Effect of gamma irradiation on viability and DNA of *Staphylococcus epidermidis* and *Escherichia coli*

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Gamma irradiation is widely used for sterilization; however, its effect on elimination of amplifiable DNA, an issue of relevance to molecular diagnostic approaches, has not been well studied. The effect of gamma irradiation on the viability of *Staphylococcus epidermidis* and *Escherichia coli* (using quantitative cultures) and on their DNA (using quantitative 16S rRNA gene PCR) was evaluated. Viability was abrogated at 2.8 and 3.6 kGy for *S. epidermidis* and *E. coli*, respectively. The radiation dose required to reduce viable bacteria by one log₁₀ (D_{10} value) was 0.31 and 0.35 kGy for *S. epidermidis* and *E. coli*, respectively. D_{10} values for amplifiable DNA extracted from bacteria were 2.58 and 3.09 kGy for *S. epidermidis* and *E. coli*, respectively, whereas D_{10} values for amplifiable DNA were significantly higher for DNA extracted from irradiated viable bacterial cells (22.9 and 52.6 kGy for *S. epidermidis* and *E. coli*, respectively; $P < 0.001$). This study showed that gamma irradiation of DNA in viable bacterial cells has little effect on amplifiable DNA, was not able to eliminate amplifiable 16S rRNA genes at a dose of up to 12 kGy and cannot therefore be used for elimination of DNA contamination of PCR reaction components or laboratory equipment when this DNA is present in microbial cells. This finding has practical implications for those using molecular diagnostic techniques in microbiology.

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INTRODUCTION

Gamma irradiation is electromagnetic radiation of short wavelength emitted by radioactive isotopes as the unstable nucleus breaks up and decays to reach a stable form. It is widely used for sterilization of medical devices, food preservation and processing of tissue allografts and blood components, obviating the need for high temperatures that can be damaging to such products (Block, 2001; Hansen & Shaffer, 2001; Kainer *et al.*, 2004; Mendonca *et al.*, 2004; Osterholm & Norgan, 2004). DNA is the principal cellular target governing loss of viability after exposure to gamma irradiation. DNA damage occurs predominantly by the indirect action of gamma rays, which interact with other atoms or molecules, particularly water, to produce reactive free radicals. Cell death (defined for proliferating cells as loss of reproductive capability) is predominantly induced by double-strand breaks in DNA, separated by not more than a few base pairs, which can generally not be repaired by the cell (Hall & Giaccia, 2006).

Although several studies have investigated the effect of gamma irradiation on the viability of micro-organisms, little information is available regarding its effect on microbial DNA. In particular, whether gamma irradiation eliminates amplifiable DNA, detectable using quantitative broad-range

PCR, is unknown. DNA may fail to amplify due to DNA degradation, such as alteration in primer binding sites or reduction of the DNA into fragments smaller than the target. If gamma irradiation effectively eliminates amplifiable DNA, it could be used widely in laboratory and clinical practice for prevention of DNA contamination of PCR reaction reagents, laboratory equipment, surgical instruments and containers for specimen collection and transportation.

We therefore studied the effect of gamma irradiation on the viability of *Staphylococcus epidermidis* and *Escherichia coli* (using quantitative cultures) and on their DNA (using quantitative PCR amplification of the 16S rRNA gene). The 16S rRNA gene was selected because this highly conserved region of bacterial DNA is often used when the infecting agent is not known and the goal is to detect and identify the presence of any bacterium (Kolbert *et al.*, 2004). The 16S rRNA gene is present as multiple copies in the genomes of most bacterial species that belong to the eubacterial kingdom, but is not present in human, viral or fungal genomes. The presence of multiple copies of this target in bacteria increases assay sensitivity when applied to infected human specimens. However, this target has been associated with false-positive results as a result of 16S rRNA gene contamination of reagents or equipment used for molecular approaches. We also evaluated differences in radiation

sensitivity of extracted DNA in comparison with DNA residing within viable bacterial cells at the time of irradiation.

