

Radiation Response of Bacteria Associated with Human Cancellous Bone

Naznin Akhtar^{1*}, Fahmida Binte Atique², Md Mamun Miah¹, and
S. M. Asaduzzaman¹

¹(Tissue Banking and Biomaterial Research Unit, Atomic Energy Research Establishment, Savar, Dhaka, Bangladesh)

²(Department of Genetic engineering and Biotechnology, North South University, Dhaka, Bangladesh)

Abstract: Cancellous bones from twenty five live tissue donors were tested for bacterial contamination and initial bioburden ranged from 4.1×10^1 to 3.1×10^3 cfu/g (average 9.0×10^2 cfu/g). Forty six representative bacterial isolates were characterized on the basis of morphological, cultural and biochemical characteristics. *Staphylococcus* spp. was found to be predominant contaminant in tissue samples (41.30%). To assess the radiation resistance all the bacterial isolates were exposed to 1 to 10 kGy gamma radiation from ⁶⁰Co gamma source. The radiation decimal reduction dose values (D_{10}) and twelve log reduction values (12 D value) of the isolates were calculated. D_{10} values of the isolates were ranged from 0.59 to 1.20 kGy. Among the studied bacterial isolates, *Streptococcus* spp. was the most radioresistant isolates (D_{10} value 0.93-1.20 kGy) and three of the *Streptococcus* spp. survived up to 8 kGy. All the bacterial isolates were killed at 9 kGy. Twelve log reduction value (12D value) of the most resistant isolate was 14.4 kGy. These results indicate that standard radiation sterilization dose (25 kGy) is satisfactory for the sterilization of the cancellous bone allografts.

Keywords- Bioburden, Cancellous bone, Radiation resistance, *Staphylococcus*, *Streptococcus*, Sterilization

I. Introduction

Bone allografts play an important role in treating bone defects and in recovering the mobility of patients suffering from bone and joint diseases. In Bangladesh, a large number of patients have been suffering from different types of bone diseases like degenerative bone diseases, congenital deformities, fractures, non-union, and bone loss from traumatic accidents etc. Consequently the demand of bone allografts is increasing sharply. Though tissue allografts can dramatically improve the quality of life of the recipients and can even save lives, there is always a potential risk of disease or infection transmission from the donor to the recipient with the use of allografts [1-3]. So, the microbiological quality of the bone graft is the most important consideration for its clinical application.

To minimize the risk of diseases transmission, gamma radiation is currently the most common method for sterilization of tissue allografts [4, 5]. Conventionally, 25 kGy gamma radiation is used for the sterilization of tissue allografts. However, there is still much discussion on the doses of radiation required and any detrimental effects of radiation on the bone [6-9]. The sterilization dose required may vary from specimen to specimen and from country to country, although there are recommended standard dose for this purpose [10]. In radiation sterilization practice, it is important to determine the radiation resistance of the bioburden, because radiation dose necessary to achieve sterility assurance level (SAL of 10^{-6}) can be calculated based on the initial microbial contamination level and the radiation resistance of the contaminant [11]. Information on the type and magnitude of microbial contamination associated with bone allografts are limited in our country. Therefore, this work was undertaken to evaluate the bacterial contaminants of human bone tissues and to assess the radiation resistance of the isolates.

II. Materials and methods

2.1 Tissue procurement

Bone samples were collected from twenty five live tissue donors from twelve hospitals of Bangladesh through the donation of femoral heads removed during total hip replacement, hemiarthroplasty and traumatic limb amputation surgery. All the tissues were collected by the written consent of donor. The ages of donors were ranged from 40 to 75 years. All donors were pre-screened for the presence of transmissible diseases (eg. HIV, HBV and VDRL) and the tissues were excised under aseptic conditions in operation theatre. Immediately after harvesting, the bones were wrapped with gamma sterilized polyethylene pack, labeled with donor ID and were kept in a freezer (-40°C) in the operation theatre. Then the bones were placed in a cool box filled with ice slabs and were transported immediately to tissue banking laboratory using AC vehicle.

