Ultraviolet C lethal effect on *Brucella melitensis*

Ayman Al-Mariri

Department of Molecular Biology and Biotechnology, Atomic Energy Commission, Damascus, Syria

SUMMARY

The gram-negative bacteria *Brucella melitensis* was investigated to evaluate its susceptibility to UVC radiation at 254 nm. At an intensity of 18.7 mW/cm^2 of UVC, the time required for inactivation of *B. melitensis* was 240 seconds in both dark and light, whereas it was 120 seconds and 240 seconds in dark and light respectively at an intensity of 19.5 mW/cm^2. The results indicate that vaccinal strain of *B. melitensis* (Rev.1) is more sensitive to UVC than wild *B. melitensis* strain.

KEY WORDS: B. melitensis, UVC irradiation, Repair in dark, Cyclobutane Pyrimidine Dimmers (CPD), Photoreactivation

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INTRODUCTION

The lethal effect of ultraviolet (UV) light on microorganisms has been known for many years. UV disinfection is also characterized by a short contact time and a more efficient antimicrobial action (Hassen *et al.*, 2000). Efficiency of UV disinfection also depends on the UV dose and the aggregation state of bacteria in solution (Miller *et al.*, 1999). Different studies have classed microorganisms and their propagules in the following increasing order of resistance: bacteria < viruses < fungi < spores < cysts (Jagger 1981; Abbaszadegan *et al.*, 1997). The ability of UV light to inactivate microorganisms is known to differ from organism to organism (Oppezza and Pizarro 2001; Abu-Chararah 1997; Didier *et al.*, 2001). The mechanisms by which UV light inactivates microorganisms differ at different wavelengths (Giese and Darby 2000). UV-induced inactivation of colony-forming ability or mutagenesis in bacteria is attributed to DNA damage which in turn is modulated by processes of repair or toleration (Setlow, 1997). The effectiveness of UV light in biological inactivation arises primarily from the fact that DNA molecules absorb UV photons between 200 and 300 nm, with peak absorption at 260 nm (Abu-Chararah, 1997). This absorption creates damage in the DNA by altering nucleotide base pairing, thereby creating new linkages between adjacent nucleotides on the same DNA strand (Lindahl and Wood, 1999). This damage occurs particularly between pyrimidine bases (Chndrasekhar and Houten, 2000). The three possible dimers that can be formed from thymine (T) and cytosine (C) are T<>T, C<>T and C<>C, in order of prevalence (Daniel and Dipankar, 2004). The principal type of cell damage from UVC (254 nm) radiation is the formation of cis-syn cyclobutane pyrimidine dimmers (CPD) in the DNA molecules (Burger *et al.*, 2002), while (6–4) photoproducts are also formed at about 10% of CPD and other kinds of lesions are also produced at lower ratios (Todo *et al.*, 1993). The lesions inhibit the normal replication of the genome and result in inactivation of the microorganisms (Linden and Darby, 1998). Also, genomes, proteins and enzymes with unsaturated bonds are

Corresponding author
A. Al-Mariri
Department of Molecular Biology
and Biotechnology
Atomic Energy Commission
P.O. Box 6091, Damascus, Syria
E-mail: scientific@aec.org.sy

known to absorb UVC and UVB, which may also result in significant damage to the organisms (Baron, 1997).

Brucella melitensis is gram-negative, facultative intracellular bacterium that can cause a highly contagious disease in sheep, goats, cattle and one-humped camels. It is also responsible for the most important zoonosis in man (Bosciroli *et al*., 2001). *B. melitensis* infections are widespread worldwide, particularly in the Mediterranean and the Middle East countries (Corbel, 1997). The disease in human is characterized by a multitude of somatic complaints including fever, sweats, anorexia, fatigue, malaise, weight loss and depression (Young 1995; Golding *et al.*, 2001), in addition to localized hepatobiliary, osteoarticular, pulmonary and nervous system symptoms (Challoner *et al.*, 1990). Without adequate and prompt antibiotic treatment, some patients develop a chronic brucellosis syndrome with many features of the chronic fatigue syndrome (Vassallo, 1996). Vaccination against *B. melitensis* infections in animals is usually performed by administration of the live attenuated smooth *Brucella* strains, *B. melitensis* strain Rev.1. (Blasco, 1997). *B. melitensis* Rev. 1 vaccine greatly decreases the prevalence of brucellosis in sheep and hence in human populations (Elberg, 1996).