METHODS

Bacterial cultures. Stock cultures of *S. epidermidis* ATCC 12228 and *E. coli* ATCC 10798 were frozen in Microbank cryovials (Pro-lab Diagnostics) and stored at -70°C until studied. One cryovial bead from each stock culture was streaked on trypticase soy agar containing 5% sheep blood (BD Diagnostic Systems) and incubated for 24 h. An isolated colony was removed aseptically from the agar plate and inoculated into 150 ml sterile trypticase soy broth. After incubation for 18 h on a rotary shaker at 150 r.p.m. at 37°C , the broth was centrifuged (5000 *g* for 10 min) and the pellet was resuspended in 150 ml normal saline to keep the bacteria viable, but minimize their replication. One millilitre aliquots of bacterial suspensions were assayed after exposure to gamma irradiation in three ways (Fig. 1). First, viability of bacteria was evaluated after irradiation of bacterial suspensions (Fig. 1a). Second, the effect of gamma irradiation was studied on amplifiable free DNA extracted from bacterial cells before irradiation (Fig. 1b). Third, the effect of gamma irradiation was evaluated on amplifiable DNA where viable cells were irradiated first and then the DNA was extracted (Fig. 1c).

DNA extraction. DNA was extracted using a QIAamp DNA mini kit (Qiagen). One millilitre aliquots of bacterial suspension were centrifuged (5000 *g* for 10 min) and the pellet was resuspended in 180 μl buffer ATL. Twenty microlitres of proteinase K was added and the mixture was vortexed and incubated at 56°C for 60 min. After incubation, 200 μl buffer AL was added and the mixture was incubated at 70°C for 10 min before 200 μl absolute ethanol (Aaper) was added and the mixture transferred to a QIAamp spin column that was centrifuged at 6000 *g* for 1 min. Five hundred microlitres buffer AW1 was then added to the column and the sample was centrifuged at 6000 *g* for 1 min. Five hundred microlitres buffer AW2 was then added to the column and the sample was centrifuged at 20 000 *g* for 3 min. After centrifugation, 1 ml distilled water was added. The sample was incubated at room temperature for 5 min and then centrifuged at 6000 *g* for 1 min to elute the DNA.

Gamma irradiation. One millilitre aliquots of bacterial suspension and extracted DNA were irradiated in triplicate in closed 1.5 ml polyethylene microcentrifuge tubes at $21 \pm 2^{\circ}\text{C}$, with rotation

during irradiation to minimize variations in the absorbed dose. Normal saline, processed in the same way as bacterial cells, served as a negative control. A self-contained ^{137}Cs gamma irradiation cell irradiator (Mark I) was used. The source strength was $\sim 6000\text{ Ci}$ ($2.2 \times 10^{14}\text{ Bq}$) with a dose rate of 9.35 Gy min^{-1} , as established by the National Institutes of Standards and Technology. Actual absorbed doses were within 3% of target doses as assessed by dosimetric measurement using 5 mm diameter alanine dosimeters (Bruker Biospin).

Assessment of irradiation effect. Bacterial suspensions were exposed to radiation doses of 0–4 kGy, in increments of 0.2 kGy (Fig. 1a). After irradiation, serial dilutions were prepared in normal saline, plated on trypticase soy agar containing 5% sheep blood and incubated at 37°C for 48 h. Viable cells were expressed as mean $\log_{10}[\text{c.f.u. (ml suspension)}^{-1}] \pm \text{SD}$ of triplicates. The gamma irradiation effect on DNA was studied at doses of 0–12 kGy, in increments of 1 kGy, using either DNA extracted from bacterial suspensions before irradiation (Fig. 1b) or DNA extracted from irradiated bacterial suspensions (Fig. 1c). Tubes with extracted DNA were stored at $21 \pm 2^{\circ}\text{C}$ for a maximum of 12 h until irradiation. DNA quantity was determined by quantitative PCR.

