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Gamma Irradiated Bone Allografts Processed from Femoral Heads

Rita Singh[1,*], Durgeshwer Singh[2]

[1]Radiation Dosimetry and Processing Group, Defence Laboratory,
[2]Defence Research and Development Organization, Jodhpur, 342011, India

Abstract Disease transmission and bacterial infection in bone allograft transplantation is of significant concern. Screening of donor for disease, bacterial testing and aseptic processing, substantially reduce risk, but do not completely eliminate the possibility of allograft associated infections. Sterilization by gamma radiation is a definitive method for eliminating microorganisms and can prevent life-threatening allograft associated infections. In the present study, microbiological evaluation of bone allografts processed from femoral heads excised during surgery was carried out. 126 femoral heads obtained from living donors were processed, freeze-dried and sterilized by gamma irradiation at 25 kGy. The bioburden and type of microbial contamination associated with bone allografts was determined based on morphological characteristics and biochemical tests. Resistance of bacterial contaminants to gamma radiation was evaluated by exposing the bacterial cell suspension to different doses of 1 to 6 kGy and analysing for the surviving bacteria. Average bioburden of processed bone allografts for different batches was found to be in the range of 1.84×10^2 to 3.88×10^3 CFU/g. 60.2% of the isolates were found to be Gram-positive organisms. The D_{10} value of bacterial isolates ranged from 0.56 to 1.68 kGy. Verification doses for different batches of processing were 5.87 to 9.46 kGy. All bone grafts exposed to the verification dose were tested culture negative. The results validate 25kGy dose for sterilization of bone allografts processed from femoral heads.

Keywords Bone Allografts, Femoral Head, Bioburden, Sterilization, Gamma Radiation

1. Introduction

Bone grafts are frequently used to revise skeletal defects by replacement or augmentation. Autografts are the most effective as they are osteoconductive as well as osteoinductive and have osteogenic cells. However, harvesting bone requires an additional incision, increasing operating time, blood loss as well as costs. There is also a significant morbidity related to the donor site. Major complications, such as cutaneous nerve damage, chronic donor site pain, vascular injury, infection and fracture are reported in autografted patients[1]. This morbidity is in direct proportion to the quantity of graft retrieved. Several studies have reported minor and major complications, with a wide range of incidences varying between 1% and 39%[2]. By eliminating the need for an additional surgical procedure, allografts reduce the operating time, expense and trauma associated with the acquisition of autografts. Further since allografts do not compromise normal structures, they avoid the significant morbidity associated with the recovery of autologous bone graft. Allografts have the added advantage of being available in large quantity. This is particularly valuable in large defects or in children where the quantity of available autografts is limited.

Bone allografts fill an important void in the surgical practice of orthopaedic surgery, and their use to replace and reconstruct musculoskeletal structures following injury or disease has gained increasing acceptance by orthopaedic surgeons[3]. Allogenic bone grafts are widely used in a variety of clinical situations. These include filling of cavities in benign tumorous conditions and infections, bridging of osteoperiosteal defects following trauma, infections or enbloc resection of malignant tumors and reinforcement of host bone prior to implantation of prosthesis. Bone allografts can improve substantially the quality of life for many patients. However, problem of bone allograft as implant material is mostly infectious disease transmission from donor to recipient.

Infections associated with contamination of allografts can result in serious morbidity and death. Bacterial transmission may occur from infected donor to recipient such as tuberculosis and syphilis or through bacterial contamination during procurement, processing and storage of the bone allograft. Viral transmission may also come from infected donor such as HIV and Hepatitis. Hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), and human T-lymphotropic virus (HTLV) have all

*Corresponding author:
singhritadr@yahoo.com (Rita Singh)
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been transmitted by tissue transplantation[4]. The estimated incidence of viremia at the time of donation is 1 in 55,000 for HBV, 1 in 34,000 for HCV, 1 in 42,000 for HIV, and 1 in 128,000 for HTLV[5]. Screening of donor for disease, bacterial testing and aseptic processing, substantially reduce risk, but do not completely eliminate the possibility of allograft associated infections.

