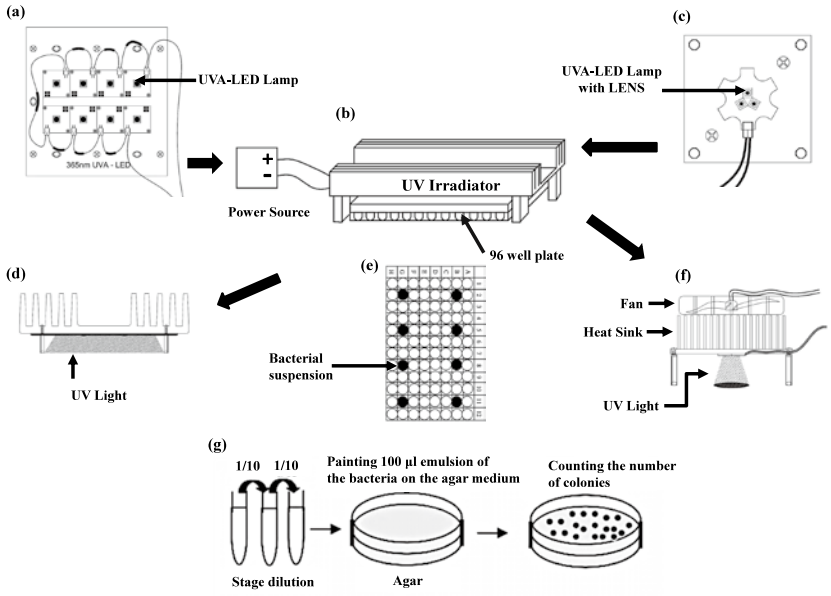

Figure 1. UVA-LED Irradiation device. The first type of UVA-LED equipped with eight 365 nm LEDs (a), and the second type equipped with three 365 nm LEDs with lens (c). The UVA-LED connected to a DC power supply (b), the illustration light during the irradiation of the first type UVA-LED light (d), and the second type (f). Colony-forming ability assay (g) before and after irradiation.

A constantly.

Bacteria were cultured in Luria-Bertani (LB) broth (1% tryptone, 1% NaCl, 0.5% yeast extract) at 37°C for 18 h. Cells were centrifuged (12,000 rpm, 3 min), washed two times with sterilized phosphate-buffered saline (PBS ; pH 7.4), and suspended in PBS at an initial concentration of 5×10^4 to 7×10^4 or 5×10^6 to 7×10^6 colony-forming units/ml. A total of 200 µl bacterial suspension were placed into a disposable 96-well plate (Fig. 1e). The distance between the UVA-LED and the surface of the bacterial solution was 2 cm. Then, 96-well plates were inserted under the sterilization device, and UVA-LED was irradiated (Fig. 1d and 1f). UVA-LED irradiation was performed at room temperature (25°C) in the same room for various periods for 15, 30, and 45 min in the first type and 5, 10, and 15 min in the second type. The UVA-LED devices equipped with a fan and heat sink (Fig. 1f) for the cooling system. The total energy (J/cm^2) was calculated as irradiance (W/cm^2) × exposure time (s) (Table 1).

Table 1. Energy produces by UVA-LED in the first type (a) and the second type (b) by different output power and irradiation time.

(a)

Power (Ampere)	Irradiation Time (Minutes)	Irradiance (mW)	Total Energy (J/cm^2)
Low Power (0.23)	15	6.93	6.23
	30	6.93	12.47
	45	6.90	18.63
High Power (0.50)	15	12.22	13.70
	30	15.05	27.08
	45	15.10	40.76

(b)

Power (Ampere)	Irradiation Time (Minutes)	Irradiance (mW)	Total Energy (J/cm^2)
0.23	5	129.5	38.85

Determination of the inactivation level

A colony-forming assay determined the inactivation level (Fig. 1g). After UVA-LED irradiation, bacterial suspensions were diluted appropriately, plated on LB agar plates, and incubated at 37°C for 18 h. After incubation, the number of colonies was counted, and a log survival ratio or an inactivation percentage was calculated using the following equation :

$$\log \text{survival ratio} = \log (N_t/N_0)$$

where N_t is the colony count of the UV irradiated sample and N_0 is the colony count of the sample before UV irradiation. The inactivation efficiency reached a minimum -3 log reduction. These data indicate that UVA-LED irradiation can inactivation the bacteria (25-26).