Quantitative 16S rRNA gene PCR. Real-time PCR (LightCycler) was used to quantify the 16S rRNA gene. Universal primers (forward primer: 5'-TGGAGAGTTTGATCCTGGCTCAG-3'; reverse primer: 5'-TACCGCGGCTGCTGGCAC-3') spanning positions 5–532 (inclusive) of *E. coli* K-12 (GenBank accession no. NC_000913) were used (Kolbert *et al.*, 2004; Tang *et al.*, 1998, 2000). Each PCR mix consisted of 2 μl target DNA added to 18 μl mastermix (LightCycler FastStart DNA Master SYBR Green I; Roche Applied Science), containing final concentrations of 2.5 mM MgCl_2 , 0.04 μM each primer and 0.05 U thermolabile uracil-*N*-glycosylase (Roche Applied Science). Cycling parameters consisted of one cycle at 95°C for 10 min (pre-incubation), followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 62°C for 5 s and elongation at 72°C for 20 s. These PCR conditions were optimized to produce the least non-specific signal by primer dimers, as evaluated by post-amplification melting curve analysis. Mastermix on its own was used as a negative control for PCR. For quantification, the second derivative maximum method with Savitzky–Golay polynomial estimation was used. The standard curve was determined by depicting the amplification threshold cycle number (crossing point) against the logarithm

Fig. 1. Study design for evaluation of the effect of gamma irradiation on bacterial viability (a), free (extracted) DNA (b) and DNA in viable bacterial cells subsequently subjected to DNA extraction (c). Samples for quantification of bacterial cells (a) were irradiated with 0–4 kGy in increments of 0.2 kGy and those for quantification of DNA (b, c) with 0–12 kGy in increments of 1 kGy.

of the initial target concentration (Shepley & Wolk, 2004; Wittwer & Kusukawa, 2004). Standard curves for *S. epidermidis* and *E. coli* were generated from five serial dilutions of known quantities of *S. epidermidis* [limit of detection, 150 c.f.u. (ml bacterial suspension)⁻¹; coefficient of determination (r^2), 0.97; amplification efficiency (E), 1.78] and *E. coli* [limit of detection, 25 c.f.u. (ml bacterial suspension)⁻¹; r^2 , 0.95; E , 1.82]. Amplification efficiency was calculated according to the formula $E = 10^{-1/k}$, where k represents the slope of the quantification standard curve. DNA quantity was expressed as c.f.u. equivalent (ml bacterial suspension)⁻¹. Random amplification products after irradiation were sequenced in both 5'–3' and 3'–5' directions with BigDye terminator version 1.1 *Taq* kit and an ABI 3730XL DNA sequencer (Applied Biosystems), using the above universal PCR primers as sequencing primers. Sequence data were analysed using MicroSeq software and GenBank. The strain of *S. epidermidis* used has five copies of 16S rRNA genes and the *E. coli* strain has seven copies.

Radiation dose–response curves and D_{10} values. Responses to gamma irradiation were expressed as the logarithm of the ratio of survivors (N/N_0), where N represents the mean c.f.u. ml⁻¹ or c.f.u. equivalent ml⁻¹ of irradiated bacterial suspension or DNA, as appropriate, and N_0 the mean number of c.f.u. ml⁻¹ or c.f.u. equivalent ml⁻¹ of non-irradiated control. The $\log_{10} N/N_0$ (outcome variable, y) was plotted against the corresponding radiation dose (explanatory variable, x) to obtain the semi-logarithmic dose–response curve. D_{10} values, defined as the radiation dose (in kGy) required to reduce the number of c.f.u. ml⁻¹ or c.f.u. equivalent ml⁻¹ by one $\log_{10}$, were determined by calculating the negative reciprocal of the slope of the linear regression curve (Aziz *et al.*, 1997; Bari *et al.*, 2003; Lamb *et al.*, 2002; Rajkowski *et al.*, 2003; Sommers & Fan, 2003; Thayer & Boyd, 1993, 2001; Thayer *et al.*, 2003).

Statistical analysis. Variables in the dose–response curve were fitted using a simple linear regression model, as determined by least-squares analysis (Woodward, 1999). The zero radiation value was excluded from the linear regression analysis to avoid a possible shoulder effect. The analysis was limited to the linear portion of the curve and r^2 values were calculated. The 95% confidence intervals (CIs) for the regression curve were weighted by standard deviations of triplicate samples. Regressions were tested for differences by analysis of covariance (Woodward, 1999). SD and 95% CI were calculated for D_{10} values. A P value of <0.05 (for a 2-sided test) was considered statistically significant. All calculations were performed using the statistical software package JMP (version 6.0; SAS Institute). Origin software (version 7.5; OriginLab) was used for graphic analysis.

