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# Mycoplasma Removal from Cell Culture Using Antimicrobial Photodynamic Therapy

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## Abstract

**Objective:** The objective of this research was to determine the effectiveness of antimicrobial photodynamic therapy (aPDT) in the removal of mycoplasmas from contaminated cells. **Background data:** Mycoplasmas often contaminate cell cultures. The cell-contaminating mycoplasmas are removed by antibiotics, but the use of antibiotics usually induces antibiotic-resistant bacteria. aPDT is expected to be a possible alternative to antibiotic treatments for suppressing infections. **Materials and Methods:** *Mycoplasma salivarium* (Ms)-infected human embryonic kidney (HEK) 293 cells were irradiated using a red light-emitting diode (LED) in the presence of methylene blue (MB) as a photosensitizer. The Ms viable count was determined using culture on agar plates or using a mycoplasma detection kit. **Results:** aPDT performed using red LED irradiation was effective in decreasing live Ms in the presence of MB without damaging the HEK293 cells. aPDT removed live Ms from the infected cells after washing the cells with sterilized phosphate-buffered saline (PBS) to decrease the initial number of live Ms before aPDT. **Conclusions:** This study suggests that aPDT could remove mycoplasmas from contaminated cells.

## Introduction

CELL CULTURE IS IMPORTANT IN BASIC STUDIES and in clinical and practical research in medical and dental studies. For example, cell culture is used to produce influenza vaccine instead of using eggs.<sup>1</sup> Cell culture has also been used in tissue engineering. Recently, cell sheets derived from healthy donor periodontal ligaments have been reported to regenerate periodontal tissues.<sup>2</sup> One of the largest problems in cell culture is bacterial contamination. The most popular way to prevent cells from becoming contaminated with bacteria is to add antibiotics to the culture medium. However, bacterial contamination occurs occasionally in most laboratories in spite of the use of antibiotics, because their presence may only suppress bacteria susceptible to the antibiotics and permit the existence of a chronic or latent infection by bacteria that are not susceptible or resistant to the antibiotics. Therefore, it is valuable to develop a method to remove bacteria from cell culture without using antibiotics.

Mycoplasmas are the smallest self-replicating prokaryotes that lack a cell wall. *Mycoplasma salivarium* (Ms) is a member

of the human normal microbial flora, and preferentially resides in dental plaques and gingival sulci.<sup>3,4</sup> As some of mycoplasmas, including Ms, are normal human inhabitants, they are likely to contaminate cell cultures. It has been reported that 5–30% of cell culture systems are infected with mycoplasmas.<sup>5</sup> This contamination is mainly the result of the characteristics of mycoplasmas, such as their small size compared with bacteria and their insusceptibility to antibiotics that target cell-wall synthesis.

Photodynamic therapy (PDT) has been developed as a minimally invasive therapeutic modality mainly applied to cancer treatments.<sup>6</sup> Although the mechanism of PDT-induced cell death is not completely understood, it is considered to be as follows. A photosensitive molecule, which is called a photosensitizer, is localized to tumor cells, and then light having a specific wavelength is irradiated at the targeted site to activate the photosensitizer. As a result of the irradiation, reactive oxygen species (ROS) are generated, which are toxic to tumor cells.<sup>6</sup> Antimicrobial PDT (aPDT) is applied to destroy bacteria instead of tumor cells. In aPDT, bacteria stained with a dye, which functions as a photosensitizer,

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should be killed by the ROS induced by light irradiation of the dye. Therefore, aPDT is expected to be a possible alternative to antibiotic treatments for suppressing infections.<sup>7,8</sup> The goal of this study was to remove Ms from Ms-infected cells using aPDT and a high-power red light-emitting diode (LED).

## Materials and Methods

### Organism and its culture conditions

*M. salivarium* ATCC 23064 (Ms) was grown as described.<sup>9</sup> Briefly, Ms was grown in PPLO broth (Difco/BD bioscience, Carlsbad, CA) supplemented with 20% (v/v) horse serum (Gibco/Invitrogen, Grand Island, NY), 1% (w/v) yeast extract (Difco), 1% (w/v) L-arginine hydrochloride and 50  $\mu\text{g}/\text{mL}$  ampicillin. Cultures were incubated at 37°C for 72 h, at which point the growth was in the mid-logarithmic phase, and centrifuged at 15,000g for 15 min. The cell pellets were washed three times with sterilized phosphate-buffered saline (PBS), re-suspended in PBS to make aliquots, and then stored at -80°C.

