ORIGINAL

Combined treatment of UVA irradiation and antibiotics induces greater bactericidal effects on *Vibrio parahaemolyticus*

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Abstract : The presence of antibiotics in the environment and their subsequent impact on the development of multi-antibiotic resistant bacteria has raised concerns globally. Consequently, much research is focused on a method to produce a better disinfectant. We have established a disinfectant system using UVA-LED that inactivates pathogenic bacteria. We assessed the bactericidal efficiency of a combination of UVA-LED and antibiotics against *Vibrio parahaemolyticus*. Combined use of antibiotic drugs and UVA irradiation was more bactericidal than UVA irradiation or antibacterial drugs alone. The bactericidal synergy was observed at low concentrations of each drug that are normally unable to kill the bacteria. This combination has the potential to become a sterilization technology. J. Med. Invest. 63 : 63-67, February, 2016

Keywords **:** *UVA, LED, bactericidal effect, synergistic benefit, antibiotics*

INTRODUCTION

Vibrio parahaemolyticus is a gram-negative, marine bacterium that can causes gastroenteritis commonly after the consumption of raw or undercooked seafood. *V. parahaemolyticus* is one of the most frequently reported food-borne pathogens in many Asian countries (1, 2), and sporadic outbreaks have been seen worldwide, including countries in North and South America (3, 4), Europe (5) and Africa (6).

Antimicrobials are commonly used in the treatment and control of prolonged and severe pathogens infection. However, extensive usage of antimicrobials has resulted in the development of antimicrobial resistance in seafood pathogens and rendered many known antimicrobials ineffective. There were reported that *V. parahaemolyticus* have drug resistance and it will became a problems for regulating the infection, recently (7, 8). Therefore development of a more effective sterilization technology is wished for.

Ultraviolet (UV) systems are widely used for effective sterilization. The low-pressure mercury lamp emitting UVC (254 nm) is used generally as a germicidal light because it is the most effective wavelength for damaging DNA, whose maximum absorbance is about 260 nm. The cost of UVC disinfection, however, is relatively high because the life span of the lamp is rather short and it consumes a large amount of energy. We previously established a disinfectant system using a UVA emitting LED (UVA-LED) that has the ability to inactivate pathogenic bacteria (9-11). UVA induces cellular membrane damage and growth delay (12-14) indirectly by increasing levels of reactive oxygen species (ROS), including superoxide anion radicals (O^{2-}), hydroxyl radicals (OH^{-}), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) (9, 10, 15, 16). A UVA-LED sterilization system is easy to combine with other disinfection systems and is more cost effective than the usual UVC method.

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We reported that the simultaneous irradiation with UVA-LED and UVC had a synergistic bactericidal effect on *V. parahaemolyticus* (17). We also showed that the combined use of a soluble organic biocide and UVA light increased the bactericidal effect compared to either treatment alone (18). These combinations exhibited strong bactericidal activity against pathogens, even though the concentrations of the compound, intensity of UVC, or intensity of UVA were not bactericidal on their own. This evidence may provide the basis for novel treatments for disinfection of contaminated liquids or of articles intended for use in the environment.

In this study, we assess the bactericidal efficiency of a combination of UVA-LED and antibiotics on *V. parahaemolyticus*. Antibiotic drugs fall into two general categories : bactericidal drugs that kill bacteria with an efficiency of > 99.9% and bacteriostatic drugs that inhibit bacterial growth (19). We use three bactericidal drugs (ampicillin, gentamicin, and norfloxacin) and one bacteriostatic drug (chloramphenicol). Lastly, we discuss the possible advantages and utility of the combination of UVA-LED and antibiotic treatments for sterilization.

MATERIALS AND METHODS

1. Culture and growth of bacteria

Wild type *Vibrio parahaemolyticus* RIMD 2210633 was purchased from Takara Bio Inc. (Otsu, Japan) and stored at -80°C. Thawed bacteria were incubated overnight at 37°C in 2 ml of 3% Luria-Bertani (LB) broth (3% NaCl, 1% tryptone, 0.5% dried yeast extract) with shaking at 170 rotations per minute (rpm). Then bacterial cells were washed three times in sterilized PBS with centrifugation for 3 min at 12000 rpm between each wash. The final supernatant was discarded, and the cell pellet was resuspended in PBS. The bacteria solution was diluted in PBS until the OD_{600} reached 0.49-0.51.