2.2 Bioburden assessment

Bacterial contaminants of the bone samples were determined by direct inoculation of the tissue wash on nutrient agar medium. Swab test and membrane filtration were not considered due to the known poor efficiency of recovery for Swab-based method [12-14] and the cleaning of bone tissue was not suitable for filtration. Because the cleaning of skeletal tissue yields turbid oily solutions containing blood, marrow and fat residues, which tend to occlude the 0.45 µm pore membranes quite rapidly. Therefore, for the assessment of bioburden, bones were first thawed to room temperature and then aseptically immersed in 200 ml sterile distilled water in sterile glass beakers separately. The tissue containing jars were shaken at 100 rpm for 15 minutes using mechanical shaker. Bioburden was determined from the tissue wash by pour plate and spread plate method using nutrient agar plate (three plates for each). After 24-72 hours of incubation at 37°C, bacterial colonies were counted and the results were expressed in cfu/g of bone. Representative bacterial isolates were sub-cultured on nutrient agar to obtain pure culture.

2.3 Characterization of bacterial isolates

Bacterial isolates were characterized on the basis of gram staining, cell shape and cell arrangement. Preliminary identification of bacterial isolates was carried out on the basis of morphological, cultural and biochemical behavior according to Bargey's "Manual of Determinative Bacteriology" [15]. The percent occurrence of different types of bacteria was also calculated.

2.4 Radiation response of the isolates

Bacterial isolates were tested for their resistance to different doses of gamma radiation. The bacterial cultures were grown in nutrient broth to a final density of about 10⁸ cells per ml. The cells were suspended in saline water and were exposed to 1-10 kGy gamma radiation at dose rate of 5.84 kGy/hr. ⁶⁰Co gamma irradiator of Institute of Food and Radiation Biology (IFRB), at Atomic Energy Research Establishment (AERE), Savar, Dhaka was used as gamma source. For each irradiation dose, bacterial colony was counted before and after gamma irradiation. Survival fraction (S) of bacteria, for each radiation dose, was calculated by dividing the number of viable cells after irradiation (N) by the initial viable cell number (N₀). Survival curve of each group of bacteria was obtained by plotting log S versus irradiation doses. D₁₀ values were calculated according to the equation: D₁₀ value= D/ (log₁₀ N₀-log₁₀ N), Where D = Radiation dose, N₀ = Untreated viable cell number, N= Irradiated viable cell number.

III. Results

All the studied bone samples (twenty five) were contaminated by bacteria. Bacterial count varied from 4.1×10¹cfu/g to 3.1×10³ cfu/g (average 9.0×10² cfu/g). Types of bacterial contaminants are presented in Table-1. Most of the isolates were gram-positive cocci (82.22%). Gram-negative bacteria were not found. Forty six bacterial isolates from the bone samples were characterized based on their morphological, cultural character and biochemical tests. Most frequently found bacterial contaminant was staphylococci (41.30%) and streptococci (36.95%). Eight gram positive bacilli (B6, B24, B25, B29, B30 and B38-B40) were identified as *Bacillus* spp. Among the thirty eight gram positive cocci, nineteen isolates (B4, B8-B10, B13, B18-B21, B27, B34-B37, B42, B43, B46, B47 and B50) were identified as *Staphylococcus* spp and two isolates (B15 and B41) were *Micrococcus* spp. Seventeen catalase negative cocci (B2, B3, B5, B7, B11, B12, B14, B17, B22, B23, B28, B31-B33, B44, B45 and B48) were identified as *Streptococcus* spp.

Table-1: Type of bacterial contaminants in bone samples

| Microorganism | Isolation frequency Number (%) |
|----------------------------|-----------------------------------|
| Gram-negative cocci | Nil |
| Gram-negative rod | Nil |
| <i>Bacillus</i> spp. | 8 (17.39) |
| <i>Staphylococcus</i> spp. | 19 (41.30) |
| <i>Micrococcus</i> spp. | 2 (4.35) |
| <i>Streptococcus</i> spp. | 17 (36.95) |

All the forty six bacterial isolates, comprising 4 genera, were screened for radiation resistance with ⁶⁰Co gamma rays at ambient temperature (25 ± 2°C) in normal saline and percentage of bacteria survived at different doses of gamma irradiation were calculated (Table-2). Among the 46 bacterial isolates, 45 (97.82%) isolates were survived up to 5 kGy, 31 (67.39%) isolates were survived up to 6 kGy, 21 (45.65%) isolates were survived up to 7 kGy and 3 (6.52%) isolates were survived up to 8 kGy gamma irradiation. All the bacterial contaminants were

completely eliminated after exposure to 9 kGy gamma irradiation. *Streptococcus* spp were the most radioresistant bacteria and three isolates (B2, B7 and B45) of this genus were survived up to 8 kGy. Isolate B9, which belonged to the genus *Staphylococcus*, was the most radiosensitive bacteria and was killed by 5 kGy gamma irradiation.