The objective of this study was to compare the repair potential of *B. melitensis* wild and attenuated strain following UVC exposure in order to use the inactive *Brucella* as an antigen.

MATERIALS AND METHODS

Microorganism

B. melitensis isolated from milk cultures at the Microbiology/Immunology Laboratory, AECS was used in this study. *Brucella* was grown under optimal conditions in 2YT (agar [20 g], peptone [10 g], sodium chloride [5 g], meat extract [5 g], distilled water [1 litre]) at 37°C in a water bath (Grant water bath shaker, model OLS200; Cambridge, UK) to ensure sufficient cell density. Various antibiotics were added to inhibit the growth of organisms other than *Brucella*, as in the medium of Kuzdas and Morse; namely, cycloheximide (100 mg), bacitracin (25000 units), polymyxin B sulphate (5000 units), vancomycin (20 mg), nalidixic acid (5 mg) and nystatin (100000 units).

To prepare solid selective media, the basal medium is melted and then cooled to 56°C in a water bath and stock solutions of the antibiotics were added. The bacteria which were oxidase and urease positive were chosen and tested for agglutination with monospecific anti-*Brucella* serum (anti-A and anti-M) (ARCOMEX, Amman, Jordan). Strains identified as *B. melitensis* were stored in brucella broth (BD, Spark, USA) at -20°C until susceptibility testing. The laboratory personnel working with these isolates wore impermeable protective clothing, gloves and a face mask during contact with the organism. *B. melitensis* vaccine strain Rev.1 was obtained from Arab Company for Medical Diagnostics, Amman, Jordan.

A 48 h culture in stationary phase was used in the experiment. The suspension was centrifuged (Eppendorf, 5810R, Hamburg, Germany) at 350 x g for 10 min, and the supernatant was aseptically drawn off. The pellet was resuspended in phosphate-buffered saline (PBS) to obtain a bacteria concentration of approximately 10^9 CFU/ml. The sample was vortexed (IKA, Wilmington, USA), and 5 ml was poured directly into a 50-mm plastic Petri dish (TPP, Switzerland). Prior to irradiation, a portion of the bacteria suspension was removed and serially diluted to determine the initial cell concentration. Due to sample volume limitations and sampling time constraints, the replication was difficult to establish within each experiment. To compensate, two last experiments were performed for each irradiation dose and each dilution was plated in triplicate as described below. Data from representative experiments with standard deviations are shown.

Sample irradiation

A bench-scale collimated beam apparatus (Uvitec, Cambridge, UK) was used to irradiate the samples. This apparatus contains an interchangeable low-pressure lamp (5 x 8 W). All samples were exposed at room temperature (20 to 22°C) to UVC dose irradiation (240 nm). After being UV-irradiated, each sample was separated and transferred into two separate plastic Petri dishes. One of the two Petri dishes was exposed to light to examine the photorepair effect [the intensity of light at the sample surface was approximately 16,900 lx], and the other was covered with aluminum foil to examine the dark repair effect. Both samples were

continuously mixed on an orbital shaker inside the incubator. One sample was aseptically drawn from each dish periodically for each time point. A portion of the irradiated sample was removed, serially diluted, and plated in triplicate on *Brucella*-Agar (BD, Spark, USA) to determine the number of organisms following exposure. The plates were incubated at 37°C for 24-48 h. All *Brucella* samples were processed by the standard plate count technique (Zervos and Bostic, 1997). All samples used to investigate dark repair were diluted in foil-covered tubes to ensure no light exposure.

Statistical analysis

Each experiment was typically performed in triplicate. Significant differences between means were determined by either Student's *t* test or analysis of variance. Both tests were conducted using Microsoft Excel, version 4, statistics package. Differences were considered significant when $p < 0.05$.

RESULTS

At an intensity of 18.7 mw/cm^2 of UVC radiation, the time needed to inhibit the growth of *B. melitensis* was 240 seconds for all times of plating whether in light or dark (Table 1, Figure 1A).

At this intensity, the bacterial count following 10 seconds of exposure was $10^{3.2}$ CFU/ml, when plating was done directly after exposure. Whereas bacterial count increased to 10^9 CFU/ml when plating was done 24, 96, or 144 hours following exposure to UVC irradiation (in light), in the dark, the bacterial count was 10^4, 10^8, 10^9 CFU/ml when plating was done after 24, 96 or 144 h respectively.

