Exposure to sublethal clinical radiotherapeutic doses of ionizing \(\gamma\)-radiation gives rise to mutants of Gram-negative and Gram-positive clinical pathogens with increased antibiotic resistance

Previously published data from our group (McMahon et al., 2007) and others (Gilbert & McBain, 2003) have demonstrated that when bacteria are exposed to environmental stresses, such as altered pH, heat shock and increased salinity, they are able to alter their antibiotic susceptibility by becoming either more susceptible or, conversely, more resistant to antibiotic agents. For example, when we stressed *Staphylococcus aureus* organisms with increased but sublethal salt or pH levels, we observed up to a fourfold increase in MICs of gentamicin and erythromycin against these organisms (McMahon et al., 2007). \(\gamma\)-Radiation has the ability to kill bacteria, mainly through DNA damage (Cerutti, 1976), depending on the dose absorbed by target bacterial cells. Such radiation, therefore, has the potential to induce mutations, ranging from mild, or ‘silent’, mutations through to catastrophic mutations that lead to terminal events within the bacterial cell. In nature, mutagenesis is hypothesized to play roles in adaptation and subsequent propagation via chromosomal rearrangement, alteration in target sites on cells and deregulation of enzyme synthesis. What has not been examined, to date, is the effect of sublethal doses of \(\gamma\)-radiation on clinical pathogens carried in or on patients undergoing radiotherapy, particularly in relation to the effect it has on the bacteria’s susceptibility to antibiotics.

Radiotherapy is an important intervention for the treatment of malignant disease. In the UK, approximately 120,000 patients each year undergo some form of radiotherapy for the management of their disease (Anonymous, 2011) and it is recommended that just over half of all people with cancer should be receiving radiotherapy as part of their treatment plan. Overall in the UK, this equates to 48,000 radiotherapy fractions (courses) per million people in the population (Anonymous, 2011). One consequence of such treatment is an associated immunosuppression, which has been reported previously (Wara et al., 1975; Oldham, 2009). In addition, employment of radiotherapy usually accompanies chemotherapy, which utilizes cytotoxic agents that are also immunosuppressive to the host. As a result of chemotherapy and/or radiotherapy, many patients become immunosuppressed with lowered neutrophil counts, thus placing a great importance on the employment of effective antibiotics, either for the prophylaxis of sepsis or the management of a treatment-related infection. In such scenarios, failure of antibiotic treatment due to the presence of an infecting pathogen with increased antibiotic resistance could lead to patient fatality.

Therefore, it is important to examine the effect of \(\gamma\)-radiation in doses equivalent to those received by patients undergoing radiotherapy for malignancies. It was the aim of this *in vitro* study to examine the effect of clinical \(\gamma\)-radiation exposure on the antibiotic susceptibility of two bacterial pathogens in order to determine if clinical levels of radiation could increase the antibiotic resistance of these organisms.

Two wild-type clinical isolates were used in this study, one Gram-positive organism, a strain of methicillin-resistant *Staphylococcus aureus* (MRSA), and one Gram-negative organism, Klebsiella aerogenes. These isolates were part of the Northern Ireland Public Health Laboratory (NIPHIL) Strain Repository (MicroARK) and were recovered from storage at \(-80^\circ\text{C}\). All isolates were subcultured at least three times onto Columbia blood agar (CM0331; Oxoid) supplemented with 5% (v/v) defibrinated horse blood and incubated for 24h at 37 \(^\circ\text{C}\) under aerobic conditions. Particular attention was given to purifying the isolates from single colonies on at least three occasions, in order to ensure a single clonal type of each organism was used in downstream analyses.

Fresh (24 h) cultures of both organisms were subcultured separately onto fresh Columbia blood agar, as detailed above. A fresh 0.5 McFarland standard was prepared separately for both organisms in 1/4 strength Ringer’s solution (BR0049G; Oxoid) and 100 \(\mu\text{l}\) of each bacterial suspension was added to 20 ml tryptone soya broth (TSB; CM0129; Oxoid) in thin-walled sterile plastic containers. Inoculated containers were irradiated with \(\gamma\)-radiation using 60Co as a source in a Gammabeam-650 irradiation unit (MDS Nordion) at an environmental temperature of 4 \(^\circ\text{C}\) to give a total dose of 100 Gy, equating to the upper dose range in humans (http://www.rcr.ac.uk/docs/ oncology/pdf/Dose-Fractionation_Final.pdf). Isolates were selected for further downstream processing and two time points for testing were established: (i), before exposure to \(\gamma\)-radiation and (ii), after exposure to \(\gamma\)-radiation.