Sterilization is a definitive method for eliminating microorganisms and can prevent life-threatening allograft associated infections[6]. Gamma radiation is used at commercial scale to sterilize healthcare products. Radiation process is a cold sterilization and is the preferred method for sterilization of biological tissues because of the several advantageous factors[7]. One of the principal advantages of radiation sterilization arises from its ability to destroy contaminating microorganisms with an insignificant rise in the temperature of the irradiated materials, thereby preserving the properties and characteristics of tissues. Gamma irradiation sterilization has been proven to eliminate viruses, bacteria, fungi and spores from tissue without affecting the structural or biomechanical attributes of tissue grafts[8]. The efficacy of allograft sterilization is supported by the absence of bacterial or viral allograft-associated infections in tissue processed by this method[9].

The behaviour of the microbial population on exposure to ionizing radiation is of greatest relevance in radiation sterilization practice. Since the destruction of microorganisms by gamma irradiation follows an exponential rule, the probability of survival is a function of the number and species of microorganisms present on the allograft and the lethality of the gamma irradiation process. The level of viable microorganisms on the product before sterilization and the radiation resistance of the contaminants determine the dose required for sterilization. The present study was thus carried out with the aim of microbiological evaluation of bone allografts processed from femoral heads and sterilized by gamma radiation. The incidence of femoral head microbial contamination and the efficacy of gamma radiation for allograft sterilization was examined. The type and magnitude of microbial contamination associated with bone allografts and their resistance to gamma radiation was evaluated. Sterilization of bone allografts using gamma irradiation was validated.

2. Materials and Methods

2.1. Procurement of Bone

Femoral heads excised during surgery were obtained from living donors after necessary consent. Donor screening was carried out to exclude infectious diseases of bacterial or viral origin including HIV and Hepatitis, neurological disorders and diseases of unknown origin. The femoral heads were stored in an ultra low temperature freezer at -80^{0}C until processing. The records were kept for the donors and the femoral head received.

2.2. Processing of Bone

126 femoral heads were processed in 6 different batches. Femoral heads were dissected using surgical instruments to remove soft tissues. Dissected femoral heads were then cut into different forms like chips and cubes according to the surgeons requirements. The cut bones were washed to remove bone marrow and blood remains and pasteurized in a water bath at 58^{0}C. The bone allografts were frozen at -80^{0}C and subsequently freeze-dried to remove 95% of the moisture. The freeze-dried grafts were double packed and sealed in polyethylene packets, in a laminar airflow biosafety cabinet.

2.3. Radiation Sterilization

Processed bone grafts were sterilized by exposure to 25 kGy of ^{60}Co gamma rays. Irradiation was carried out in the Gamma chamber GC-5000 at dose rate of 6.175 kGy/h. The freeze-dried irradiated grafts were stored at room temperature.

2.4. Bioburden Estimation

Bone allografts from 6 batches of processing were checked for the bioburden. 10 random samples of bone allografts from each batch were selected and weighed. The samples were shaken in 0.01% saline polysorbate for 30 min. The solution was filtered through Millipore membrane filters. The filters were placed on soyabean casein digest medium and thioglycollate medium and incubated. The plates were observed for growth up to 7 days. Counts were calculated per gram of bone.

2.5. Isolation and Characterization of Microbial Contaminants

Representative types of bacteria were isolated from different batches of bone during processing. Bacterial cultures were successively re-isolated on nutrient agar to obtain pure cultures. A total of 93 bacteria were isolated and maintained. Occurrence of different types of bacteria on bone was determined. The bacteria were characterized with reference to their gram-staining and their morphology as cocci and bacilli. The percentage occurrence of different morphological types was calculated. Preliminary identification of 6 bacterial isolates was carried out on the basis of various morphological, cultural and biochemical characteristics by standard methods[10].