To test the effects of the photosensitizer and light irradiation on Ms growth, Ms was allowed to grow in the broth in a flat-bottomed 3 mL vial with or without photosensitizers, and a red LED light was irradiated from its bottom.

### Photosensitizer

Methylene blue (MB) was purchased from Sigma-Aldrich (St Louis, MO). MB was dissolved in MilliQ water to give a 1 mg/mL stock solution that was filter sterilized, and stored at 4°C in the dark until used.

### Light source and irradiation

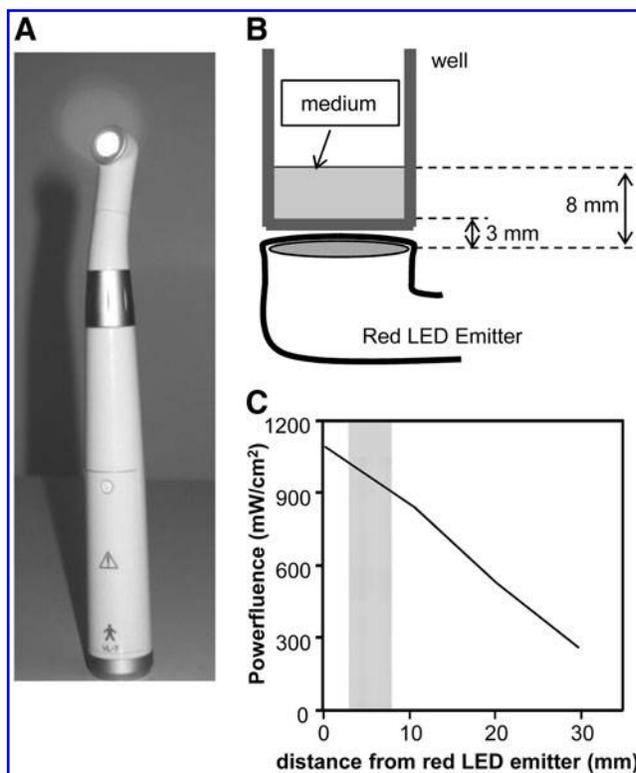
A portable red LED prototype emitter was created (Fig. 1 A) that supplied 5W of power in an extremely small package. The red LED was an LZ1-00R205 Deep Red LED (LedEngin, Santa Clara, CA), which emits specific red wavelengths (600–700 nm), with a peak at 650 nm and a power density of 1100 mW/cm<sup>2</sup>. The light intensity of the emitter was confirmed by a power meter (Nova II) (Ophir, North Andover MA). Irradiation distances to medium or broth were ~3–8 mm (Fig. 1 B). As the directionality of this LED emitter was cone shaped, ~0.5 or 0.78 cm<sup>2</sup> were irradiated from a distance of 3 or 8 mm, respectively.

### Cell cultures

Human embryonic kidney (HEK) 293 cells obtained from ATCC (CRL-1573) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin G (100 U/mL) and streptomycin (100  $\mu\text{g}/\text{mL}$ ) (DME complete). The cells were cultured in a 24 well plate with the bottom of the wells coated with 0.01% poly-L-lysine solution (Sigma-Aldrich).

### Ms infection and the effect of aPDT

Ms infection to HEK293 cells was effected by adding live Ms to HEK293 cells in a 10 cm cell culture dish. To test the effects of MB and/or LED irradiation on the growth rate of HEK293 cells, the infected cells were grown in 500  $\mu\text{L}$  of DME complete medium with or without MB in a 24 well



**FIG. 1.** (A) The red light-emitting diode (LED) emitter used in this experiment. (B) Schematic explanation to show how red LED irradiation was performed. (C) The relationship between power density of the red LED emitter (mW/cm<sup>2</sup>) and distance (mm) from the emitter. Measurement was performed by a power meter at 650 nm. The gray area shows irradiation distances of 3–8 mm.

plate, and then a red LED light was irradiated from the bottom of the plate. The cell viability was determined by staining the cells with 0.4% trypan blue solution (Sigma-Aldrich).