Statistical analysis

Every isolated strain was regarded as a sample. Each data in this study represents the average ± standard deviation of three replicates. Analysis of covariance was used to analyze the differences among group means in each sample strain. $p < 0.05$ was considered statistically significant. Statistical analysis was performed using IBM SPSS Statistics version 25 software.

RESULTS

Characteristics of ESBL-Ec

The incidence rate of ESBL-producing Enterobacteriaceae in the last 10 years from 2010 to 2018 in our hospital has fluctuated (10–25%) ; however, overall, it tended to increase. The percentage of ESBL-Ec was 19.4% in 2014, 17.8% in 2015, 14% in 2016, 19.7% in 2017, and 22.2% in 2018 (data not shown).

PCR analysis showed that 12 ESBL-Ec clinical isolates (Table 2a) positive for ESBL production harbored *bla*CTX-M-9 (75% ; n = 9) and 3 isolates harbored a combination of *bla*TEM and/or *bla*CTX-M-1 (25% ; n = 3), whereas 6 ESBL-Ec food isolates (Table 2b) harbored *bla*TEM (66.67% ; n = 4) and 2 isolates harbored a combination of *bla*TEM and/or *bla*CTX-M-2 (33.33% ; n = 2).

Six *E. coli* clinical isolates, 12 ESBL-Ec clinical isolates, and 6 ESBL-Ec food isolates were tested with 13 antimicrobial agents. Six *E. coli* clinical isolates were susceptible to 11 antimicrobial agents, except ampicillin (16.67%) and ciprofloxacin (16.67% ; data not shown). All tested ESBL-Ec clinical and food isolates displayed resistance to ampicillin, cefazolin, and ceftriaxone. In contrast, all strains were susceptible to piperacillin-tazobactam, flomoxef, imipenem, and meropenem. ESBL-Ec clinical isolates were mainly resistant to aztreonam (83.3%),

Table 2. Genotyping and antimicrobial resistance of ESBL- *E. coli* (ESBL-Ec). 12 samples of ESBL-Ec from clinical isolates (a), and six samples of ESBL-Ec from domestic chicken meats (b).

(a)

ESBL No.	ESBL Genotyping
E1	CTX-M9
E2	TEM, CTX-M1
E3	CTX-M9
E4	CTX-M9
E5	CTX-M9
E6	CTX-M9
E7	CTX-M9
E8	CTX-M9
E9	CTX-M9
E10	CTX-M9
E11	TEM, CTX-M1
E12	TEM, CTX-M1
n = 12	CTX-M9 : 9 (75%) TEM, CTX-M1 : 3 (25%)

(b)

ESBL No.	ESBL Genotyping
C1	TEM
C2	TEM, CTX-M2
C3	TEM, CTX-M2
C4	TEM
C5	TEM
C6	TEM
n = 6	TEM : 4 (66.67%) TEM, CTX-M2 : 2 (33.33%)

ciprofloxacin (75%), sulfamethoxazole-trimethoprim (50%), cefmetazole (8.33%), and gentamicin (4.17%), whereas ESBL-Ec food isolates from domestic chicken meats were resistant to aztreonam (100%), sulfamethoxazole-trimethoprim (33.3%), and gentamicin (16.7%). ESBL-Ec food isolates were still susceptible to cefmetazole, cefoperazone-sulbactam, and ciprofloxacin.

Inactivation of ESBL-Ec by the first type of 365 nm UVA-LED

In the first experiment, *E. coli* and ESBL-Ec were inactivated using the first type of 365 nm UVA-LED. Figure 2 shows the difference in the log survival ratio of *E. coli* and ESBL-Ec based on the irradiance [low power (0.23 A) and high power (0.50 A)] and irradiation time (15, 30, and 45 min).

At the circuit current of 0.23 A for 45 min, the total energy produced was 18.63 J/cm^2 (Table 1a), and the log survival ratio of bacteria was different in each isolated strain. The log survival ratio was -0.75 ± 0.15 in *E. coli* clinical isolates (Fig. 2a), -0.74 ± 0.05 in ESBL-Ec clinical isolates (Fig. 2c), and -1.39 ± 0.15 in ESBL-Ec food isolates (Fig. 2e). The circuit current was increased to 0.50 A for 45 min, and the total energy produced was 40.76 J/cm^2 (Table 1a). The log survival ratio was -3.37 ± 0.29 in *E. coli* clinical isolates (Fig. 2b), -3.87 ± 0.46 in ESBL-Ec clinical isolates (Fig. 2d), and -3.89 ± 0.41 in ESBL-Ec food isolates (Fig. 2f). These results indicated that 40.76 J/cm^2 was the minimum energy dosage required to inactivate *E. coli* and ESBL-Ec at a minimum irradiation time of 45 min, and the killing ability was significantly increased.