RESULTS AND DISCUSSION

Irradiation effect on viability of bacterial cells

The effect of gamma irradiation on the viability of stationary-phase cells of *S. epidermidis* and *E. coli* is shown in Fig. 2(a) and (b), respectively. Non-irradiated bacterial suspensions contained a mean $\pm$ SD of $8.78 \pm 0.12 \log_{10}(\text{c.f.u. ml}^{-1})$ for *S. epidermidis* or $9.47 \pm 0.07 \log_{10}(\text{c.f.u. ml}^{-1})$ for *E. coli*. Bacterial viability was abrogated at 2.8 kGy for *S. epidermidis* and 3.6 kGy for *E. coli*. D_{10} values for bacterial cells were 0.31 kGy for *S. epidermidis* and 0.35 kGy for *E. coli* ($P > 0.1$) (Table 1).

Gamma irradiation at 4 kGy sterilized stationary-phase populations of both *S. epidermidis* and *E. coli*. The calculated

E. coli D_{10} values in our study are comparable to those reported by others, ranging from 0.20 to 0.65 kGy (Block, 2001; Osterholm & Norgan, 2004). The radiation susceptibility of cells is known to be affected by a number of factors, including replication rate, intracellular water content, amount of DNA, medium composition, temperature, pH, oxygenation status and the ability to repair radiation-induced DNA damage (Thayer & Boyd, 1993, 2001; Thayer *et al.*, 2003). To our knowledge, D_{10} values have not been reported previously for *S. epidermidis*, as most studies have focused on micro-organisms important in the food industry

Fig. 2. Effect of gamma irradiation on *S. epidermidis* (a) and *E. coli* (b). This is shown as the effect on bacterial viability after irradiation of bacterial suspensions (determined by subsequent quantitative cultures; ■), on amplifiable free DNA extracted from bacterial cells before irradiation (determined by subsequent quantitative PCR; ▲) and on amplifiable DNA where viable cells were first irradiated and then the DNA was extracted and subjected to quantitative PCR (*). Dotted lines represent the 95% CIs for the estimates of each regression.

Table 1. Radiation D_{10} values for bacterial cells and DNA

Irradiation procedure	D_{10} [kGy (95 % CI)]*	r^2
<i>S. epidermidis</i>		
Irradiated bacteria	0.31 (0.29–0.34) ^a	0.985
Irradiated extracted DNA	2.58 (2.37–2.83) ^b	0.984
Irradiated bacteria, followed by DNA extraction	22.9 (18.9–28.9) ^b	0.920
<i>E. coli</i>		
Irradiated bacteria	0.35 (0.32–0.38) ^a	0.981
Irradiated extracted DNA	3.09 (2.78–3.46) ^b	0.976
Irradiated bacteria, followed by DNA extraction	52.6 (34.5–111.1) ^b	0.638

*Determined from the slope of the simple linear regression analysis: *a*, from 0.2 to 4 kGy; *b*, from 1 to 12 kGy.

(Block, 2001). However, *S. epidermidis* is the predominant pathogen causing device-associated infections and is clinically important in the implant industry (Zimmerli *et al.*, 2004).

Irradiation effect on amplifiable DNA

Table 1 shows radiation D_{10} values for DNA extracted from bacteria before irradiation and for DNA extracted from irradiated bacterial cells. Fig. 2 shows that gamma irradiation at 4, 8 and 12 kGy reduced the free amplifiable DNA quantity (extracted before irradiation) by 1.20 ± 0.06 , 2.65 ± 0.02 and 4.44 ± 0.03 $\log_{10}$ (c.f.u. equivalent ml^{-1}) for *S. epidermidis*, respectively, and by 0.55 ± 0.05 , 1.80 ± 0.08 and 3.60 ± 0.04 $\log_{10}$ (c.f.u. equivalent ml^{-1}) for *E. coli*, respectively. D_{10} values for extracted DNA were lower for *S. epidermidis* than for *E. coli* (2.58 versus 3.09 kGy, $P=0.02$).