2.6. Determination of Radiation Resistance of Bacterial Isolates

6 representative bacterial isolates from bone identified as *Bacillus, Clostridium, Staphylococcus, Enterococcus, Pseudomonas* and *Klebsiella* 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 1x10^8 colony-forming units (CFU)/ml. The cells were suspended in phosphate buffer and exposed to different doses of 1, 2, 3, 4,

5 and 6 kGy. Thereafter, plating was carried out after making appropriate dilutions. For each irradiation dose, the survival fraction (S) was estimated dividing the number of viable cells after irradiation (N) by the initial viable cell number (No). Survival curves relating log S with irradiation dose in kGy were obtained for all the strains. The radiation decimal reduction dose values (D_{10}) for the strains were obtained from the gradient of the linear portion of the inactivation curves.

2.7. Validation of Radiation Sterilization

Verification dose experiment was carried out according to ISO 13409[11]. Test sample size was selected as per batch size. For production batch with uniformed samples between 20 and 79, 20 samples are selected for experiment. 10 samples were used for bioburden determination and 10 samples for verification dose experiment.

2.8. Determination of Sterility

10 random samples of bone from each of the 6 batches exposed to verification dose were tested for sterility. Samples were aseptically transferred to saline blank containing 0.01% polysorbate and shaken for 30 min. The solution was passed through the sterile membrane filters and the filters were placed on soyabean casein digest agar plates. The plates were incubated at 30 ± 2^0C and observed for growth up to 14 days.

3. Results and Discussion

3.1. Processing of Femoral Heads as Bone Allografts

Femoral heads removed during hip replacement surgery are the most common source of allograft bone. 126 femoral heads excised during surgery were included in the present study. These included 61 male and 65 female donors. Number of donors in relation to age is presented in Table 1. Since the bones from femoral heads are processed for filling cavities or bone gaps and not for structural purposes, the donors from all age groups were included. Maximum numbers of donors were above the age of 50 years. 40 donors were in the age group 71-80 years followed by 28 in the age group 51-60 years and 26 in the age group 61-70 years. There was only one donor below 20 years.

The repair of bony defects resulting from trauma or disease remains a major problem in trauma and orthopaedic surgery. Autologous bone grafts, though ideal, have the drawback of secondary surgery for autograft retrieval, complications of infection and donor site morbidity. Autologous bone is most commonly harvested from the iliac crest[12] or greater trochanteric region[13]. There is a substantial incidence of morbidity associated with the harvest procedure. Reported complications of harvest of the iliac crest bone graft include deep infection, osteomyelitis, haematoma, neurological injury, vascular injury, iatrogenic iliac wing or sacroiliac joint injury, persistent pain and cosmetic defects[1]. In particular, residual pain has been reported to occur in as much as 31% of the cases. Harvest of autologous bone from the greater trochanteric region may decrease bone strength of the donor site and increase risk of proximal femur fracture, which is a devastating complication for the patients[13]. Bone allografts obviate these difficulties and once replaced by invading osteoblasts can be as good as the patient's own bone. Allografts also offer many advantages compared to metallic implants including joint reconstruction, incorporation of the graft to the host bone, and longevity. Synthetic biomaterials may be rejected if infected and can also cause erosion of underlying bone, as well as can extrude by eroding soft tissues. An allograft presents a more biological approach. However, extensive use of bone allografts has been limited due to the availability of safe and reliable processed bones.