2. $UVA_{365\,nm}$-LED irradiation

200 µl of OD-adjusted *V. parahaemolyticus* solution was put into each of 5 wells of a 96-well plate. The colony-forming units (CFU) per well was $1.01\times10^8\pm0.32\times10^8$ per well. One well was used as a

non-irradiated control, and the other four wells were UVA-LED (Nichia Chemical Inc.) irradiated simultaneously for 12 min at 0.45 A current and 14 V. The distance from the solution sample to the UVA-LED was 1 cm. The intensity of UVA-LED light was 635 w/m^2, which was measured by an accumulated UV meter in a dark room (UIT-250, Ushio co., LTD, Tokyo, Japan). The surface temperature of the LED was kept constant at 30°C by a low-temperature thermostat (Yamato Scientific Co., LTD). The experiment was performed 3 times with UVA-LED alone, and 3 times combined with each antibacterial drug.

3. Antibiotic treatment

The β-lactam antibiotic ampicillin (Sigma-Aldrich) and the aminoglycoside gentamicin (Sigma-Aldrich) were dissolved in Milli-Q ultra-pure water. Norfloxacin (Sigma-Aldrich), a fluoroquinolone antibiotic, was dissolved in acetic acid, and chloramphenicol (Wako, Japan) in ethanol. Serial dilutions from 0.1 μg/ml to 100 μg/ml were prepared for each antibacterial drug ; the range of concentrations depended on the mean minimum inhibitory concentration (MIC) for *V. parahaemolyticus* and the maximum solubility of the drug. For the combined treatment groups, *V. parahaemolyticus* was irradiated for 12 minutes, and then antibacterial solution was added. The bacteria were incubated in the dark with antibiotic for 4 hours (ampicillin, gentamicin, chloramphenicol) or 10 minutes (norfloxacin).

4. Plate-culture of treated V. parahaemolyticus and determination of bacterial inactivation level

Dilutions ranging from 10^{-1} to 10^{-6} were made for antibiotic and/or UVA-LED treated *V. parahaemolyticus* before overnight culture in a 37°C incubator (Sanyo, Co. LTD). 100 μl of each dilution was spread onto LB-agar plates (3% agar) and cultured for 15 to 18 hours. After incubation, CFU were counted and log survival ratios were calculated using the following formula :

Log Survival Ratio = $\log_{10}$ (N_t/N_0)

Nt is the colony number of the UVA-irradiated sample and/or antibacterial drug treated sample, and N_0 is the colony count of the sample before the UVA irradiation and/or antibacterial drug treatment.

5. Analysis of results

The bactericidal efficiency of single and combined treatments was assessed by determining bacteria reduction with the log survival ratio shown above. The synergy value of combined UVA-LED irradiation and antibacterial drugs was calculated by the following equation :

Synergy value = log reduction by combined UVA/antibacterial drug − (log reduction by single UVA + log reduction by single antibacterial drug)

Synergy value was bigger than zero, which indicated exist of synergy of bactericidal effect in combined use. Each datum in this study represents the average value of four replicates.

RESULTS

Combined treatment of ampicillin and UVA irradiation

Ampicillin is a bactericidal drug that works by using its amino group to penetrate the outer membrane of Gram-negative bacteria. It inhibits binary fission, the third and final stage of bacterial cell wall synthesis, which ultimately leads to cell lysis (20). MIC of ampicillin for *V. parahaemolyticus* ranges from 10 to 100 μg/ml (21, 22).

UVA irradiation alone was bactericidal, but 10 μg/ml or 100 μg/ml of ampicillin did not kill *V. parahaemolyticus* (Figure 1). When irradiated for 12 minutes at 38.1 kJ/m^2 and treated with ampicillin for 4 hours, the bactericidal effect was greater than with UVA irradiation only ; similar results were seen when 10 or 100 μg/ml ampicillin was used. The calculated synergy values between UVA irradiation and both of 10 μg/ml ampicillin or 100 μg/ml ampicillin were 1.3, respectively.