To determine the ratio of apoptotic cells induced by aPDT, uninfected cells were prepared in DME base medium in a 24 well plate. Apoptosis was assessed using an Annexin-V-FLUOS staining kit (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, 24 h after aPDT, the cells were harvested, washed with PBS, and then the cells were stained simultaneously with annexin V and propidium iodide (PI). As necrotic cells may also be stained with annexin V according to the loss of membrane integrity, PI was used to discriminate apoptotic cells and necrotic cells. The percentages of annexin- and annexin-PI-stained cells were determined using a flow cytometer, FACS Calibur (BD Biosciences). For flow cytometric analysis, data for 30,000 cells falling within appropriate forward-scatter and side-scatter gates were collected from each sample. The results were analyzed by using FlowJo software (Tree Star, Ashland, OR).

### Detection of viable Ms

The number of viable Ms was determined by counting colonies on an agar plate, and the viable counts were expressed using the colony-forming unit (CFU). The viable Ms were also detected using a MycoAlert<sup>®</sup> mycoplasma

detection kit (Lonza, Rockland, ME) according to the manufacturer's instructions. The MycoAlert assay is a selective biochemical test that exploits the activity of mycoplasmal enzymes, suggesting the presence of live mycoplasmas. The kit can show a possible mycoplasmal infection in cells by producing contamination level (expressed as B/A ratio) > 1.

#### Statistical analysis

Statistical analysis was done by Student's *t*-test.

## Results

### LED irradiation

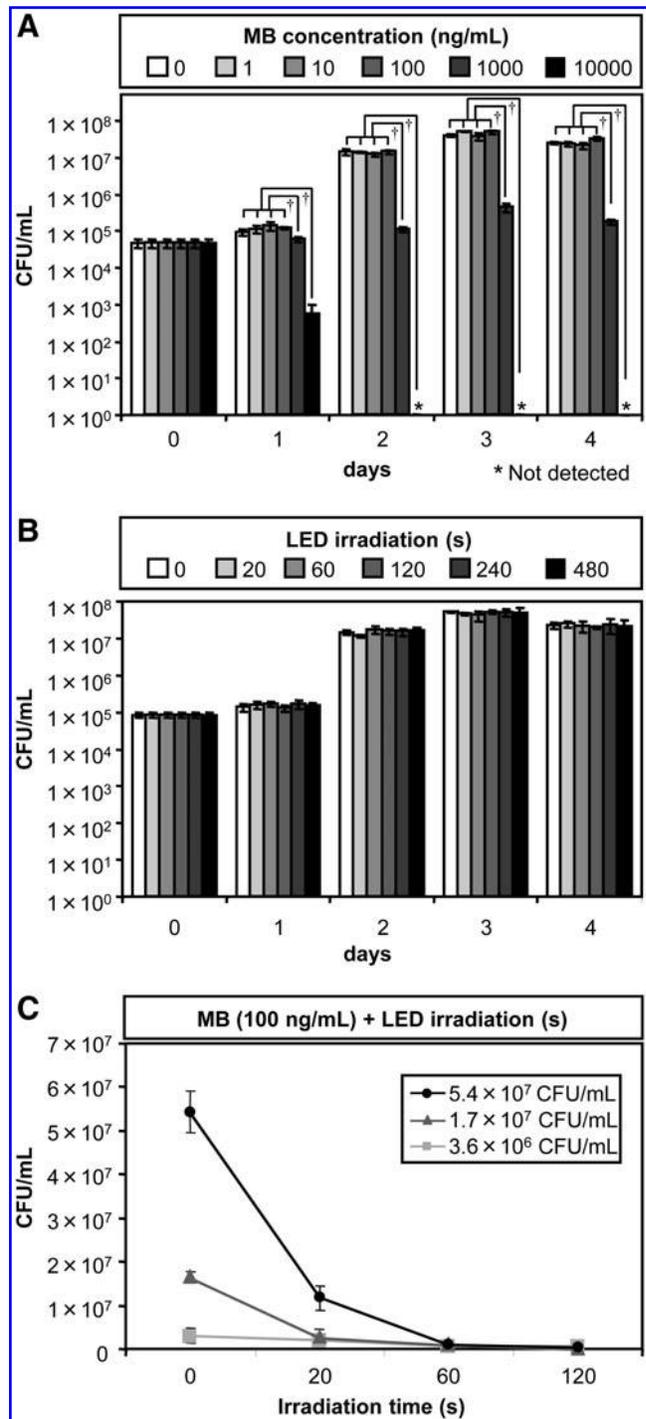
At 650 nm of the wavelength, the power meter gave readings of  $\sim 1,000$  and  $850 \text{ mW/cm}^2$  at distances of 3 and 8 mm from the light source, respectively (Fig. 1C). Therefore, the energy doses that reached the medium were estimated to be  $\sim 60$  and  $51 \text{ J/cm}^2$  for 60 sec of irradiation respectively at irradiation distances of 3 and 8 mm.

