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COMPARATIVE BACTERICIDAL ACTIVITIES OF LASERS OPERATING AT SEVEN DIFFERENT WAVELENGTHS

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ABSTRACT

Seven laser instruments, delivering radiation at a selection of wavelengths in the range of 0.355 to 118 μm, were investigated for their ability to kill *Escherichia coli* as a lawn of the bacteria on nutrient agar culture plates. Easily the most effective was a 600-W CO_2 laser operating at 10.6 μm, which produced 1.2-cm^2 circular zones of sterilization at energy densities of around 8 J cm^{-2} in a 30-msec exposure. Circular zones with an area of 0.7 cm^2 were achieved with 200 W from a Nd:YAG laser delivering 8-ms, 10-J pulses of 1.06 μm radiation at 20 Hz. The exposure time, however, was 16 s and the energy density (1940 J cm^{-2}) was more than 240 times higher than with the CO_2 laser. This difference is believed to be partly due to the much higher absorption of radiation at 10.6 μm than at 1.06 μm, by water in the bacterial cells and the surrounding medium (nutrient agar). Sterilization was observed after exposure to frequency-tripled Nd:YAG laser radiation at 355 nm (3.5 J cm^{-2}). Lasers that were totally ineffective in killing *Escherichia coli* (with their wavelength and maximum energy densities tested) were the far infrared laser (118 μm; 7.96 J cm^{-2}), the laser diode array (0.81 μm; 13,750 J cm^{-2}), and the argon ion laser (0.488 μm; 2210 J cm^{-2}). The speed at which laser sterilization can be achieved is particularly attractive to the medical and food industries.

Keywords Ar ion; CO_2; *Escherichia coli*; far infrared; frequency doubled; frequency tripled; inactivation; laser; laser diode array; Nd:YAG; Q-switched; sterilization.

1 INTRODUCTION

In 1963 Saks and Roth[1] demonstrated that ruby lasers had significant biocidal capacity against *Spirogyra* and *Amoeba*. Since then, a number of laser sources have been used to sterilize a range of bacteria and yeasts, with most applications occurring in dentistry and medicine. For example, Adrian and Gross[2] demonstrated that within 1.5 min, a 10 W CO_2 laser could sterilize metal scalpel blades contaminated with spores of *Bacillus subtilis* and *Clostridium sporogenes*. A comparison of the sterilization efficacy of CO_2, Nd:YAG, and argon ion lasers was made by Powell et al.,[3] who concluded that the argon ion laser provided the best sterilization efficiency for dental instruments in that it required an exposure of 120 s at 1 W. The beam area, however, was not given and therefore the energy density required for sterilization was not determined. Schultz et al.[4] found that *Pseudomonas aeruginosa* was more sensitive than *Escherichia coli* and *Staphylococcus aureus* to exposure from an Nd:YAG laser; moreover, the addition of methylene blue reduced the sterilization threshold energy density.

Medical applications of laser sterilization have centered on reducing wound infections during surgery. Clinical trials comparing laser sterilization and iodine for controlling infection in amputee cases indicated that the former proved most effective.[5] Mullarky, Norris, and Goldberg[6] used a CO_2 laser to sterilize skin seeded with bacteria, and showed that contamination by the laser plume was only a small risk. Ruby, Nd:YAG, and He-Ne lasers were found to have no effect on *S. aureus* and *P. aeruginosa* by McGuff and Bell.[7] However, low-

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power lasers have proved effective in sterilization if they are used in conjunction with photosensitizers. For example, Wilson et al.[8] evaluated 27 compounds to sensitize *Streptococcus sanguis*, *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, and *Fusobacterium nucleatum* to exposure from a 7.3-mW He-Ne laser, and found that these bacteria were killed after 30 s exposures with toludine blue O, azure B chloride, and methylene blue. Black-pigmented bacteria, for example *P. gingivalis*, were more sensitive to light than nonpigmented strains.