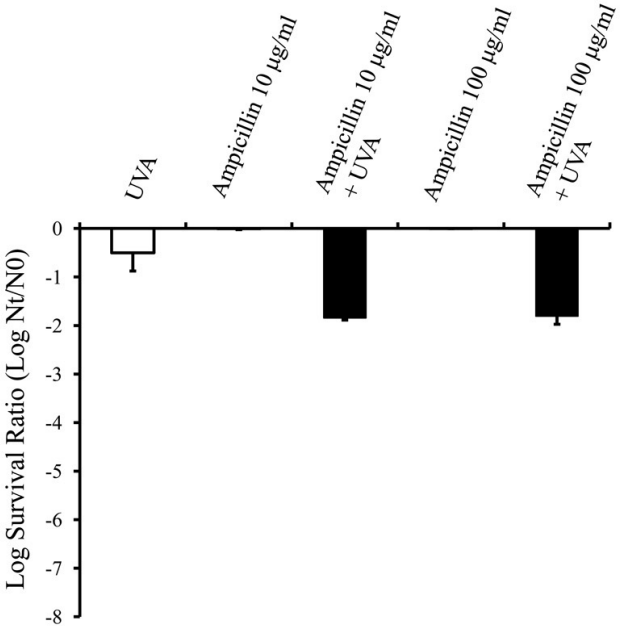

Figure 1. Combined bactericidal effect of UVA_{365nm}-LED irradiation and ampicillin on *V. parahaemolyticus*.
□ ; UVA irradiation, ▒ ; ampicillin treatment, ■ ; combination with UVA irradiation and ampicillin treatment. *V. parahaemolyticus* samples were exposed to UVA-LED with 3.81×10^4 J/m^2/min. Ampicillin powder was dissolved in Mill-Q ultra-pure water, and then mixed with UVA-irradiated bacteria samples. The bacteria were incubated with antibiotic for 4 hours in the dark. All values are expressed as the mean ± SD of four independent experiments.

Combined treatment of gentamicin and UVA irradiation

Gentamicin, a bactericidal antibiotic, interrupts protein synthesis by irreversibly binding the 30S subunit of the bacterial ribosome. MIC of gentamicin for *V. parahaemolyticus* is reported to be between 0.1 and 10 μg/ml (22, 23).

The bactericidal effect of 0.1 or 1 μg/ml of gentamicin alone was weak ; however, 10 μg/ml of gentamicin had a significant bactericidal effect by itself (Figure 2). The calculated synergy values between UVA irradiation and 1 μg/ml gentamicin or 10 μg/ml gentamicin were 3.0 or 4.1, respectively. This indicated a bactericidal synergy with the combination of UVA irradiation and gentamicin (Figure 2). Furthermore, the bactericidal effect of UVA irradiation and 10 μg/ml gentamicin was higher than that of UVA irradiation and 1 μg/ml gentamicin. This result indicates that the synergy phenomenon between UVA irradiation and gentamicin is dependent on the concentration of gentamicin (Figure 2).

Combined treatment of norfloxacin and UVA irradiation

Norfloxacin is a broad-spectrum antibiotic, i.e., it is active against both Gram-positive and Gram-negative bacteria. It kills bacteria by inhibiting DNA gyrase, an enzyme required to separate bacterial DNA strands, thereby inhibiting cell division (24, 25). The average MIC of norfloxacin for *V. parahaemolyticus* is from 1 to 10 μg/ml (23-25).