Inactivation of ESBL-Ec by the second type 365 nm UVA-LED

In the second type of UVA-LED, the UVA-LED tools system was improved. The difference in irradiance produced by the second type of UVA-LED was measured. At the circuit current of 0.23 A for 5 min, the total energy produced was 38.85 J/cm^2 (Table 1b), and the log survival ratio of bacteria was different in each isolated strain. The log survival ratio was -1.78 ± 0.16 in *E. coli* clinical isolates (Fig. 3a), -3.25 ± 0.29 in ESBL-Ec clinical isolates (Fig. 3b), and -2.97 ± 0.15 in ESBL-Ec food isolates (Fig. 3c). Figures 2 and 3 indicate that UVA-LED can inactivate *E. coli* and ESBL-Ec from both clinical and environment isolates.

Inactivation of bacteria was dependent on the energy dosage produced by UVA-LED and irradiation time

The log survival ratio is the ability of *E. coli* and ESBL-Ec to survive after UVA-LED radiation. *E. coli* and ESBL-Ec using UVA-LED with 365 nm wavelength required high dosage energy for inactivation. High energy required high output power of irradiance and long irradiation time. The increase of energy produced by the UVA-LED system led to the decrease of the log survival ratio. Figure 4 shows the inactivation of *E. coli* and ESBL-Ec in an UVA-LED dose-dependent manner. There were no significant differences in the three regression lines between *E. coli* clinical isolate strains (Fig. 4a) and ESBL-Ec from both clinical (Fig. 4b) and food (Fig. 4c) isolate strains (data not shown).