### Effect of MB and/or red LED irradiation on the growth rate of Ms

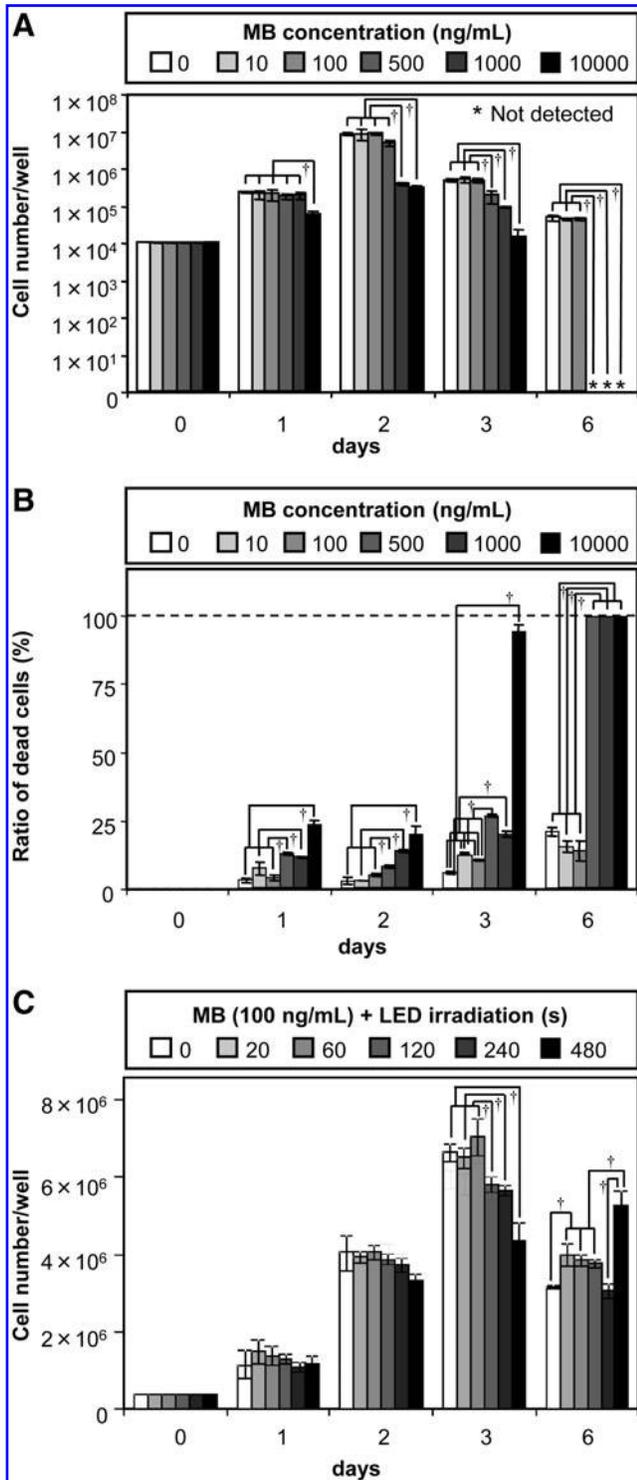
At first, the effect of MB on the Ms growth rate was determined. Ms was inoculated in broth with various MB concentrations, and their viable counts were monitored in the presence of MB for 4 days. There were no significant differences in the Ms growth rate among 0–100 ng/mL of MB, whereas there were significant delays in the Ms growth rate in both 1,000 and 10,000 ng/mL of MB (Fig. 2A). This result showed that MB alone had no effect on the Ms growth rate at concentrations < 100 ng/mL. The effect of red LED irradiation alone on the Ms growth rate was also examined, and irradiation time using red LED had no effect on the growth rate (Fig. 2B). On the basis of these results, the effect of red LED irradiation on the Ms growth rate was examined in the presence of MB (100 ng/mL). Vials containing various Ms densities in 100  $\mu\text{L}$  of broth were prepared and were irradiated from the bottom with red LED. A 60 sec irradiation was able to destroy Ms almost completely compared with the initial Ms densities of between  $3.0 \times 10^6$  and  $5.4 \times 10^7$  CFU/mL (Fig. 2C).