Results demonstrated the absence of bacteria following the application of UVC at an intensity of 19.5 mw/cm^2 for 120 seconds (Table 2, Figure 1B), where plating was done after 24, or 96 h after exposure; while it increased to 10^3, 10^4 CFU/ml 144 h after exposure (in light or dark). If the plating was done at 144 h following exposure

TABLE 1 - *Average of log_{10} count of B. melitensis following exposure to 18.7 mw/cm^2 of UVC and growth under light or in the dark.*

Time of exposure to UVC radiation (seconds)	*Time of plating of B. melitensis after exposure to UVC radiation*	*Log CFU/ml mean ± standard division*	
		Light	*Dark*
10	direct*	3.2±0.01	
45		0	
60		2±0.0	
240		0	
10	24 h†	9±0.28	4±0.10
45		3.4±0.07	3±0.04
60		3.2±0.02	2.6±0.01
240		0	0
10	96 h‡	9±0.18	8±0.13
45		7±0.20	4±0.09
60		6±0.12	4±0.07
240		0	0
10	144 h	9±0.30	9±0.22
45		9±0.27	7±0.24
60		9±0.18	5.2±0.16
240		0	0

*p<0.06 between culture done directly and that done 24 h (in light). P<0.03 between culture done directly and that done 96 h or 144 h (in light). P<0.05 between culture done directly and that done 96 h or 144 h (in dark). †p<0.09 between culture done 24 h and that done 96 h or 144 h (in light). P<0.07 between culture done 24 h and that done 96 h (in dark). P<0.03 between culture done 24 h and that done 144 h (in dark).‡p<0.09 between culture done 96 h and that done 144 h (in light). P<0.06 between culture done 96 h and that done 144 h (in dark).

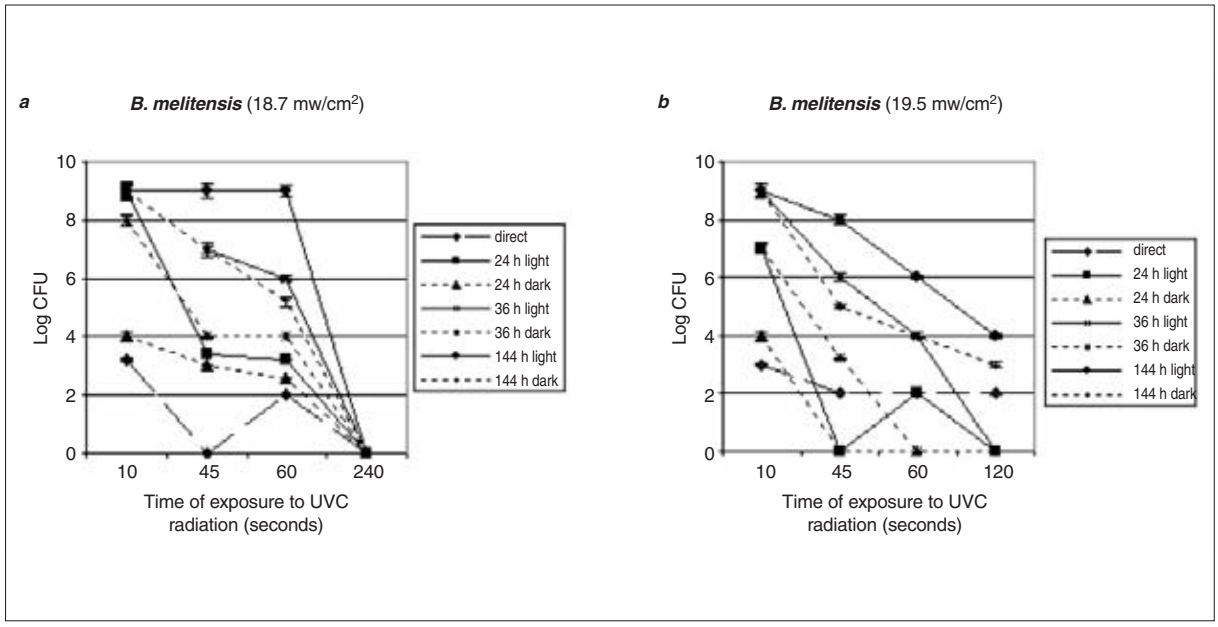

FIGURE 1 - *Log_{10} count of B. melitensis following exposure to 18.7 mw/cm² (a) or 19.5 mw/cm² (b) of UVC and grow under light or dark. The data represent the mean ± the standard deviation of the triplicate for each experiment.*

TABLE 2 - *Average of log_{10} count of B. melitensis following exposure to 19.5 mw/cm² of UVC and growth under light or in the dark.*