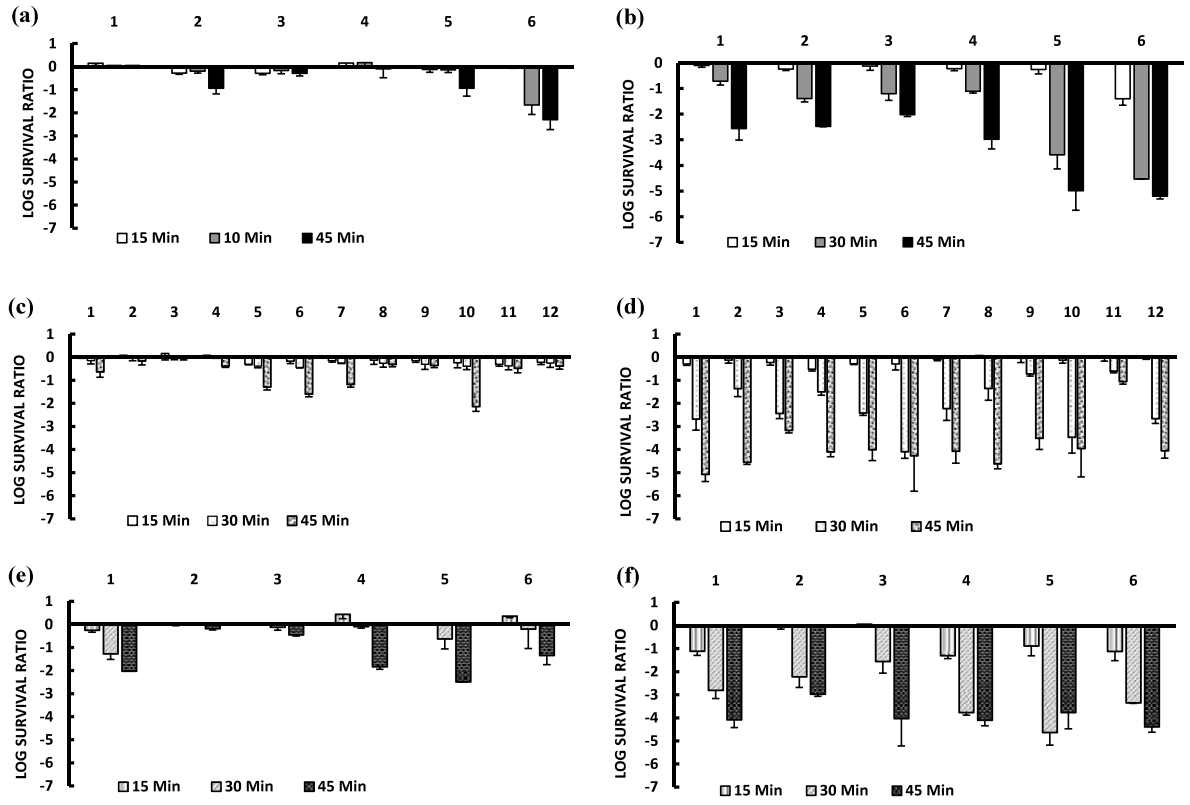

Figure 2. The log survival ratio depends on different output power and irradiation time using the first type of 365 nm UVA-LED. *E. coli* clinical isolate group in the first line (a and b), ESBL-Ec clinical isolates group in the second line (c and d), and ESBL-Ec food isolates group in the third line (e and f). The left side (a, c, e) at the low power (0.23 A) from 15 min (6.23 J/cm^2), 30 min (12.47 J/cm^2), and 45 min (18.63 J/cm^2). The right side (b, d, f) at the high power (0.50 A) from 15 min (13.70 J/cm^2), 30 min (27.08 J/cm^2), and 45 min (40.76 J/cm^2). Values are shown as means ± SD (n = 3, n = number of independent replicates).

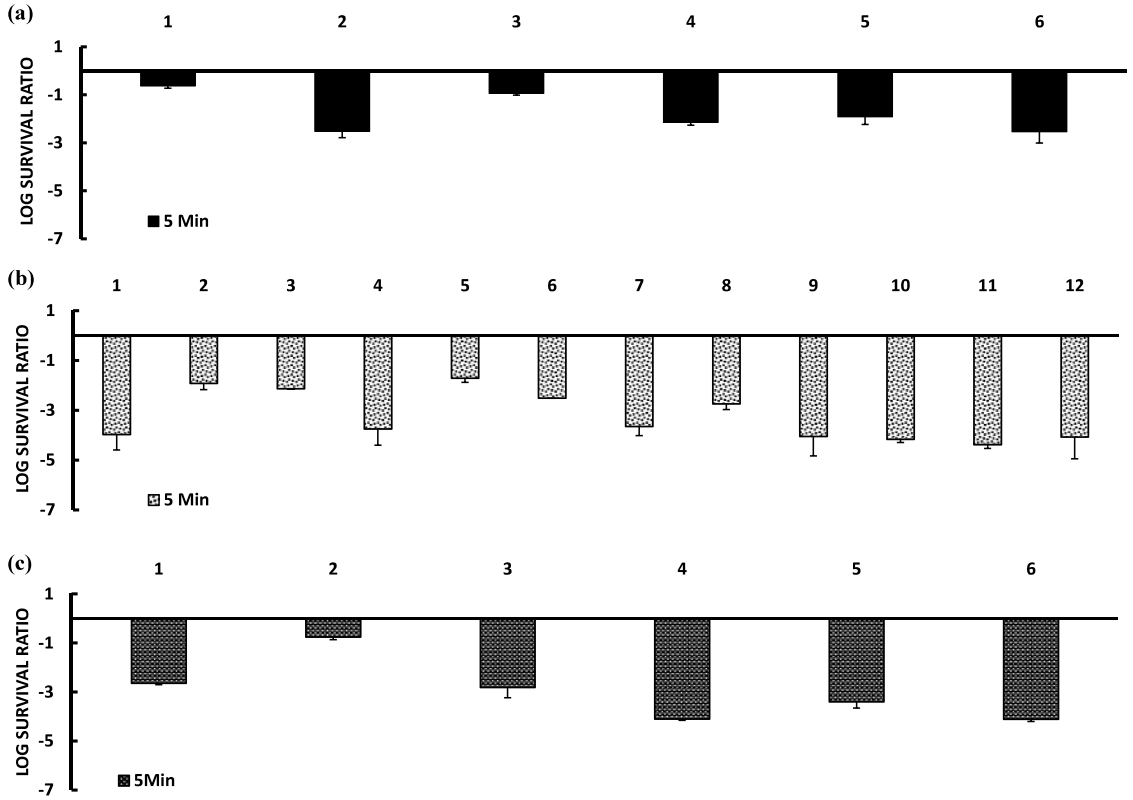

Figure 3. The log survival ratio of *E. coli* clinical isolates group (a), ESBL-Ec clinical isolates group (b) and ESBL-Ec food isolates group (c), at the low power (0.23 A), in 5 min irradiation (38.85 J/cm^2) using the second type of 365 nm UVA-LED. Values are shown as means ± SD ($n = 3$, n = number of independent replicates).