It is clear from these reports that lasers have the capacity to achieve sterilization in remarkably short periods of time compared with some conventional methods, such as autoclaves, which typically require a 15-min exposure at 121 °C. From the results reported here, sterilization by laser was achieved with exposure times as short as tens of milliseconds. The present study investigated the performance of seven different laser wavelengths to find the rate of sterilization for the different laser devices and the optimum laser sterilization or inactivation source.

2 MATERIALS AND METHODS

The wavelengths ranged from 118 μm for a CW far-infrared (FIR) laser to 355 nm with a Q-switched, frequency-tripled Nd:YAG operating with 5-ns pulses. A standardized assay was developed to assess each laser's bactericidal capacity. Plates that had been lawned with *E. coli* were exposed to various energy densities of laser irradiation, incubated for 24 h, and examined for growth. Any cleared zones on the surface indicated that the laser sterilization had been successful. Because of the nonuniform spatial distribution of energy within the laser beam, the energy density delivered to the sample had a spatial variation. Consequently, for a given exposure, the area of clearing was indicative of how effective the laser was at sterilization. The laser's effect was quantified by measuring the area of the cleared zones as a function of the energy density.

2.1 LASER SOURCES

Table 1 shows the laser characteristics and their manufacturers, namely; wavelength and mean power and where applicable the pulse energy, pulse duration, peak power, and frequency. The mean power of the lasers ranged from 0.04 to 600 W, the peak power from 150 to 108 MW, and the minimum pulse duration was about 4 ns.

2.2 PREPARATION OF LAWNED PLATES

Escherichia coli B10537 was obtained from the culture collection at the University of Glasgow, where it is maintained on nutrient agar (Difco) slopes at 4 °C and subcultured once monthly. The culture was grown in nutrient broth (Difco) and incubated overnight at 37 °C. Aliquots (1.5 ml) of the culture (approximately 10^8/ml) were pipetted onto nutrient agar plates and allowed to flood the surface; the excess culture was decanted. The plates were dried for 30 min in a Petric Class III microbiological safety cabinet. The approximate concentration of *E. coli* on the surface of the lawned plates was 8.5×10^5 cm^{-2}.

2.3 EXPOSURE OF BACTERIA TO LASER RADIATION

Plates lawned with *E. coli* were irradiated with various laser beams and exposures; typically four or five separate exposures were made on each plate, and two exposures were made for each condition. The laser beam and petri dish were stationary during each exposure. A detailed statistical analysis has been done on Nd:YAG laser sterilization for a range of bacteria and yeasts by Ward et al.;[9] these results indicate the high degree of repeatability of this process. After exposure, the plates were incubated for 24 h at 37 °C and analyzed for growth. Unlawned plates were kept for control for up to 14 days, and because only a small part of each plate was exposed, the nonexposed area served as an additional control. As further control, unlawned plates were exposed to the range of energy densities, then lawned and incubated in the usual manner. This control was designed to see if the laser exposure affected the nutrients in the agar, leading to subsequent death of the bacteria. If the laser had a significant effect on the *E. coli*, then after the incubation period an area free from bacterial growth was observed and the average area was calculated. If the plate had no such areas after incubation, the laser was deduced to have had no significant effect. The zones of sterilization were measured as a function of the applied energy density. An imaging system consisting of a Sun workstation (ARS, Edinburgh, UK) and an ITEX frame grabber (Bedford, MA) was used to reduce errors in the measurements of the areas.

To compare the bactericidal capacity of the seven lasers, the energy density at which bacteria were killed over an area greater than 15% of the beam area, over an area less than 15% of the beam area, and where no killing was observed was plotted. Of the lasers where sterilization/inactivation was observed, the average zones of clearing were plotted as a function of energy density. To compare the relative performance and time over which sterilization could be achieved for these lasers, these data were normalized to the laser beam area and applied energy density, and plotted as a function of time.