Time of exposure to UVC radiation (seconds)	*Time of plating of B. melitensis after exposure to UVC radiation*	*Log CFU/ml mean ± standard division*	
		Light	*Dark*
10	direct*	3±0.05	
45		2±0.0	
60		2±0.0	
120		2±0.0	
10	24 h†	7±0.19	4±0.12
45		0	0
60		2±0.0	0
120		0	0
10	96 h‡	9±0.24	7±0.17
45		6±0.13	3.2±0.04
60		4±0.09	0
120		0	0
10	144 h	9±0.27	9±0.22
45		8±0.17	5±0.07
60		6±0.06	4±0.10
120		4±0.05	3±0.12

*p<0.01 between culture done directly and that done 144 h (in light). P<0.006 between culture done directly and that done 144 h (in dark). †p<0.07 between culture done 24 h and that done 96 h (in light). P<0.01 between culture done 24 h and that done 144 h (in light). P<0.09 between culture done 24 h and that done 96 h (in dark). P<0.001 between culture done 24 h and that done 144 h (in dark). ‡p<0.05 between culture done 96 h and that done 144 h (in light). P<0.01 between culture done 96 h and that done 144 h (in dark).

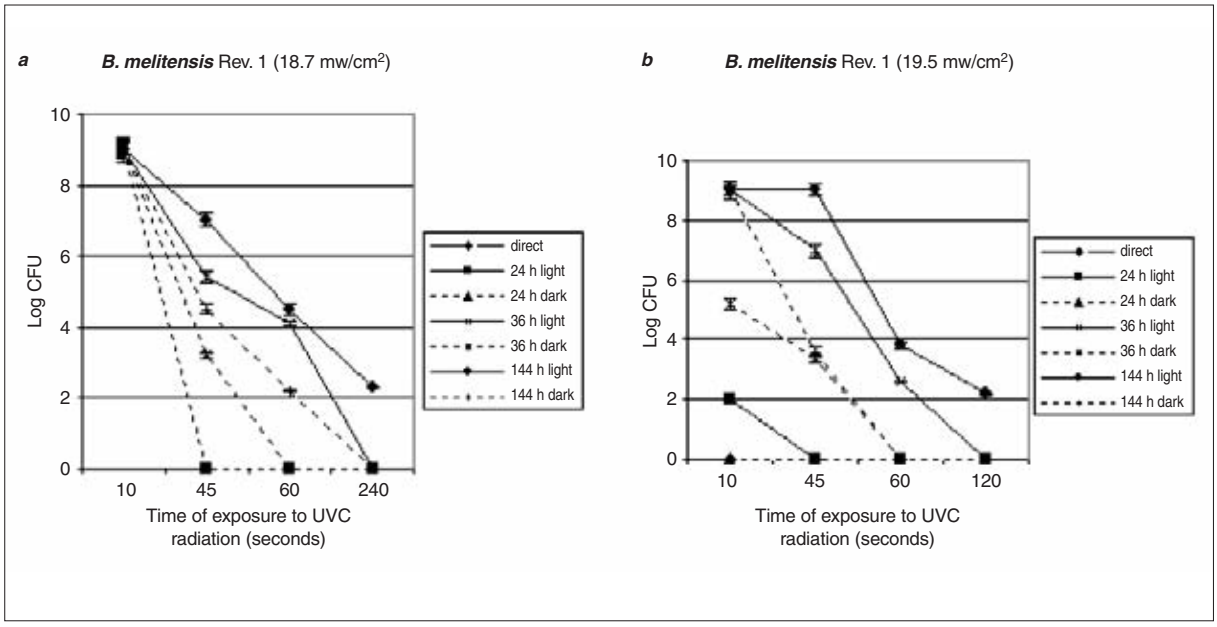

FIGURE 2 - *Log_{10} count of B. melitensis Rev.1 following exposure to 18.7 mw/cm² (a) or 19.5 mw/cm² (b) of UVC and grow under light or dark. The data represent the mean ± the standard deviation of the triplicate for each experiment.*

TABLE 3 - *Average of log_{10} count of Rev. 1 strain following exposure to 18.7 mw/cm² of UVC and growth under light or in the dark.*