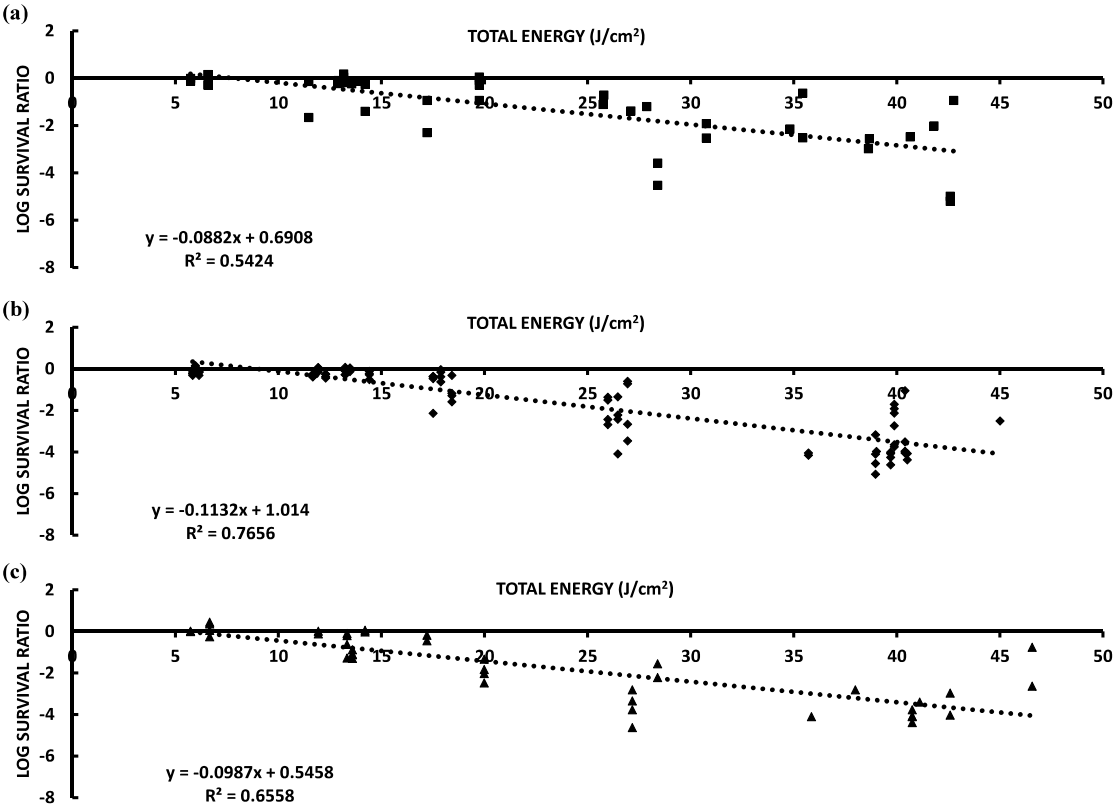

Figure 4. UVA-LED Irradiation inactivation of *E. coli* clinical isolates (a), ESBL-Ec clinical isolates (b), ESBL-Ec food isolates (c), in an UVA-LED dose-dependent manner. There were no significant differences in the three regression lines ($P > 0.05$) between *E. coli* clinical isolate strains and ESBL-Ec, both clinical and food isolate strains.

DISCUSSION

In this study, we focused on the inactivation of ESBL-Ec from both clinical and food isolates, and we also tried to inactivate *E. coli* from clinical isolates using UVA-LED with 365 nm wavelength. This is the first report that applied UVA-LED on *E. coli* and ESBL-Ec from clinical and food isolates.

We applied the first type of 365 nm UVA-LED device that was developed in our previous work (17-20,28). The killing ability of *E. coli* and ESBL-Ec was significantly increased with high energy dosage and long irradiation time. In the second type of UVA-LED, we improved the UVA-LED tools system equipped with three 365 nm LEDs with lenses. The lenses played a vital role in affecting the quality of the lighting itself. The light had more brightness and was more concentrated (Fig. 1f) compared to the first type of UVA-LED (Fig. 1d).