2 RESULTS

Table 2 shows the beam diameters, mean power, exposure times, and energy densities that were applied to *E. coli* for the different lasers under investigation. The maximum applied energy density was

Table 1 Laser characteristics.

Laser/ model	Manufacturer	Wavelength (μm)	Pulse energy (J)	Pulse duration (s)	Frequency (Hz)/CW	Mean power (W)	Peak power (W)
FIR/ FIRL 100	Edinburgh Instruments, Edinburgh, UK	118	NA	NA	CW	0.150	0.150
CO_2/MFKP	Laser Ecosse, Dundee, UK	10.6	NA	NA	CW	600	600
Nd:YAG MS830	Lumonics, Rugby, UK	1.06	10	8×10^{-3}	20	200	1.3×10^{3}
Nd:YAG/ Minilite	Continuum, Santa Clara, CA,	1.06	2.5×10^{-2}	5×10^{-9}	10	0.25	5.0×10^{6}
Doubled		0.532	1×10^{-2}	4×10^{-9}	10	0.10	2×10^{6}
Tripled		0.355	4×10^{-3}	4×10^{-9}	10	0.04	1×10^{6}
Nd:YAG/ Surelite II-10		1.06	0.65	6×10^{9}	10	6.5	1.08×10^{8}
Doubled		0.532	0.3	5×10^{-9}	10	3	6.0×10^{7}
Tripled		0.355	0.1	5×10^{-9}	10	1	2.0×10^{7}
Laser Diode Array OPC-AO15	Opto Power Corporation, CA	0.810	NA	NA	CW	15	15
Ar ion/ Beamlok 2060	Spectra Physics, Hemel Hempstead, UK	0.488	NA	NA	CW	2	2

Note: NA=not applicable; CW=continuous wave.

13,754 J cm^{-2} from Opto Power Corporation's laser diode array. The minimum and maximum beam diameters were 1.5 and 40 mm respectively; both of these diameters were for the Ar ion laser. A number of exposures were made on each lawned plate, except for the 40-mm Ar ion beam, where only one exposure per plate was made. In all other cases the beam area was kept constant for each laser.

Sterile areas were observed after exposing plates to radiation from the CO_2, Lumonic's Nd:YAG MS830, and the frequency-tripled output from Continuum's Nd:YAG Minilite 10 and Surelite II-10, indicating that these lasers had significant biocidal effect at these energy densities. For Lumonic's Nd:YAG, the energy density had to be above a minimum value, about 1200 J cm^{-2}, to ensure growth inhibition. It was found that the zones of inhibition after exposure from the CO_2 and Lumonic's Nd:YAG lasers were strongly dependent on the applied energy density. The laser-exposed plates were incubated for up to 14 days, but in no case was delayed growth observed. Similarly, no further growth occurred when sections of the laser-exposed areas were removed and imprinted on fresh agar. In every case growth was observed on the controls—unexposed lawned plates—indicating that the laser exposure was the cause of the observed zones of clearing. Growth was observed after exposure from the following lasers: FIR, both Q-switched Nd:YAG lasers operating at 1.06 μm and 532 nm, the laser diode array, and the Ar ion laser, indicating that these lasers had no effect at these applied energy densities.

Figure 1 compares the performance of each laser investigated by plotting the energy density against the observed response, i.e., no killing; bacteria killed, but only over an area less than 15% of the beam area; and bacteria killed over an area greater than 15% of the beam area. It is seen that the frequency-tripled Minilight laser only produced sterilization areas below 15% of its beam area, whereas the tripled Surelite sterilized areas were all greater than 15% of its beam area. The Nd:YAG MS830 produced areas above 15% of its beam area

Table 2 Bactericidal effect of exposure of *E. coli* colonies to various lasers.