### Effect of MB and/or red LED irradiation on HEK293 cell growth

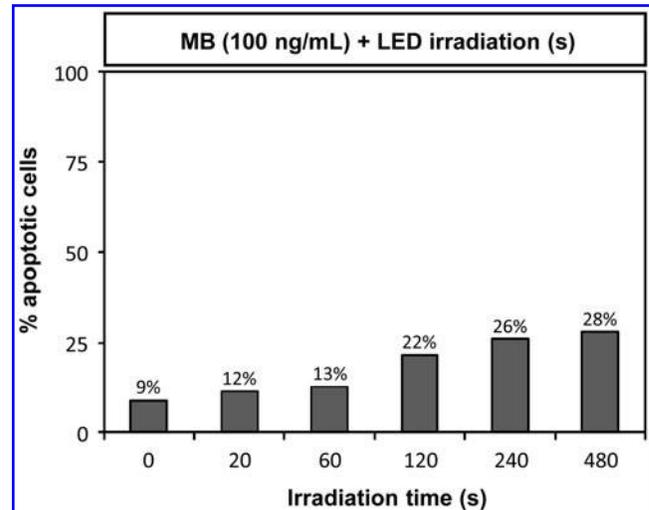
HEK293 cells were prepared in a 24 well plate in DME complete medium with various MB concentrations. The growth rate was monitored by counting the live and dead cells in the presence of MB. MB had no effect on HEK293 cell growth at concentrations < 100 ng/mL (Fig. 3A), whereas there was a significant cytotoxic effect at concentrations > 500 ng/mL after a 6 day incubation (Fig. 3B). Hereafter, the MB concentration used in this study was fixed at 100 ng/mL. Based on these results, the effect of red LED irradiation on the HEK293 cell growth rate was examined in the presence of MB, because red LED irradiation alone had already been shown to have no effect on the growth rate (data not shown). The cells were irradiated with red LED (0–480 sec) in the presence of MB (100 ng/mL) and then the growth rate was monitored. The result showed that a 60 sec irradiation had no significant effect on the HEK293 cell growth rate,



**FIG. 2.** Effects of methylene blue (MB) and/or red light-emitting diode (LED) on the viability of *Mycoplasma salivarium* (Ms). (A) Ms were treated with various concentrations of MB (0–10,000 ng/mL) in PPLO broth in 3 mL vials. (B) Ms in the vials were irradiated with red LED at various time periods (0–480 sec). (C) Vials containing Ms were irradiated using red LED for various time periods in the presence of MB (100 ng/mL). Initial Ms densities were  $5.4 \times 10^7$  colony-forming units (CFU)/mL (black circle),  $1.7 \times 10^7$  CFU/mL (dark gray triangle) and  $3.0 \times 10^6$  CFU/mL (bright gray square). † $p < 0.05$ . Viable counts are expressed as CFU/mL of Ms in each vial. Results are expressed as the mean  $\pm$  SD of three determinations.