The energy produced by the first type of UVA-LED at 0.50 A after 45 min was similar to the power provided by the second type of UVA-LED at 0.23 A after 5 min irradiation. In the second type of UVA-LED, higher irradiance and shorter exposure time led to higher inactivation efficiency at the same UVA dose compared to the first type of UVA-LED. Therefore, we concluded that the second type of UVA-LED saved more time and was energy efficient in the deactivation of *E. coli* and ESBL-Ec. The inactivation of *E. coli* and ESBL-Ec in UVA-LED was dose-dependent. The high energy produced by output power and irradiation time of the UVA-LED system increased the killing ability ; thus, the log survival ratio of bacteria decreased.

Our UVA-LED irradiation system can inactive *E. coli* and ESBL-Ec at least 38.85 J/cm^2 to achieve -3 log inactivation to reach safe levels (25-26). The pathogenic *E. coli* group has differences in an infective dose range that causes illness in healthy adults. The infectious dose of enterotoxigenic *E. coli* (ETEC) is estimated to be 10^8 organisms. Enteroinvasive *E. coli* (EIEC) at least 10^6 organisms and enteropathogenic *E. coli* (EPEC) is estimated to be 10^6 organisms (27). Our result indicated that UVA-LED radiation could inactive the bacteria -3 log (10^8 to 10^5 organisms) reduction of *E. coli* or ESBL-Ec. It lower than the infective doses range of pathogenic *E. coli* group. There is no scientific standard that exists in the probability of ESBL-Ec infection. Many resources identifying the requires UVA-LED doses for inactivation various pathogens can be found in numerous publications and studies. All existing requirements for disinfection to achieve at least -3 log inactivation of *E. coli*.

Interestingly, UVA-LED sensitivity in this study was different in each genotype isolated from clinical and food isolate strains. However, we were not able to find a significant correlation between genotyping and UVA-LED sensitivity. Therefore, for the next step, we plan to increase the number of ESBL-Ec from another isolate, such as vegetables, to get a variety of ESBL-Ec genotyping. Furthermore, we would want to explore the sensitivity mechanism of UVA-LED on ESBL-Ec. The irradiation of UVA-LED induces oxidative damage of intracellular DNA or protein and increases 8-OHdG, a DNA oxidation product. The oxidative effects of UVA-LED-mediated reactive oxygen species are significant for bacterial inactivation (28).

In addition, the UVA-LED radiation has been reported effected for inactivation on biofilms such as *Candida albicans* or *Escherichia coli*. Exposure pulsed of UVA-LED radiation at 365 nm wavelength had more inactivation efficiency than continuous mode (29). The UVA-LED irradiation system has been reported can killing the methicillin-resistant *Staphylococcus aureus* (MRSA), *Candida aureus*, bacteriophage MS2, and bacteriophage Phi X174. The UVA-LED irradiation at 365 nm wavelength shown a significant reduction of the pathogenic microorganism from in-use medical equipment (24). These findings suggest that healthcare-associated infections (HAIs) caused by antibiotic resistance, such as ESBL-producing Enterobacteriaceae and MRSA, can be reduced by UVA-LED irradiation. The results of this study confirm that UVA-LED radiation is a viable technology as a disinfection system in healthcare settings.

The present study is a brief report on the ESBL-Ec incidence at Tokushima University Hospital. Data showed the prevalence and characteristics of ESBL-Ec clinical isolates at Tokushima University Hospital from December 2017 to June 2018 and ESBL-Ec food isolates in retail domestic chicken meats from May to June 2018 in Tokushima City. Our study presents several limitations. The number of processed samples was small, and systematic surveillance was not used, so this study does not represent the overall data of ESBL-Ec incidence in Tokushima Prefecture.

CONCLUSIONS

This study can assist in developing the UVA-LED irradiation system as an innovative non-antibiotic approach to be applied for the disinfection and inactivation of pathogen-associated infectious diseases in the hospitals or healthcare facilities and food safety systems, as this system is highly energy efficient, reliable, free of mercury, simple to apply, and cost-effective and has a much longer lifespan.

This is the first report that applied UVA-LED on *E. coli* and ESBL-Ec from clinical and food isolates. In this study, we demonstrated that UVA-LED was effective to inactivate *E. coli* and ESBL-Ec from clinical and food isolate strains. The inactivation of bacteria was dependent on the output power of the UVA-LED device and irradiation time.

CONFLICT OF INTEREST

There is no actual or potential conflict of interest that exist in this manuscript.

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