Laser/ model	Beam area (cm^2)	Mean power (W)	Exposure time (s)	Energy density ($J\ cm^{-2}$)	Area of zone of bacterial killing (cm^2)
FIR/ FIRL 100	0.385	0.15	60[a]	7.96[a]	0
CO_2/MFKP	2.30	600	0.005	1.31	0.160
			0.010	2.63	0.660
			0.020	5.26	1.04
			0.030	7.88	1.21
Nd:YAG/ MS830	1.65	200	9	1090	0
			10	1210	0.0380
			12	1460	0.310
			14	1700	0.540
			16	1940	0.715
Nd:YAG/ Minilite 10	0.283	0.25	10[a]	8.84[a]	0[c]
Doubled Nd:YAG/Minilite 10	0.283	0.1	15[a]	5.31[a]	0[c]
Tripled	0.283	0.04	5	0.710	0.0107
Nd:YAG/			15	2.12	0.0178
Minilite 10			20	2.82	0.0277
			30	4.22	0.0362
			60	8.49	0.0365
Nd:YAG/ Surelite II-10	0.283	6.5	3[a]	69[a]	0[d]
Doubled Nd:YAG/ Surelite II-10	0.283	3.0	3[b]	31.8[b]	0[c]
Tripled	0.283	1.0	1	3.54	0.121
Nd:YAG/			2	7.07	0.112
Surelite II-10			3	10.6	0.123
Laser diode Array OPC-AO15	0.196	15	120[a]	13750[a]	0
Ar ion/ Beamlok 2060	12.56[e]	0.65	60[a]	2210[a]	0

[a] Shorter times and smaller energy densities were also nonbactericidal.

[b] A single-shot exposure produced a visible but unquantifiable effect.

[c] The plastic of the petri dish was burnt.

[d] The plastic of the petri dish was melted.

[e] Minimum beam diameter tested was 0.15 cm.