**FIG. 3.** Effects of methylene blue (MB) and/or red light-emitting diode (LED) on the viability of human embryonic kidney (HEK) 293 cells. **(A, B)** HEK293 cells were seeded in a 24 well plate with various concentrations of MB (0–10,000 ng/mL) and **(A)** growth rates and **(B)** the ratio of dead cells were determined. **(C)** HEK293 cells in a 24 well plate were irradiated using red LED for various time periods in the presence of MB (100 ng/mL).  $\dagger p < 0.05$ . Results are expressed as the mean  $\pm$  SD of three determinations.



**FIG. 4.** Ratio of apoptotic cells in HEK293 cells induced by antimicrobial photodynamic therapy (aPDT). Uninfected HEK293 cells were prepared in Dulbecco's modified Eagle's (DME) base medium, and then aPDT was performed in the presence of MB (100 ng/mL) with red light-emitting diode (LED) irradiation (0–480 sec). Representative result of three independent experiments is shown.

whereas the delay in the growth rate shown by irradiating HEK293 cells for >120 sec was probably because of the decrease in the initial number of live HEK293 cells (Fig. 3C).

#### *A mechanism of cell death induced by aPDT*

To further examine the effect of aPDT on HEK293 cells, the mechanism of cell death induced by aPDT was investigated. It was speculated that apoptosis was triggered by aPDT, because it has been reported that MB-mediated PDT can induce apoptosis to some cells.<sup>10,11</sup> Uninfected HEK293 cells were prepared in DME base medium and then aPDT was performed in the presence of MB (100 ng/mL) with red LED irradiation (0–480 sec). It was found that the ratio of apoptotic cells increased in a time-dependent manner, and the difference of ratio of apoptotic cells between unirradiated cells and 60 sec irradiated cells was  $\sim 4\%$ , whereas that of unirradiated cells and 120 sec irradiated cells was  $\sim 13\%$  (Fig. 4). It was also shown that treatment with MB alone was able to induce apoptosis, as apoptosis was induced in 9% of unirradiated cells. This result suggests that apoptosis was induced in HEK293 cells by aPDT.

#### *Removal of Ms from Ms-infected HEK293 cells*

Based on these results, this study attempted to remove Ms from Ms-infected cells. HEK293 cells were infected with Ms, and the contamination level was monitored for 1 week using MycoAlert. A contamination level (B/A ratio, Fig. 5) >1 indicated that the cells were contaminated with mycoplasmas.

The cells, which were contaminated or uncontaminated, were then prepared in a 24 well plate in the presence or absence of MB, and some wells were irradiated using red LED for 60 sec. The contamination level was examined 1 and 3 days after irradiation. The contamination level decreased in all of the cells 1 day after irradiation (Fig. 5). However, these decreases were considered to be caused by temporal

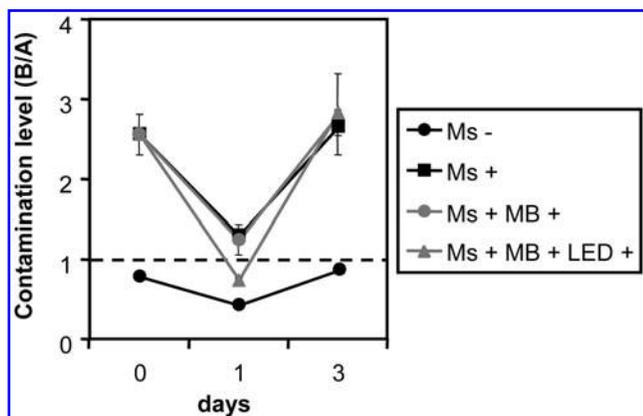


FIG. 5. First trial of *Mycoplasma salivarium* (Ms) removal from human embryonic kidney (HEK) 293 cells by red light-emitting diode (LED) irradiation in the presence of methylene blue (MB). HEK293 cells were seeded without (Ms-) or with Ms (Ms+) in the presence of MB (100 ng/mL) with (Ms+MB+red LED+) or without 60 sec red LED irradiation (Ms+MB+). The level of contamination was determined using MycoAlert<sup>®</sup>. Results are expressed as the mean  $\pm$  SD of three determinations.

decreases of viable Ms that resulted from changing cell culture media after irradiation. Three days after irradiation, the contamination level of the uninfected control cells remained  $<1$ , whereas those of other cells became  $>1$ , suggesting that it is difficult to remove Ms from Ms-infected HEK293 cells using a 60 sec irradiation. Therefore, the irradiation time was prolonged to 120, 150, and 180 sec, but Ms remained in the Ms-infected HEK293 cells (data not shown).