Table-2: Radiation response of bacterial isolates

| Type of bacteria | Total number of isolates | Survive up to radiation dose (kGy) | | | | | |
|----------------------------|--------------------------|------------------------------------|---------------|---------------|---------------|--------------|--------------|
| | | 4 | 5 | 6 | 7 | 8 | 9 |
| <i>Bacillus</i> spp. | 8 | 8 | 8 | 8 | 4 | Nil | Nil |
| <i>Staphylococcus</i> spp. | 19 | 19 | 18 | 5 | Nil | Nil | Nil |
| <i>Micrococcus</i> spp. | 2 | 2 | 2 | 1 | Nil | Nil | Nil |
| <i>Streptococcus</i> spp. | 17 | 17 | 17 | 17 | 17 | 3 | Nil |
| Total | 46 | 46 | 45 | 31 | 21 | 3 | Nil |
| % Survival | | 100% | 97.82% | 67.39% | 45.65% | 6.52% | 0.00% |

Survival curves for bacterial isolates following exposure to several doses of gamma radiation were obtained by plotting the logarithm of survival fractions ($\log_{10} S$) versus radiation doses (kGy). $\log_{10} S$ values of the isolates at each data point were very close, so overlapping lines were obtained in the survival curve (Fig.1-4). The radiation decimal reduction dose values (D_{10} value) and twelve log reduction values (12 D value) of the isolates are presented in Table-3. D_{10} values of *Bacillus* spp. were ranged from 0.82-0.98 kGy. In case of *Staphylococcus* spp. D_{10} values were in the range of 0.59-0.89 kGy. D_{10} values of *Micrococcus* spp. were 0.70 and 0.86 kGy. Relatively higher D_{10} values (0.93-1.20 kGy) were recorded for *Streptococcus* spp. The lowest D_{10} value (0.59 kGy) was recorded for the isolate B9 (*Staphylococcus* sp.) and the isolate B7 (*Streptococcus* sp.) was the most resistant isolate with a D_{10} value of 1.20 kGy. To achieve sterility assurance level (SAL) 10^{-6} , twelve log reduction of the contaminants is required for tissue samples contaminated with 10^6 cfu per gram. Therefore, 12 D-value of the isolates were also calculated. 12 D value of the bacterial isolates was 7.08 to 14.44 kGy (Table-3).

Table 3: D_{10} values and 12 D values of bacterial isolates

| Bacteria | D_{10} value (kGy) | 12 D value (kGy) |
|----------------------------|----------------------|------------------|
| <i>Bacillus</i> spp. | 0.82-0.98 | 9.84-11.76 |
| <i>Micrococcus</i> spp. | 0.70-0.86 | 8.40-10.32 |
| <i>Staphylococcus</i> spp. | 0.59-0.89 | 7.08-10.68 |
| <i>Streptococcus</i> spp. | 0.93-1.20 | 11.16-14.44 |