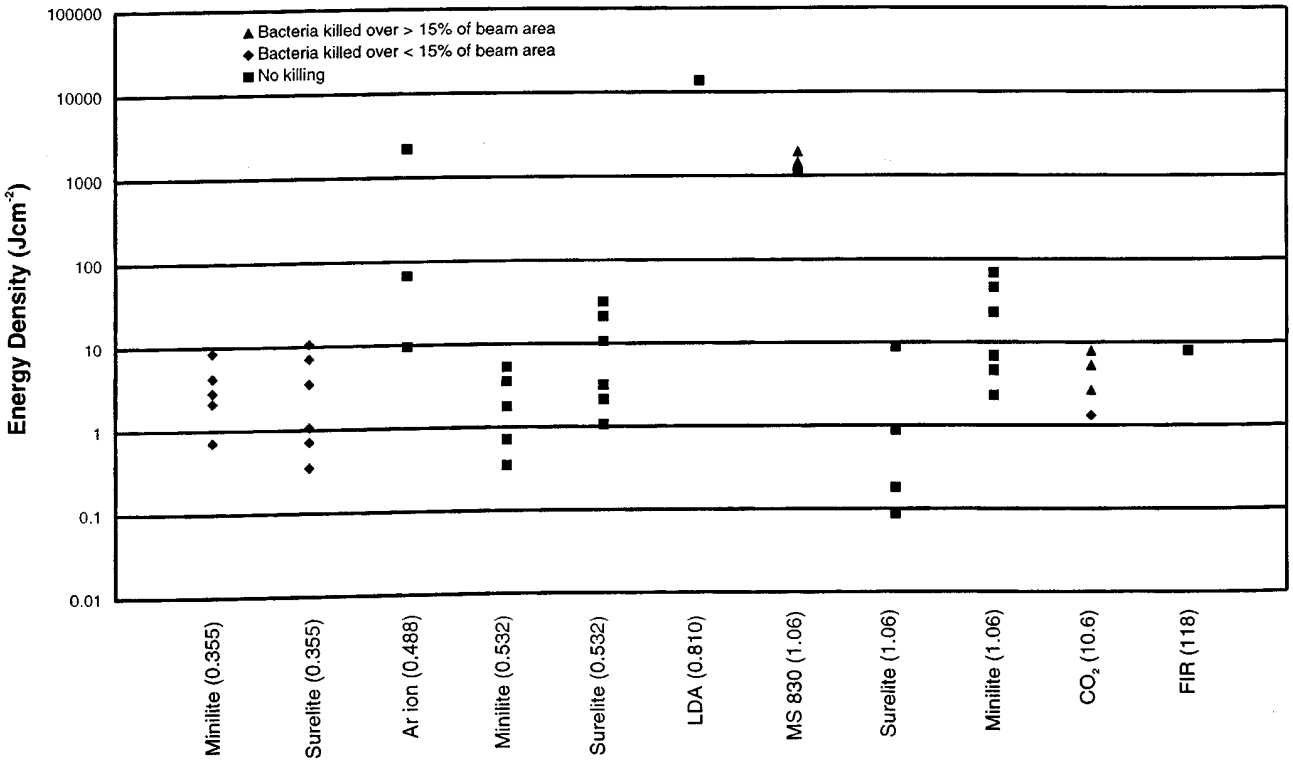

Fig. 1 Comparison of the bactericidal capacity of the seven lasers investigated. Energy densities are shown at which there was no killing observed, killing at less than 15% of the total beam area, and killing over 15% of the total beam area.

for energy densities above 1460 J cm^{-2}, and the CO_2 laser produced areas greater than 15% for energy densities above 2.63 J cm^{-2}.

Figure 2 shows a graph of the zones of clearing as a function of the applied energy density for each laser that demonstrated a biocidal capacity. To achieve similar areas of no growth, the energy densities were over two orders of magnitude greater for Lumonic's Nd:YAG laser than the CO_2 laser. For example, with the CO_2 laser and an energy density of 2.63 J cm^{-2}, a clear area of 0.66 cm^2 was measured; this increased to an area of 1.215 cm^2 for an applied energy density of 7.88 J cm^{-2}. Areas approximately 18% lower were produced with Lumonic's Nd:YAG laser after applying energy densities about 650 times larger than those used for the CO_2 laser. For the frequency-tripled lasers, the areas of sterilization did not vary much as the energy density was increased. The areas, however, were greater for the Surelite II-10, which had the same beam diameter as the Minilite (as quoted by the manufacturer) but a greater pulse energy. Areas of partially inhibited growth were observed after exposure to a single pulse from the frequency-tripled Nd:YAG laser (Surelite II-10), but these areas were not quantifiable.

Figure 3 shows the zones of inhibition normalized to the average applied energy density and the beam area as a function of exposure time. The greatest fractional value was observed for the Surelite II-10 laser operating at 355 nm, closely followed by the CO_2 laser (600 W), the minilite (355 nm), and Lumonic's Nd:YAG (200 W). The CO_2 laser provided the most rapid sterilization, achieving zones of inhibition of 1.2 cm^2 in 30 msec (7.88 J cm^{-2}), whereas a 16 s exposure from the Nd:YAG laser produced a zone of inhibition of about 0.7 cm^2 (1940 J cm^{-2}). It is interesting to note that even though the peak powers were over three orders of magnitude greater for the Q-switched lasers, operating at 1.06 and 0.532 μm, than Lumonic's Nd:YAG, no effect on the *E. coli* was observed for these devices. The Q-switched irradiances were sufficiently high for the petri dish to melt or burn at the junction between the agar and the dish and on the rear side of the dish. No such effect was observed on the dishes exposed to any of the other lasers tested.