Table 1. Number of Donors in Relation to Age

No.	Age (years)	Number of Donors	
		Male	Female
1.	< 20	0	1
2.	21 – 30	1	1
3.	31 – 40	4	0
4.	41 – 50	9	8
5.	51 – 60	15	13
6.	61 – 70	10	16
7.	71 – 80	18	22
8.	81 – 90	4	4

In the present study, freeze-dried bone allografts were processed from femoral heads that were obtained from living donors. Bone allografts are generally required to have no immunogenicity, possess good osteogenesis potential, maintain sufficient strength until incorporation, and not transmit a disease. Fresh allografts are less frequently used than processed allografts. The host immune response to freeze-dried allografts is less intensive than the response to fresh or fresh-frozen allografts[3]. Antigenicity of bone is believed to decrease or disappear as a result of the destruction of cellular membrane in freezing or freeze drying. Allografts are primarily osteoconductive, but they retain a variable number of osteoinductive proteins. The freeze-dried, irradiated bone acts as a scaffold for deposition of new bone by the host bed. New bone is formed by osteoconduction, a process in which mesenchymal cells migrating from the recipient site together with new capillaries grow into the grafted bone. This leads to a slow process of creeping substitution of the graft. The ideal allograft incorporation involves the envelopment of the necrotic graft by the new host bone containing a remodeling unit consisting of haematopoietic cells and osteoblasts. Allograft integration takes place through ingrowth (creeping substitution) or apposition of new host bone[14]. This requires optimal osteoclast-mediated bone resorption as well as bone formation. Human bone allografts have been proved

clinically to be a viable alternative to autografts.

3.2. Bioburden Assessment and Characterization of Microbial Contaminants

Microbiological evaluation of bone allografts processed from femoral heads was carried out. The bioburden of 10 random bone samples from each batch was assessed. Microbial load of bone allografts from 6 different batches of processing is presented in Figure 1. The counts for the different batches ranged from 0.98 to 3.87 log CFU/g. Maximum counts were recorded for the fourth batch of processing which ranged from 3.04 to 3.87 log CFU/g. Lowest microbial level of 1.09 to 2.49 log CFU/g were observed for the first batch. Type of microbial contaminants associated with bone allografts was determined. Ninety three bacterial isolates were obtained from different batches of bone processed. The bacterial isolates from different bone samples were categorized based on their morphology and Gram staining. Percent occurrence of different morphological types is presented in Figure 2. 41.9% of the isolates were found to be Gram-positive bacilli and 36.6% were found to be Gram-negative bacilli. 18.3% of the isolates detected were Gram-positive cocci followed by 3.2% Gram-negative cocci. 6 representative bacterial contaminants from different batches were identified based on biochemical tests. Four Gram-positive bacteria were identified as *Bacillus, Clostridium, Staphylococcus* and *Enterococcus*. Two Gram-negative isolates were *Pseudomonas* and *Klebsiella*.

Microbiological analyses for aerobic bacteria, anaerobic bacteria and fungal contamination of bone allografts processed from femoral heads were carried out. Contaminant microbes consisted of mostly aerobic bacteria. No yeasts and molds were found. Other studies[15-17] have also reported a range of microorganisms isolated from femoral head bone retrieved from living donors during surgery.