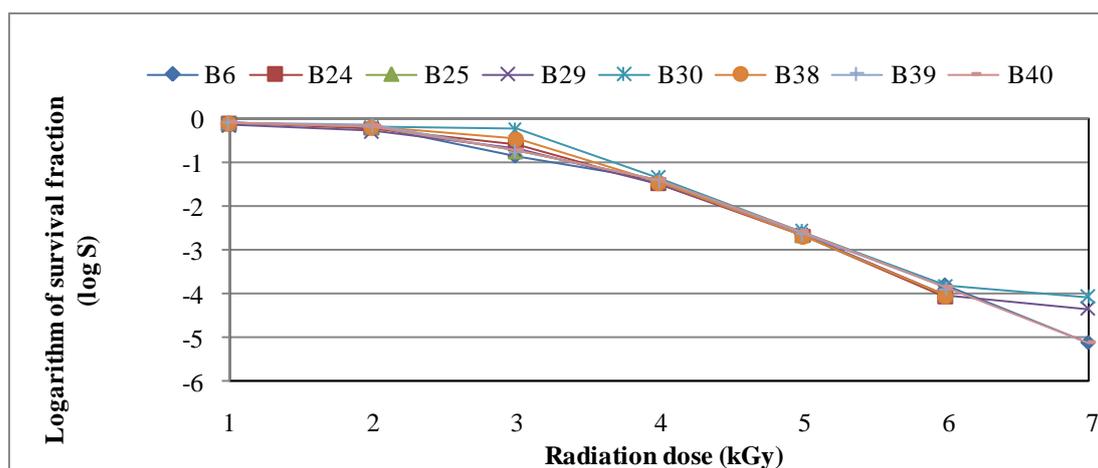


Fig-1: Survival curve of eight isolates (B6, B24, B25, B29, B30 and B38-B40) of *Bacillus* spp. Four isolates (B24, B25, B38 and B39) survived up to 6 kGy and another four (B6, B29, B30 and B40) survived up to 7 kGy.

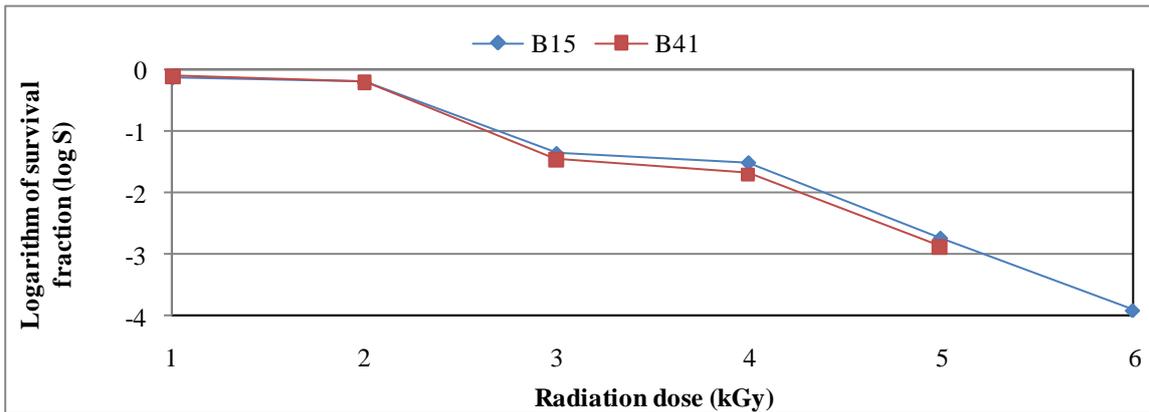


Fig.-2: Survival curve of two isolates (B15 and B41) of *Micrococcus* spp. Isolate B41 survived up to 5 kGy and B15 survived up to 6 kGy.