3 DISCUSSION

Although there is now a substantial literature on the bactericidal activity of laser radiation, there have been few studies in which different laser wavelengths were compared for activity in relation to energy density with a standardized bacterial tar-

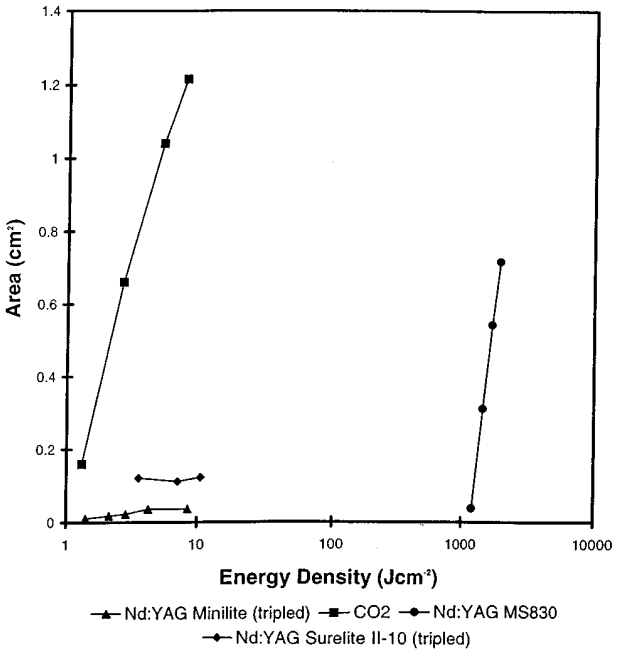

Fig. 2 Zones of clearing as a function of the applied energy density for each laser that demonstrated a bactericidal capacity against *Escherichia coli* grown on nutrient agar culture plates.

get. In the present study, where seven wavelengths of laser radiation were explored, by far the most effective was the 10.6-μm radiation delivered by a 600-W CO_2 laser. This instrument, operating with a beam area of 2.3 cm^2, was able to sterilize a circular 1.21-cm^2 patch on the *E. coli* colony in 30 msec. The energy delivered to the patch under these conditions was about 18 J.

The sterilization mechanism will differ for lasers operating in the UV and IR. Interestingly, sterilization was not observed for the Q-switched lasers operating at 1.06 and 0.532 μm, where the intensities were above the damage threshold of the plastic petri dishes but below that required to kill the bacteria. Because high peak power radiation at 1.06 μm had no bactericidal effect, whereas high mean power at this wavelength did, it is apparent that the sterilization mechanism at this wavelength is probably thermally dominated, i.e., it is not dependent on rapid photochemical or ablative processes occurring over short pulse durations, as was observed for exposure at 355 nm, where even a high-power single pulse of 4 ns duration affected the growth of the bacteria. As far as the authors are aware, there are no reports on laser sterilization at 355 nm; how-