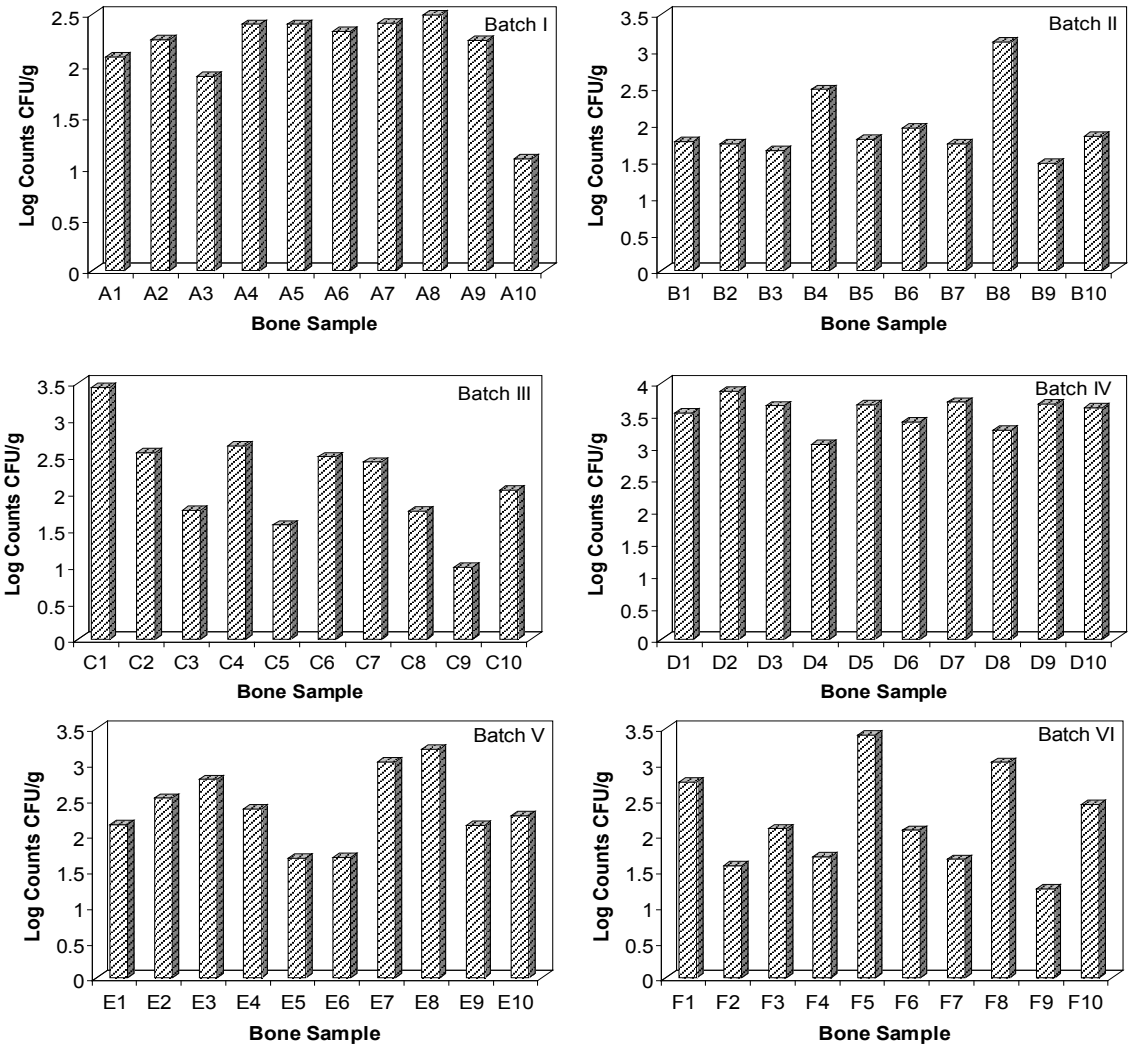

Figure 1. Microbial load of bone allografts from different batches of processing

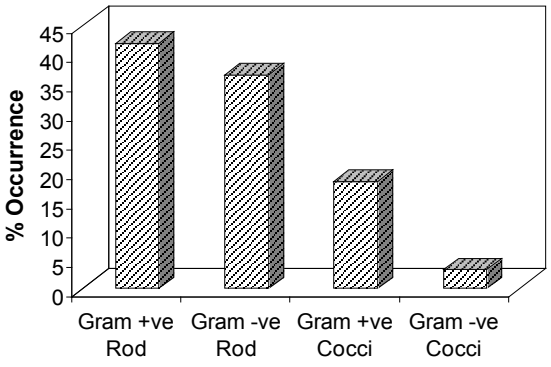

Figure 2. Type of microbial contamination in bone

The greatest numbers of isolates reported are Gram-positive cocci, predominantly coagulase-negative staphylococci. The second group most frequently isolated was Gram-positive bacilli, predominantly diphtheroids. Varettas and Taylor[18] have reported coagulase-negative staphylococci as the predominant organism isolated from femoral head allografts of living donors. However, organisms such as *Clostridium* have become particularly important following report by Malinin et al.[19] who showed a significant number of clostridial contamination in musculoskeletal allografts. Deijkers et al.[20] analysed the bacterial contamination of bone allografts retrieved under sterile operating conditions. Organisms of low pathogenicity were cultured from 50% of the grafts and of high pathogenicity from 3%. The major source of contamination was exogenous and strongly influenced by the procurement team. Most organisms of low pathogenicity are skin commensals (e.g. coagulase-negative staphylococci, *Corynebacterium* species and *Propionibacterium acnes*) and probably represent external contamination at the time of procurement. Organisms of high pathogenicity probably originate from endogenous sources in the donor. They are usually contaminants from the upper respiratory or gastrointestinal tract (e.g. Streptococci, *Staphylococcus aureus* and *Escherichia coli*) and are more likely to cause infection in the recipient of the allograft[29]. Dennis et al.[21] have reported *Propionibacterium*, coagulase-negative *Staphylococcus*, *Pseudomonas aeruginosa, Klebsiella oxytoca, Lactobacillus* species, *Peptostreptococcus asaccharolyticus* and *Streptococcus sanguinis* as the most frequently cultured organisms from the musculoskeletal allograft tissues. As in other studies[15, 16] the organisms isolated from this study were predominantly skin flora. In living donors, contamination with regard to incidence and type of microorganisms is similar to that observed in surgical theatres during routine surgery[22].