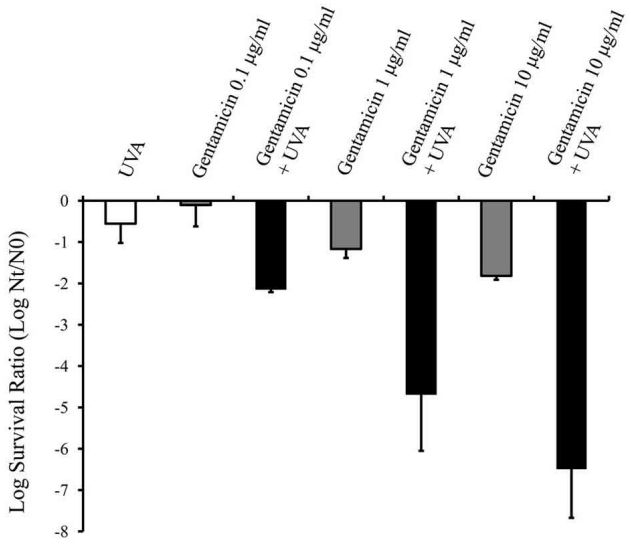

Figure 2. Combined bactericidal effect of UVA_{365nm}-LED irradiation and gentamicin on *V. parahaemolyticus.*
□; UVA irradiation, ▒; gentamicin treatment, ■; combination with UVA irradiation and gentamicin treatment. *V. parahaemolyticus* samples were exposed to UVA-LED irradiation with 3.81×10^4 J/m^2/min. Gentamicin solution was mixed with UVA-irradiated bacteria samples and incubated for 4 hours in the dark. All values are expressed as the mean± SD of four independent experiments.

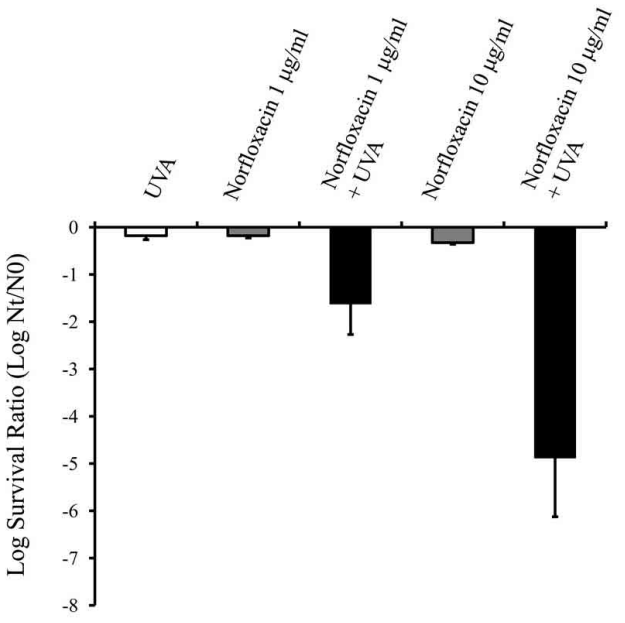

Figure 3. Combined bactericidal effect of UVA_{365nm}-LED irradiation and norfloxacin on *V. parahaemolyticus.*
□; UVA irradiation, ▒; norfloxacin treatment, ■; combination with UVA irradiation and norfloxacin treatment. *V. parahaemolyticus* samples were exposed to UVA-LED irradiation with 3.81×10^4 J/m^2/min. Norfloxacin powder was dissolved with acetic acid and then added to UVA-irradiated bacteria samples. The bacteria were incubated with antibiotic for 10 minutes in the dark. All values are expressed as the mean± SD of four independent experiments.

The combination of 1 or 10 μg/ml norfloxacin with UVA irradiation was more bactericidal for *V. parahaemolyticus* than UVA irradiation or norfloxacin exposure alone. The calculated synergy values between UVA irradiation and 1 μg/ml norfloxacin or 10 μg/ml norfloxacin were 1.3 or 4.4, respectively. This suggests that an antibacterial synergy against *V. parahaemolyticus* solution exists between UVA irradiation and norfloxacin treatment (Figure 3).

Combined treatment of chloramphenicol and UVA irradiation

Chloramphenicol prevents protein chain elongation by stopping the peptidyl transferase activity of the bacterial ribosome, thereby inhibiting the growth of, but not killing, bacteria. It specifically binds to A2451 and A2452 residues in the 23S rRNA of the 50S ribosomal subunit, preventing peptide bond formation (26).