Antibiotic susceptibility testing was performed in accordance with CLSI guidelines (CLSI, 2005) on all isolates at each of the two time points using a standard antibiotic disk susceptibility testing method. Cultures were scored on whether or not the zone of inhibition was bigger before or after exposure to radiation. Susceptibility to the following antibiotics was tested (\(\mu\text{g}\) per disk): amoxicillin with clavulanic acid (3), ampicillin (10), aztreonam (30), chloramphenicol (10), erythromycin (5), cefoxitin (30), fusidic acid (10), levofloxacin (5), moxifloxacin (1), oxacillin (1), penicillin (2), quinupristin–dalfopristin (15), tetracycline (30) and tobramycin (10). Antibiotic concentrations on each disk were selected to simulate
levels that are therapeutically achievable in the patient and represent notional clinical breakpoints. In addition, quantitative MIC susceptibility tests were performed on antibiotic resistant mutants by using E-test strips (bioMérieux) containing the following antibiotics: cefotaxime, ceftriaxone, ceftazidime, cefepime, and piperacillin. In both methods, cultures were spread onto Columbia Blood agar, supplemented with 5% (v/v) defibrinated horse blood, using a cotton swab charged with inoculum equivalent to a 0.5 McFarland standard. After drying, a standard diffusion assay was performed with either an E-test strip or a susceptibility disk. The plates were incubated aerobically at 37 °C for 24 h prior to reading. In the disk susceptibility tests, the diameter (mm) of the zone of inhibition was measured manually, and in the E-tests, the MIC was determined as instructed by the manufacturer.

No differences in zone sizes were seen between isolates before and after γ-radiation with each antibiotic tested. However, individual antibiotic-resistant colonies did appear after radiation exposure and were detected around the cefotaxin (30 μg) disk for the MRSA isolate and the tetracycline (30 μg) disk for the K. aerogenes isolate. These resistant colonies were not present in the original non-irradiated population of either organism. MICs for these resistant populations were compared against those of the non-radiated wild-type populations for both organisms. MICs of four β-lactam agents, namely piperacillin, ceftriaxone, cefotaxime, ceftazidime, as well as to the fluoroquinolones, ciprofloxacin, are shown in Table 1. Overall, the antibiotic MICs for these resistant colonies were approximately threefold greater than those seen for both organisms before exposure to radiation, with a fivefold increase in the MIC of piperacillin against the MRSA isolate and a fourfold increase in the MIC of ciprofloxacin against the Klebsiella isolate.

Infection is an important complication when managing patients with malignancy who are undergoing treatment regimens that can result in immunosuppression. Thirumala et al. (2010) recently reviewed cases of infection arising as complications in critically ill patients with cancer. The choice of pathogens in the current study reflects clinically significant organisms commonly isolated from peripheral blood in patients with sepsis, including both a Gram-negative organism (K. aerogenes) and a Gram-positive organism (MRSA), the latter of which is one of the most common nosocomial infections. In the current study, a wide range of antibiotics were selected for examination that would be part of the oncologist’s antibiotic formulary to deal with post-treatment infections, particularly with respect to the fluoroquinolones and the third-generation cephalosporins.

In the present study, a 100 Gy dose was employed as this represented the absolute upper limit of radiotherapy dosing regimens. Most doses that are commonly employed for treating patients with malignancies are generally below this value. We chose to use such an upper limit; however, future studies should be aimed at examining the effects of actual clinical doses in combination with potential pathogens at specific sites, e.g. the effect of radiation dosage on antibiotic resistance in Streptococcus pneumoniae in patients with lung cancer who are undergoing palliative radiotherapy regimens.

Several stresses induce the mar (multiple antibiotic resistance) operon (Alekshun & Levy, 1999), which regulates the expression of several genes, including those which encode a broad-specificity efflux pump (Rickard et al., 2004). In addition, stress hardening (Rowan, 1999) may lead to cross-protection against a range of apparently unrelated stress challenges, including resistance to antibiotics. Therefore, bacterial cells have several mechanisms to select for mutants from within sublethally stressed bacterial populations to minimize further stress, and to maximize continued cell viability to ensure survival following the removal of the stress conditions. Recently, the term ‘stressosome’ has been proposed, which describes a signal transduction cascade that increases the expression of stress-response genes, in which stress signals may be integrated by a multiprotein signalling hub that responds to various signals to effect a single outcome (Marles-Wright et al., 2008). Previously, we have shown that exposure of organisms to sublethal doses of radiation of lesser energy, namely UVC light and X-rays, does not have a significant effect on altering the antibiotic resistance profiles of Gram-positive or Gram-negative organisms (Moore, unpublished data). It is not until higher energy γ-radiation is employed that we begin to observe physiological events that result in the development of antibiotic-resistant populations. As yet, we do not know the mechanisms by which these resistant populations are produced or their mode of resistance.

In conclusion, these data indicate that exposure to γ-radiation at therapeutic doses, such as those used in radiotherapy regimens in cancer patients, may increase the frequency of antibiotic-resistant

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<tr>
<th>Organism</th>
<th>Before/after exposure to radiation</th>
<th>MIC (μg ml⁻¹)*</th>
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<tr>
<td></td>
<td></td>
<td>Cefotaxime</td>
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<td>MRSA</td>
<td>Before</td>
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<td></td>
<td>After</td>
<td>32</td>
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<tr>
<td>K. aerogenes</td>
<td>Before</td>
<td>0.032</td>
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*As determined from E-test strips (bioMérieux).
organisms in endogenous bacterial populations. A comprehensive in vivo study would now be required to examine this phenomenon using endogenous organisms from patients undergoing such treatments.

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