Bone allografts are a vital option for skeletal insufficiency in trauma, joint reconstruction, musculoskeletal tumors, or other reconstructive procedures. Microbial contamination of bone allografts can be a serious cause of morbidity and mortality in recipients. Reported cases of fatal and nonfatal bacterial infections in recipients of contaminated allografts have called attention to the importance of avoiding tissue donors suspected of carrying infectious disease, of not processing donated tissue carrying virulent bacteria, the occurrence of falsely negative final sterility tests, and the need to sterilize tissues. Contamination can arise from an infected donor, during tissue removal, from the processing environment, and from contaminated supplies and reagents used during processing. Stringent allograft handling procedures are recommended to minimize the chances of contamination. Final sterility testing can be unreliable, especially when antibiotics remain on tissues. Aseptic processing practices can reduce but not eliminate microbial contamination of tissue. Bone allograft material must be treated with sterilization methods to prevent the transmission of diseases from the donor to the recipient. Sterilization of tissue allografts using gamma radiation has been suggested as an answer to concerns about sterility. Microbial load play a key role in the practical application of radiation sterilization technology. The chance of one organism surviving after irradiation decreases logarithmically with increasing dosages. However, it is important to consider microbial population characteristics that define a products pre-sterilization bioburden. Relevant characteristics include the magnitude of the population and the resistance of the population to radiation. Microbiological assessment is thus an important part of the quality system and plays a key role in achieving sterility.

3.3. Radiation Resistance of Bacterial Isolates

Sterility is a dose dependent measure of probability which is determined by the initial microbial concentration, the radiation dosage administered and the unique radioresistance of a given organism. Representative bacterial isolates characterized from bone were tested for their resistance to gamma radiation. Survival curves (log N/N_0) for the *Bacillus, Clostridium, Staphylococcus, Enterococcus, Pseudomonas* and *Klebsiella* at different doses of gamma radiation are presented in Figure 3.