In contrast, irradiation of DNA in viable bacterial cells, which were subsequently subjected to extraction, had less effect on amplifiable DNA than did irradiation of extracted DNA ($P<0.001$). Even at the highest radiation dose tested (12 kGy), a reduction in the quantity of amplifiable DNA in irradiated viable bacterial cells corresponding to just 0.43 ± 0.05 $\log_{10}$ (c.f.u. *S. epidermidis* ml^{-1}) or 0.10 ± 0.06 $\log_{10}$ (c.f.u. *E. coli* ml^{-1}) was achieved (Fig. 2). D_{10} values for DNA extracted from irradiated viable bacterial cells were 22.9 and 52.6 kGy for *S. epidermidis* and *E. coli*, respectively. The DNA quantity after amplification of normal saline without bacteria (negative control) was below the detection limit. Sequence data of 15 randomly chosen amplification products with positive signals confirmed the specific target with >99 % identity.

Comparison of effects on viability and amplifiable DNA

We have demonstrated that gamma irradiation of viable bacterial cells has a smaller effect on amplifiable 16S rRNA genes than does irradiation of extracted DNA. Importantly, gamma irradiation did not eliminate amplifiable DNA at the

highest radiation dose tested (12 kGy). Potential reasons for the radiation resistance of DNA in viable cells are manifold. DNA in viable cells may be more resistant to irradiation than free (extracted) DNA because of low molecular mass scavengers that mop up free radicals in cells, physical protection of DNA by packaging in cells and/or cellular repair of damaged DNA (Hall & Giaccia, 2006). In dying cells, DNA fragmentation may also occur because of the action of nucleases. Less likely, irradiated bacteria may be more easily lysed than non-irradiated bacteria; consequently, larger amounts of extracted DNA would be available for PCR. However, it is unlikely that relatively small differences in DNA extraction efficiency in irradiated and non-irradiated cells could explain the significant differences in D_{10} values of cell-associated and free DNA. DNA extraction is less efficient for Gram-positive bacteria than for Gram-negative bacteria. Failure to extract the DNA from *S. epidermidis* may make it appear easier to eliminate.

Importantly, the amplification assay used in this study quantified amplifiable DNA using universal primers annealing to 16S rRNA genes present as multiple copies in the genomes. Whether or not the use of a specific rather than broad-range PCR assay, targeting a single copy gene, would yield different results is unknown. However, broad-range PCR is commonly used in diagnostic microbiology and was therefore chosen for study. Different results may arise with different sizes of target; for example, a shorter partial 16S rRNA gene target may have yielded greater residual amplifiable DNA.

The results of our study indicate that gamma irradiation cannot be used for elimination of DNA contamination of PCR reaction components, surgical instruments or laboratory equipment, when this DNA is present in microbial cells. This subject is important in clinical practice as molecular amplification techniques are increasingly deployed in microbiological diagnostics due to their high sensitivity, rapidity and ability to detect organisms that are not growing because of prior antimicrobial therapy or are not culturable on conventional growth media. Possible strategies to enhance elimination of DNA residing in viable cells by

gamma irradiation include inactivation of cellular repair mechanisms using low temperatures for irradiation, exposure to high temperatures before irradiation or DNA extraction before irradiation. Alternatively, other methods for DNA elimination, such as chemical (e.g. bleach) or enzymic (e.g. nuclease) treatment, might be considered. Finally, radiation resistance of DNA in microbial cells may be beneficial for diagnostic purposes if the goal is to reduce the infectivity of the specimen while preserving microbial DNA as a target for molecular diagnostics. This strategy has been validated for herpes viruses and *Bacillus anthracis* using autoclaving (Espy *et al.*, 2002), but has not yet been described with gamma irradiation.

In summary, our observations have important implications for those using molecular techniques in diagnostic microbiology. The inability of gamma irradiation to eliminate microbial DNA in viable cells needs to be taken into account when using irradiated specimens.

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