An attempt was made to decrease the initial number of live Ms by detaching Ms-contaminated HEK293 cells from the wells and washing them with sterilized PBS three times. The cells were incubated in 100  $\mu$ L of PBS as a suspension in the presence or absence of MB, and red LED was irradiated in some wells for 60 sec. The contamination level was found to be  $<1$  in both uninfected and Ms-infected red LED-irradiated cells containing MB, and the contamination levels of both of the cells were maintained  $<1$  for up to 60 days (Fig. 6A). On the other hand, the contamination level of Ms-infected cells and Ms-infected cells with MB without red LED irradiation were all  $>1$  (Fig. 6A).

To further confirm that Ms was removed from Ms-infected HEK293 cells, the Ms viable count was determined in the cell culture supernatant. Similar to the results obtained using MycoAlert, no Ms colonies were detected in the supernatant collected from Ms-infected and red LED-irradiated cells with MB up to 60 days (Fig. 6B), whereas many colonies were detected in the supernatant from Ms-infected cells and Ms-infected cells with MB without red LED irradiation.

Thus, this study showed that red LED irradiation in the presence of MB (100 ng/mL) was able to remove Ms from Ms-infected HEK293 cell suspension by washing the cells to decrease the initial number of live Ms before aPDT.

## Discussion

Antibiotics are the cornerstone drugs of modern medicine, and antibiotics have saved the lives of humans who have been infected with pathogenic bacteria. However, soon after

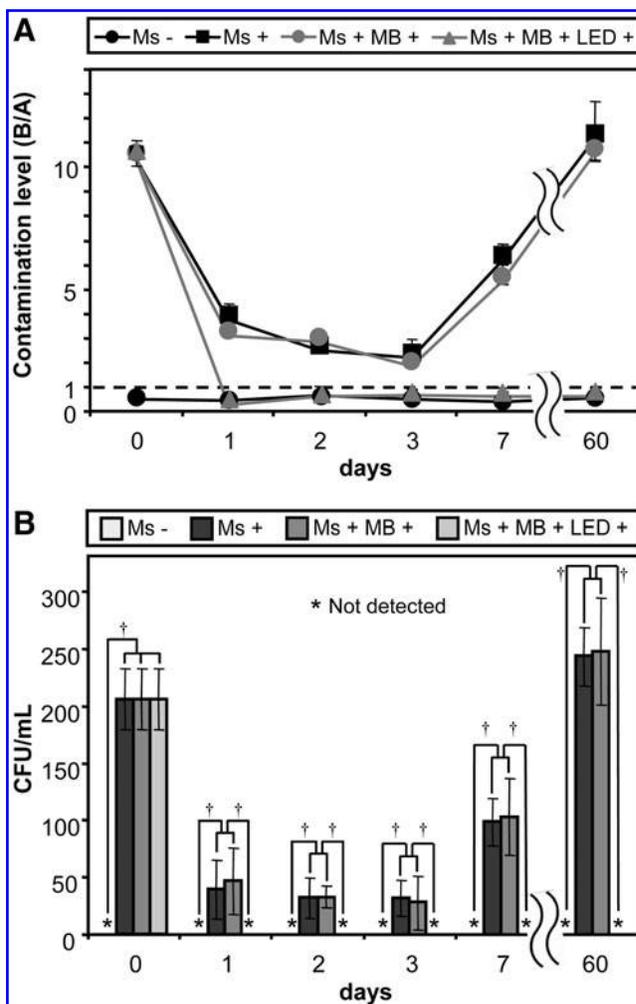


FIG. 6. *Mycoplasma salivarium* (Ms) removal from human embryonic kidney (HEK) 293 cells by red light emitting diode (LED) irradiation in the presence of methylene blue (MB). HEK293 cells incubated with (Ms+) or without Ms (Ms-) in the presence of MB (100 ng/mL) with (Ms+ MB+ red LED+) or without 60 sec red LED irradiation (Ms+ MB+). Red LED irradiation was performed after the cells were detached and washed three times with PBS. (A) The contamination level was determined using MycoAlert<sup>®</sup>. (B) Counts of viable Ms in HEK293 cell culture supernatants were determined. † $p < 0.05$ . Results are expressed as the mean  $\pm$  SD of three determinations.