The reported average MIC of chloramphenicol is 10 μg/ml to 100 μg/ml for *V. parahaemolyticus* (22, 23). The calculated synergy values between UVA irradiation and 1 μg/ml chloramphenicol or 10 μg/ml chloramphenicol were 0.1 or 2.8, respectively. Treatment with chloramphenicol alone at 10 μg/ml, or 100 μg/ml had few bactericidal effects (Figure 4).

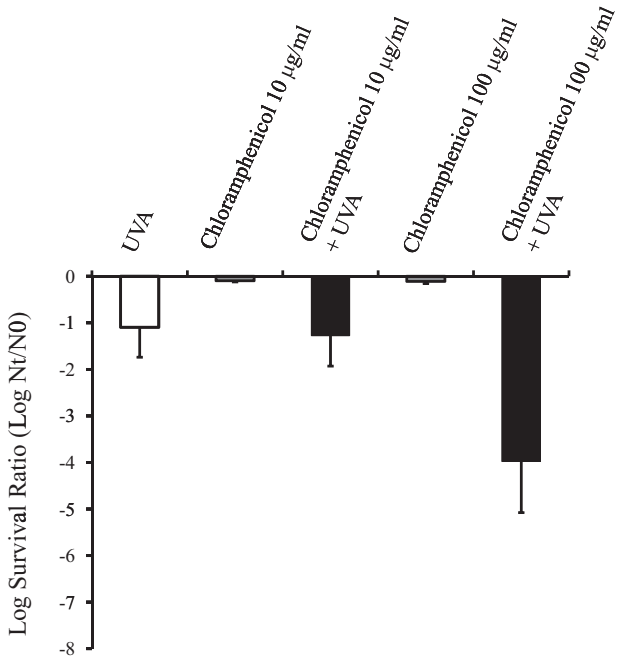

Figure 4. Combined bactericidal effect of UVA_{365nm}-LED irradiation and chloramphenicol on *V. parahaemolyticus.*
□; UVA irradiation, ▒; chloramphenicol treatment, ■; combination with UVA irradiation and chloramphenicol treatment. *V. parahaemolyticus* samples were exposed to UVA-LED irradiation with 3.81×10^4 J/m^2/min. Chloramphenicol powder was dissolved with ethanol and then mixed with UVA-irradiated bacteria samples. The bacteria were incubated with antibiotic for 4 hours in the dark. All values are expressed as the mean± SD of four independent experiments.

DISCUSSION

The study presented here revealed that the combination of antibacterial drugs with UVA_{365nm}-LED irradiation induced more bacterial death than UVA irradiation or antibacterial drugs alone. This is the first report on the bactericidal effects of combined UVA irradiation and antibiotic treatment. UVA irradiation indirectly leads to intracellular membrane injury and growth delay by increasing ROS (9, 10, 16, 17). On the other hand, most clinically used antibiotics target cell wall assembly, protein synthesis, or DNA replication. A recent report, however, suggests that oxidative stress is an important contributor to antibiotic leathality (27). Thus, there are possibility that the bactericidal synergy seen in this study is

dependent on the combination of damage by UVA-induced ROS and antimicrobial drugs.

With the combination of UVA irradiation and ampicillin treatment, bactericidal synergy was observed but it was not dose-dependent. There is a possibility that *V. parahaemolyticus* had relatively resistance to ampicillin (28-30) or the particularity by the mechanism of action of ampicillin. Future experiments will be needed to explore the relatively low bactericidal synergy induced by ampicillin and UVA.

Development of the UVA-LED disinfection system introduced in this study was based on water sterilization equipment, as previously reported (9-11). Because the UVA-LED sterilization device has many advantages compared with traditional low-pressure mercury lamps (UVC), such as no mercury waste products, low energy consumption, and a theoretically permanent life span, we expect that it will be applied as a new type of water sterilization device (9-11, 17, 18). Given that bactericidal synergy was observed using low concentrations of each antibiotic, it is possible that this combination may also be developed for use in water treatment.

Altogether, we demonstrated that the combination of UVA irradiation and exposure to antibiotics produced a greater bactericidal effect for *V. parahaemolyticus.* The UVA-LED and antibiotic combination is a potential high-efficiency disinfecting technology for *V. parahaemolyticus.*

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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