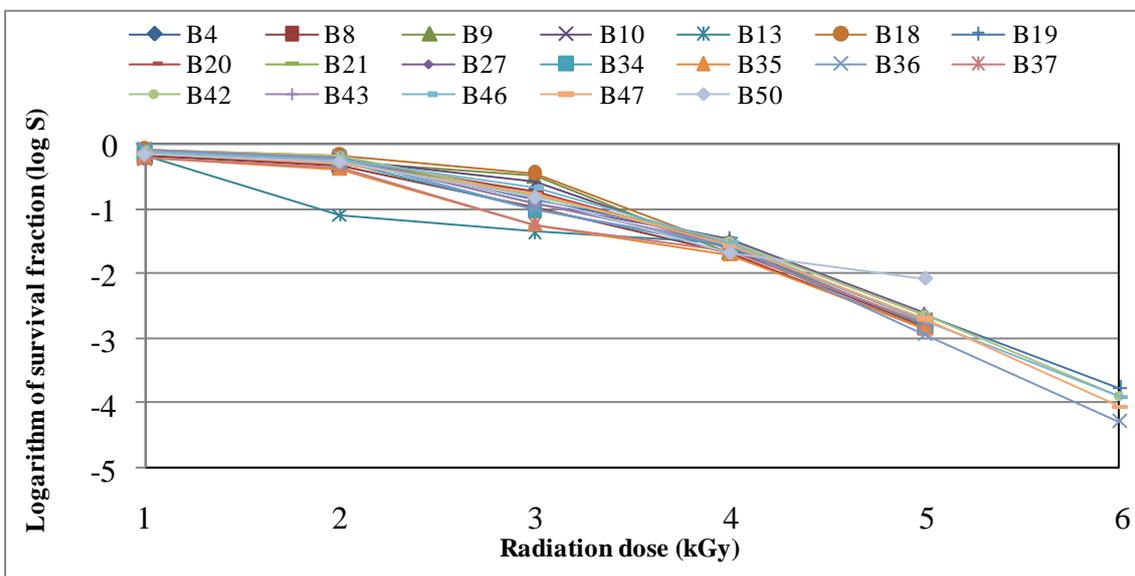


Fig.-3: Survival curve of nineteen isolates (B4, B8-B10, B13, B18-B21, B27, B34-B37, B42, B43, B46, B47 and B50) of *Staphylococcus* spp. One isolate (B9) survived up to 4 kGy, thirteen ((B4, B8, B10, B13, B18, B20, B21, B27, B34, B35, B37, B43 and B50) survived up to 5 kGy and five (B19, B36, B42 and B47) survived up to 6 kGy.

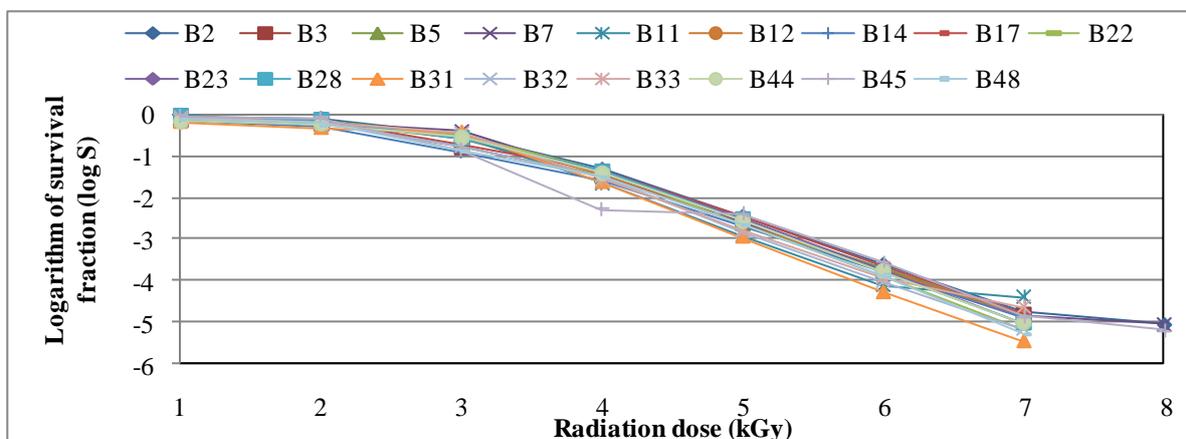


Fig.-4: Survival curve of seventeen isolates (B2, B3, B5, B7, B11, B12, B14, B17, B22, B23, B28, B31-B33, B44, B45 and B48) of *Streptococcus* spp. Fourteen isolates (B3, B5, B11, B12, B14, B17, B22, B23, B28, B31-B33, B44 and B48) survived up to 7 kGy and three isolates (B2, B7 and B45) survived up to 8 kGy.

IV. Discussion

Transmission of diseases or infections from the donor to the recipient is always a risk in allograft transplantation. Many authors reported about infection following allograft implantation [7, 12, 16, and 17]. Wang et al. [18] reported that approximately one-half of the infections associated with human tissue transplants were due to bacterial agents and of that, 90% were aerobic organisms. The best way to avoid infection or diminish its incidence is careful donor selection and the application of sterile techniques in tissue procurement, processing and storage. Varetas and Taylor [19] considered bioburden assessment as a small but integral part of the allograft bone processing, which combine with other good tissue banking practices would provide safe allograft bone for transplantation. Therefore, our focus was on the assessment of bacterial load associated with unprocessed human bone and determination of radiation response of the isolates.