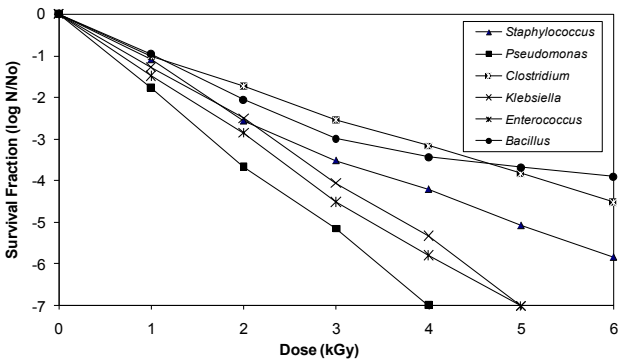

Figure 3. Effect of gamma radiation on bacterial isolates from bone

Pseudomonas was found to be the most sensitive and no viable counts were detected on gamma irradiation at 4 kGy. *Bacillus, Clostridium and Staphylococcus* had higher resistance to gamma radiation and complete inactivation was not observed at 6 kGy. The radiation resistance of a

microorganism is measured by the decimal reduction dose (D_{10} value), which is defined as the radiation dose (kGy) required to reduce the number of that microorganism by 10-fold (one log cycle) or required to kill 90% of the total number. The D_{10} value of the contaminants is presented in Table 2. The D_{10} value of Gram-positive isolates ranged from 0.63±0.06 kGy to 1.68±0.16 kGy. Lower D_{10} values of 0.56±0.01 and 0.64±0.03 kGy were recorded for Gram-negative *Pseudomonas* and *Klebsiella*.

Bacterial infection is a severe potential complication of bone allograft contamination. Post-allograft transplantation infections have been documented with unirradiated allograft[9]. To minimise the risk of this complication, gamma irradiation of all bone at 25 kGy prior to transplantation is performed. However, the amount of absorbed radiation energy required to inactivate the microorganism in a product depends on its resistance to radiation. Microorganisms differ greatly in their resistance to ionizing radiation. There are differences in resistance from species to species, and even among strains of the same species. Gram-positive microbes particularly *Bacillus* sp., *Enterococcus* sp. and *Deinococcus* sp. are reported to be the most radiation-resistance microbes. Gram-negative bacilli are usually much more sensitive to gamma radiation than Gram-positive[23]. Variation in the lipid content of cell wall of Gram-positive and Gram-negative bacteria may be responsible for the variation in the radiation sensitivity. It is also generally believed that the more sensitive organisms cannot repair double strand breaks in DNA and that many showing greater resistance are likely to have capacity to repair double strand breaks in DNA.

Table 2. D_{10} values of bacterial isolates from femoral heads

Bacteria	D_{10} Value (kGy)
Bacillus	1.68±0.16
Clostridium	1.36±0.03
Enterococcus	0.63±0.06
Klebsiella	0.64±0.03
Pseudomonas	0.56±0.01
Staphylococcus	1.03±0.26

3.4. Validation of Radiation Sterilization

Validation of radiation sterilization dose of 25 kGy for bone allografts was carried out according to ISO 13409[11]. The number of uniformed samples tested for validation per production batch size according to ISO standard was 20. 10 samples from each batch were used for bioburden determination and 10 for sterility test at verification dose. Average bioburden of freeze-dried bone grafts for different batches was found to be in the range of 1.84×10^2 to 3.88×10^3 CFU/g (Table 3). Verification doses obtained were 5.87 to 9.46 kGy. 10 samples from each batch were exposed to the verification dose and tested for sterility. According to ISO 13409, if during the sterility test of samples which are irradiated at the verification dose, positive growth is found in one sample or less after incubation for 14 days at 30^0C, the completed batch can be sterilized at 25 kGy. Sterility test results show that no positive growth was observed for 6 different batches of bone allografts processed from femoral heads. Based on ISO 13409, the results of verification dose are accepted and the radiation sterilization dose of 25 kGy is substantiated.