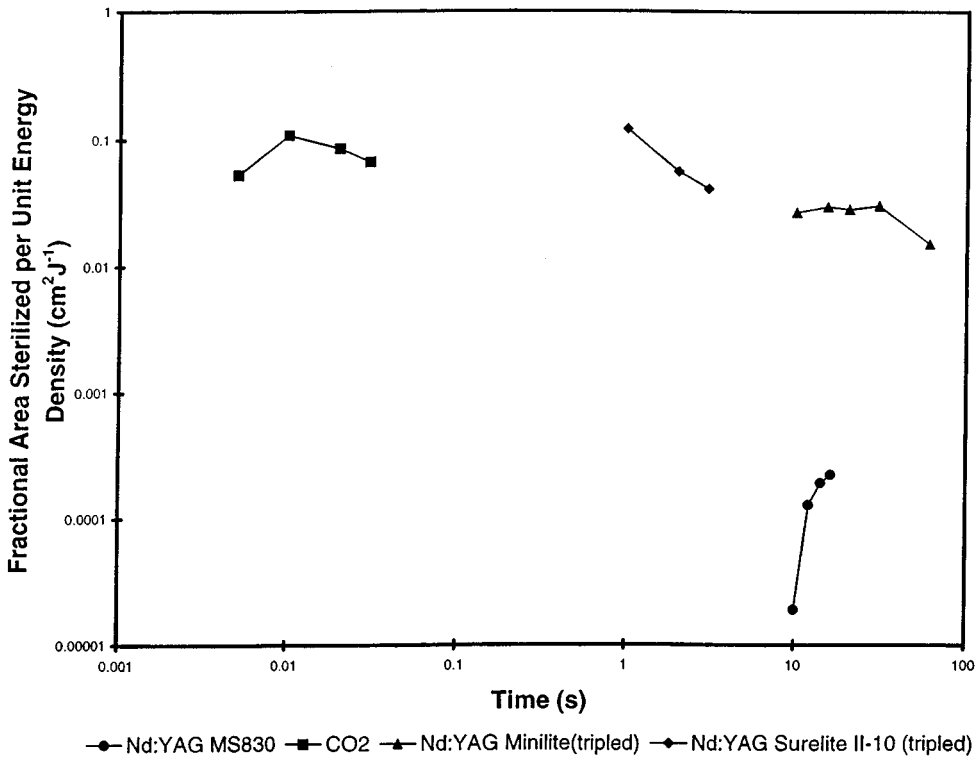

Fig. 3 Exposure times to generate zones of clearing normalized to the laser beam area and applied energy density, for each laser that demonstrated a bactericidal capacity against *Escherichia coli* grown on nutrient agar culture plates.

ever, the observed inactivation may be due to fluorescence occurring at wavelengths normally associated with UV sterilization, i.e., between 254 and 260 nm, which may be a result of a biphotonic process; this hypothesis was not examined further.

A plausible explanation for the sterilization from exposure to the IR laser is that the rapid transient temperature rise of the bacteria and the underlying agar is sufficient to kill by thermal effects. Since water absorbs strongly at 10.6 μm and the bacterial cell is about 80% H_2O, it can be calculated that 18 J absorbed by the top 0.6 μm of thickness of agar in a 1.21-cm circular patch on the culture plate would raise its temperature from 20 to 80°, which would kill the bacteria very rapidly. At energy densities 500 to 1000 times greater than those used with the CO_2 laser (i.e., around 2000 J cm^{-2}), the Nd:YAG laser at 1.06 μm was fully effective in sterilizing circular areas on the bacterial colony, and such doses were delivered in about 16 s with the laser operating at 200 W.

If the values of the fractional beam area that cause sterilization per unit energy density were known for bacterial vegetative cells and spores, environmental conditions, lasers, and laser parameters, then such information would allow development of laser sterilization systems across a number of industrial sectors. At their present stage of development, however, it is convenient to standardize the experiment on seeded agar surfaces because of the simplicity of these experiments and the ease with which the biocidal capacity of different lasers can be compared.

It is clear that lasers offer a novel way to achieve inactivation or sterilization, with a capacity to sterilize much faster than conventional methods. The rate of exposure from the frequency-tripled YAGs was limited because the highest pulse repetition frequency was only 10 Hz. In practice, the 3 s exposure was only 30 pulses, each of 5 ns, totaling an exposure of 150 ns. This represents an extremely fast and efficient sterilization system which may have useful implications for sterilization and hygiene practices in general. High-power excimer lasers, operating in the UV and at high pulse repetition frequency, are being developed[10] and could be used for extremely rapid laser sterilization systems that may have applications across a number of industrial sectors. Studies are now under way to refine and extend knowledge of the bactericidal capabilities of the CO_2 and Nd:YAG high-power lasers for a range of bacterial and ambient conditions.

4 CONCLUSIONS

Of the lasers tested, significant ability to kill *Escherichia coli* was observed with the CO_2 (600 W), frequency-tripled Nd:YAGs (1 and 0.04 W) and Nd:YAG (200 W) lasers. The CO_2 laser provided the most rapid sterilization: a zone of inhibition of 1.2 cm^2 was achieved in 30 msec, whereas 16-s exposure of Nd:YAG (200 W) irradiation produced a zone of inhibition of 0.7 cm^2. Growth inhibition was observed after exposure to laser radiation at 355 nm. No bactericidal capacity was observed with the FIR device, Q-switched lasers operating at 1.06 μm and 532 nm, the Ar ion laser, or the laser diode array.

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