the first use of antibiotics in medicine, resistant organisms were seen to arise during therapy. In the case of mycoplasma infection, *Mycoplasma pneumoniae* strains have been reported to be resistant to antibiotics.<sup>12</sup> Therefore, it is valuable to establish a method to remove bacteria and mycoplasmas from infected patients or cell culture without using antibiotics. As described, aPDT is expected to be a potent method for bacterial removal without using antibiotics. Recently, numerous studies *in vitro* have demonstrated that bacteria can be effectively killed by aPDT,<sup>13-17</sup> and this study showed that it is possible to remove mycoplasmas from cell cultures using aPDT. However, the efficiency of aPDT in killing bacteria that have host cell-invasive properties is still unknown. In mycoplasmas, *Mycoplasma fermentans*, which frequently contaminates cell culture,<sup>18</sup> and *Mycoplasma*

*penetrans* are known to have invasive properties.<sup>19,20</sup> Recently, O'Riordan et al.<sup>21</sup> have reported that 50% of intracellular *Mycobacterium bovis* were killed using aPDT, compared with extracellular bacteria, when they are co-cultured with macrophages, suggesting that aPDT may be applicable to killing intracellular bacteria. However, there are still few reports on the application of aPDT for killing intracellular bacteria, and further studies are needed to develop a method that is able to completely remove the mycoplasmas that have host cell-invasive properties from the contaminated cells.

Mycoplasma contamination in cell culture results in an alteration of the biological cell characteristics.<sup>18</sup> Therefore, it is important to monitor the contamination. One of the methods frequently used for monitoring mycoplasma contamination is polymerase chain reaction (PCR). However, we did not use PCR in this study because PCR does not differentiate between live and dead mycoplasmas, and PCR results are unable to reflect the cells' viability. Therefore, viable counts and MycoAlert were utilized, because MycoAlert only reacts with enzymes released from live mycoplasma.

Recently, attempts have been made to apply aPDT to clinical use, such as the treatments of oral candidiasis and periodontal diseases.<sup>7,22–24</sup> It is important to establish protocols that enhance the selectivity of the photodynamic process toward microbial targets for the clinical application of aPDT, with minimal collateral damage to the hosts.<sup>25,26</sup> It is also important to establish protocols for the complete removal of bacteria without damaging cells when removing bacteria from cell lines using aPDT. As described, although cancer cells are mainly damaged by ROS produced during PDT treatment, PDT has been reported to also damage cells by other possible effects. In addition to the damage induced by ROS, PDT can induce apoptosis by activating the apoptosis regulating signaling pathway.<sup>27</sup> Fortunately, the optimal condition was found for keeping HEK293 cells alive during aPDT treatment in this study, but under the same conditions, normal human gingival cell lines were easily destroyed (data not shown). Therefore, there are differences in the susceptibility among cell lines to aPDT. One of the possible ways to reduce cellular damage from aPDT might be to use antimicrobial peptides together with aPDT, because the combination of the peptides and dyes would allow the dyes to bind efficiently to the bacterial surface. For example, the combination of an anionic dye and a cationic antimicrobial peptide human  $\beta$ -defensin-2 (hBD-2) might be a good candidate. Both the efficient effect of aPDT and a synergistic effect with hBD-2 are expected, because it has been reported that hBD-2 alone plays a protective role in *M. pneumoniae* infection.<sup>28</sup>

Although there are some problems that remain to be solved, further studies might make it possible to remove mycoplasmas from any cells using aPDT.

### Conclusions

This study suggests that aPDT could remove mycoplasmas from contaminated cells.

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### Author Disclosure Statement

No conflicting financial interests exist.

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