Table 3. Average bioburden and verification dose of processed bone allografts

Batch	Average bioburden (CFU/g)	Verification dose (kGy)
I	1.84×10^2	5.87
II	2.03×10^2	5.99
III	4.38×10^2	6.90
IV	3.88×10^3	9.46
V	1.39×10^3	8.27
VI	4.60×10^2	6.96

Allograft usage among orthopaedic surgeons has risen dramatically over the past two decades, resulting in impressive life-enhancing benefits. However, tissue safety is a major concern in transplantation. The transmission of infectious agents from donor to recipient with allografts is their major risk and disadvantage. The presence of virulent microorganisms in the bone allografts can lead to potentially serious complications for graft recipients. Infection following allograft implantation has been reported by many[24, 25]. Safety issues regarding the transmission of biological infections via allograft transplantation are of critical concern to both surgeons and tissue recipients. Adequate donor screening coupled with appropriate processing are employed to reduce the risk of disease transmission. Even with adequate donor screening, there remains a risk of allograft contamination. The specific problem with aseptic processing is that it only minimizes bacteria without eradicating organisms and spores, especially in tissue that is heavily contaminated at the time of recovery. Antibiotic/antifungal solutions also do not eliminate spores of organisms such as *Clostridium* spp.[9]. Terminal sterilization of bones that does not adversely affect the functioning of tissue when transplanted into patients is the best way to eliminate the risk for allograft-associated infections. Ethylene oxide gas and gamma radiation have historically been employed to terminally sterilize the tissue prior to distribution. The use of ethylene oxide gas sterilization has fallen out of favour as residual gas concentration is a problem. Toxic effects of ethylene oxide as an industrial fumigant, and the removal of residual gases from bone grafts has been a major concern for ethylene oxide-sterilized allografts. Ethylene oxide has been associated with adverse outcomes such as synovitis or damage to musculoskeletal tissue, resulting in an unacceptably high rate of mechanical failure[26]. Gamma radiation has been established as a technique for inactivating bacteria, fungal spores, and viruses[27,28], and thus has

become a popular sterilization technique for allografts. Gamma irradiation can eliminate the danger of disease transmission through allografts terminal sterilization. The virucidal and bactericidal effects of gamma irradiation are created by two mechanisms[29]. The primary mechanism is direct alteration of nucleic acids leading to genome dysfunction and destruction. A secondary mechanism is the generation of free radicals. Gamma radiation can affect DNA directly, by energy deposition in this critical target, or indirectly, by the interaction of radiation with other atoms or molecules in the cell or surrounding the cell. In particular, radiation interacts with water, leading to the formation of free radicals (hydrogen atoms, hydroxyl radical and solvated electron) that can diffuse far enough to reach and damage DNA. The destruction of microorganisms by gamma radiation follows an exponential law. The probability of survival is a function of the number and types (species) of microorganisms present on the product (bioburden). Sterility assurance level (SAL) defines the probability of a viable microorganism being present on an individual product unit after sterilization. Sterilization is intended to provide the required or desired sterility assurance level. The bone allografts must receive a sterilization dose high enough to ensure that the probability of an organism surviving the dosage is no greater than one in one million units tested (10^{-6}). The sterilization process must be validated to verify that it effectively and reliably kills any microorganisms that may be present on the presterilized allograft. In the present study, sterilization of bone grafts, using gamma irradiation at 25 kGy was validated. Therefore, the probability of a viable microorganism being present on an allograft post-gamma irradiation is one in a million at the sterilization dose of 25 kGy. Exposure to a validated sterilization process ensures that the bone allografts are sterile and safe for clinical use.

4. Conclusions

Bone allografts processed from femoral heads were found to be contaminated and about 60% of the isolates were found to be Gram-positive bacteria. Maximum radiation resistance (D_{10} value) of 1.68 kGy was observed. Based on the average bioburden, verification doses for validation of 25 kGy gamma radiation were 5.87 to 9.46 kGy. Sterilization of bone allografts by gamma irradiation at 25 kGy was validated ensuring safety of the allografts processed from femoral heads for transplantation in orthopaedic reconstructive procedures.

Conflict of Interest

None

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