Time of exposure to UVC radiation (seconds)	*Time of plating of Rev. 1 after exposure to UVC radiation*	*Log CFU/ml mean ± standard division*	
		Light	*Dark*
10	direct*	9±0.32	
45		0	
60		0	
240		0	
10	24 h†	9±0.27	9±0.34
45		0	0
60		0	0
240		0	0
10	96 h‡	9±0.23	9±0.19
45		5.4±0.17	3.2±0.09
60		4.1±0.06	0
240		0	0
10	144 h	9±0.24	9±0.28
45		7±0.19	4.5±0.13
60		4.5±0.17	2.2±0.04
240		2.3±0.0	0

*p<0.09 between culture done directly and that done 96 h (in light). p<0.01 between culture done directly and that done 144 h (in light). †p<0.09 between culture done 24 h and that done 96 h (in light). P<0.05 between culture done 24 h and that done 144 h (in light). ‡p<0.06 between culture done 96 h and that done 144 h (in light).

to this intensity, 240 seconds of exposure were needed to kill *B. melitensis*.

Table 2 and Figure 1B show that, if culturing was done directly or at 24 h after exposure to UVC (in the light or dark), *B. melitensis* count was zero and 10^2 CFU/ml for exposure times of 45 or 60 seconds, respectively; it increased to 10^4 and 10^6 CFU/ml where plating was done at 96 h after exposure (in light), and to 1585 CFU/ml and zero, respectively (in the dark).

UVC radiation was applied on the vaccination strain Rev.1 (attenuated *B. melitensis*) to compare the effect of UVC on wild and attenuated strain. Figure 2A and Table 3 shows that the bacterial count of vaccine Rev. 1 was 10^9 CFU/ml 10 seconds following exposure to intensity of 18.7 mw/cm^2, when plantation was done immediately after exposure or 24, 96, or 144 h after exposure (in light or dark).

Bacterial count started to decrease with increasing exposure time, and became zero where plating was done immediately or 24 h after exposure to irradiation; and 251189 CFU/ml when culturing was done 96 h after exposure to the irradiation for 45 seconds.

Using the intensity of 19.5 mw/cm^2 on strain Rev. 1, no increase in the bacterial count was noticed whether culturing of the bacteria was done immediately or 24 h following UVC exposure regardless of the culturing being in the dark or under light (Table 4, Figure 2B). Whereas, growing strain Rev. 1 under light after 96 or 144 h exposure to UVC resulted in exposure time-dependent increase in bacterial count, reading 10^9 CFU/ml, 10^7 CFU/ml and 6309 CFU/ml for 10, 45 and 60 seconds, respectively. Bacterial count decreased to lower levels when the plantation was done in the dark. When plantation was done 96 h after irradiation, bacterial count reached 158489 CFU/ml after an exposure time of 10 seconds, and to 2512 CFU/ml for an exposure period of 45 seconds; and it decreased to zero when exposure was done for 60 seconds.

We found that a 120 second exposure was necessary to reach a complete inactivation of Rev. 1 growth, regardless of culturing time or whether

TABLE 4 - *Average of log_{10} count of Rev.1 strain following exposure to 19.5 mw/cm^2 of UVC and growth under light or in the dark.*

Time of exposure to UVC radiation (seconds)	*Time of plating of Rev. 1 after exposure to UVC radiation*	*Log CFU/ml mean ± standard division*	
		Light	*Dark*
10	direct*	0	
45		0	
60		0	
120		0	
10	24 h†	2±0.0	0
45		0	0
60		0	0
120		0	0
10	96 h‡	9±0.18	5.2±0.17
45		7±0.24	3.4±0.13
60		2.6±0.02	0
120		0	0
10	144 h	9±0.30	9±0.30
45		8±0.20	3.6±0.19
60		6±0.12	0
120		4±0.03	0

*p<0.05 between culture done directly and that done 96 h (in light). P<0.02 between culture done directly and that done 144 h (in light). †p<0.05 between culture done 24 h and that done 96 h (in light). P<0.02 between culture done 24 h and that done 144 h (in light). ‡p<0.04 between culture done 96 h and that done 144 h (in light).

the culturing was done in the dark or under light. For the laboratory attenuated *B. melitensis* NalR strain, it was proved that inactivation of this strain needed about 240 seconds of exposure to 18.7 mw/cm^2 of radiation, and about 120 seconds of exposure to 19.5 mw/cm^2 of radiation (results are not shown).