All the bone samples used in this experiment were collected from seronegative (HIV, HBV, and VDRL) donor and were procured under aseptic condition. In spite of following good tissue banking practice, donor tissues were not sterile. Average bacterial count was 9.0×10^2 cfu/g (ranged from 4.1×10^1 to 3.1×10^3 cfu/g). Many authors [19-21] had reported different rate of contamination in bone tissues. In our study, most of bone samples were contaminated with *Staphylococcus* spp. *Streptococcus* spp. was the second highest contaminant. Ibrahim et al. [24] also reported coagulase negative staphylococci as major contaminant in bone allografts and twelve of their bone allografts were contaminated with streptococci. In many studies, bacterial contaminant in bone tissues was predominantly skin flora [19-23]. Though the isolates found in this study are comparable with the previous reported studies, it was difficult to determine the source of contamination in these tissue samples. Microbial contamination may arise from the donor at the time of retrieval, or from the environment and the personnel during processing [25]. Deijkers et al. [26] divided contaminating organisms into low and high pathogenicity. They considered organisms of low pathogenicity to be skin commensals and organisms of high pathogenicity were thought to be originated from endogenous sources in the donor, which more likely to cause infection in the recipient.

Generally, 25 kGy gamma irradiation is used for terminal sterilization of tissue allografts. As gamma radiation adversely affects the mechanical and biological properties of bone allografts, some tissue bank prefer lower dose without compromising a SAL of 10^{-6} . Baker et al. [27] has found that sterility (10^{-6} SAL) of tissue allograft could be achieved using at least 9.2 kGy gamma radiation, and in another study 15 kGy gamma radiation was substantiated as radiation sterilization dose (RSD) for frozen bone allografts [28]. Radiation dose necessary to achieve SAL of 10^{-6} depends on the initial microbial contamination level and the radiation resistance of the contaminant. To observe the radiation effects, bacterial isolates were exposed to different doses of gamma radiation and D_{10} values was calculated in this study. We found that, D_{10} values of bacterial isolates ranged from 0.57 to 1.2 kGy. *Streptococcus* spp. was the most radioresistant isolates (D_{10} value 0.93-1.2 kGy) and three of the *Streptococcus* spp. survived up to 8 kGy. All the bacterial isolates were killed at 9 kGy.

In a sterilization process, the nature of microbiological death or reduction is described by an exponential function. Therefore, the number of microorganisms which survive in a sterilization process can be expressed in terms of probability. While the probability may be reduced to a very low number, it can never be reduced to zero. For the sterilization of medical and pharmaceutical product, maximum probability of a single nonsterile unit in 10^6 units is widely accepted. Therefore, if the bioburden on an article were one million, 12 log reduction of the initial bioburden is required to achieve a 10^{-6} probability of a nonsterile unit. Though the initial bioburden of the studied bone samples ranged from 4.1×10^1 to 3.1×10^3 cfu/g, to ensure patients safety, 12 log reduction was calculated. In this study, twelve log reduction value (12D value) of the most resistant isolate was 14.4 kGy. From these findings, it is clear that the standard sterilization dose of 25 kGy gamma radiation was adequate for the sterilization of these tissue allografts and a lower dose might also be acceptable. To determine lower radiation sterilization dose (RSD), further study may therefore be suggested using radioresistant reference strain.

V. Conclusion

Microbial contamination in musculoskeletal tissue graft is a serious concern in rehabilitative surgery. To minimize the chance of contamination, in addition to select seronegative donor, good tissue banking practice should be implemented in tissue procurement, processing and storage. However, aseptic processing practices can reduce but not eliminate all microbial contaminants of tissue. To prevent the risk of diseases transmission from donor origin, terminal sterilization is an integral part of allograft preparation. The choice of RSD depends on the microbial contamination level and the radiation response of the contaminant. In our study, average bioburden was 9.0×10^2 cfu/g and maximum radiation resistance (D_{10} value) was 1.20 kGy and 12 D value of the most resistant isolate was 14.4 kGy. Based on the average bioburden and radiation response of the contaminants, 25 kGy gamma radiation was found to be ample to ensure sterility of cancellous bone allografts.

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