DISCUSSION

UV radiation induces two of the most abundant mutagenic and cytotoxic DNA lesions namely cyclobutane–pyrimidine dimmers (CPDs) and 6-4 photoproducts (6-4 PPs) and their Dewar valence isomers (Uchida *et al*., 1997; Kumiko *et al.*, 2002). Some organisms, however, are known to possess the ability to repair their DNA by mechanisms such as photoreactivation and dark repair (Chinnapen and Dipankar 2004; Courcelle and Hanawalt, 2001). Photoreactivation, the foremost of these DNA repair mechanisms, was first discovered by Kelner in 1949 (Lindenhauer and Darby, 1994). It is an important repair mechanism which influences the performance of UV disinfection systems in both air and water (Downey *et al*., 1998). Photoreactivation is the phenomenon by which UV-inactivated microorganisms recover activity through the repair of pyrimidine dimers in DNA under near-UV light (310 to 480 nm) with the enzyme photolyase (Kalisvaart, 2001). Photoreactivation can be a very efficient DNA repair mechanism. For example, photoreactivation has been shown to repair in excess of 90% of the cyclobutane pyrimidine dimers formed in baker's yeast as a result of UV irradiation (Oguma *et al.*, 2001). Nucleotide excision repair, often referred to as dark repair, is widely distributed and conserved through evolution (Sedliakova, 1998). This repair process involves the action of more than a dozen proteins that coordinate the removal of DNA damage (Todo *et al*., 1993; Sommer *et al.*, 2000). The processes of repair and toleration presumably benefit survival by establishing a genome free of UV damage for replication (Sommer *et al*., 1998). When the cells lack excision repair activity, this condition may arise principally by the formation of daughter strand gaps and their post-replication repair by recombination (Kuzminov, 1999). Chandrasekhar and Van Houten (2000), working with *E. coli* lacI and lacZ genes, found that cyclobutane pyrimidine dimers constituted 65.3± 9.0% of the total photoproducts produced, while 6–4 photoproducts comprised only 34.7±9.0%.

The present study aimed at specifying the dose or the intensity of the UVC needed to inhibit the *Brucella* in order to use it in sterilization, to obtain attenuated vaccination strain, or to inhibit the bacteria to use it as an antigen in the immune studies of these bacteria. We chose the ultraviolet irradiation because it has a destructive influence on the DNA cell without influencing the other cellular compositions which maintains the three dimensional structure of the bacterial protein.

Our results deal with the effects of UVC irradiation on *B. melitensis* and Rev.1. We took into account the dark repair and the photoreactivation. We found that the dark or light repair of *Brucella* decreased with the increase in UVC irradiation exposure time (Tables 1, 2). So it was possible to conclude that there was no inverse relationship between the DNA repair and the time of UVC irradiation. We reveal that the *Brucella* count augments with the increase in interval between the end of UVC exposure and the culturing of the bacteria, for both photoreactivation and dark repair. Our experiments suggested that the DNA repair was better using photoreactivation repair than dark repair.

As expected, exposure to irradiation intensity of 18.7 mw/cm^2 was less effective on *Brucella* than exposure to intensity of 19.5 mw/cm^2. The results showed that DNA repair mechanisms in the presence of light were more effective than in the dark. The longer the interval between culturing and exposure to the UVC, the higher the distraction of DNA repair mechanisms, till we reach (and within our experiment conditions) 240 seconds of exposure, as it was no longer possible for these mechanisms to repair the damage caused by the exposure to the irradiation which caused a 99% of inactivation of the *Brucella*. We conclude that the exposure time needed to inhibit the *Brucella* should be at least 240 seconds. We could use this irradiation in the immune studies (Killing the *Brucella*) instead of using the physical or chemical factors (heat, aldehyde, etc.).

Instead of the bacteria (used in our experiment) being unable to grow when exposed to irradiation for more than 10 seconds, and cultured immedi-

ately after exposure, we noted that the wild *Brucella* strain has slightly more ability to repair its DNA than the attenuated vaccination strain, as the bacterial count of the wild bacteria was about 10^3 CFU/ml at 24 h following exposure to a 45-second irradiation period and zero for attenuated vaccination strain, if the plantation was done in dark or light. While the time of exposure needed to inhibit both strains was equal or more than 60 seconds, when plantation was done in light or dark. We conclude that *Brucella*, like most other bacteria, has the DNA repair mechanisms for the damage caused by the UVC in dark or light.

In conclusion, an exposure time to UVC radiation at an intensity of 19.5 mw/cm^2 was required to effectively inactivate *B. melitensis* and Rev.1, whereas the required exposure time was slightly lower when an intensity of 18.7 mw/cm^2 was used. We found that Rev.1 is more sensitive to UVC than *B. melitensis*. This is the first report, to our knowledge, to document the UVC irradiation effects in *Brucella*.

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