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Destructive dose determination of electron beam irradiation for pathogenic bacteria in water medium by 96 well plate assay

S. Visnuvinayagam[1]*, L.N. Murthy[2], U. Parvathy[4], A. Jeyakumari[3], K.P. Rawat[5], S.A. Khadar[5] and K.S.S. Sarma[6]

[1]Microbiology Fermentation and Biotechnology Division, ICAR- CIFT, Cochin-682 029, India
[2]Mumbai Research Centre of ICAR-CIFT, Vashi, Mumbai-400 703, India
[3]Fish Processing Division, Mumbai Research Centre of ICAR-CIFT, Mumbai-400 703, India
[4]Fish Processing Division, ICAR- CIFT, Cochin-682 029, India
[5]EBPS, IRAD, BARC, BRIT-BARC Complex, Mumbai-400 703, India
[6]Isotope and Radiation Applications Division, Bhabha Atomic Research Centre, Mumbai-400 085, India

***Corresponding Author Email :** visnuvinayagam@yahoo.co.in

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Abstract

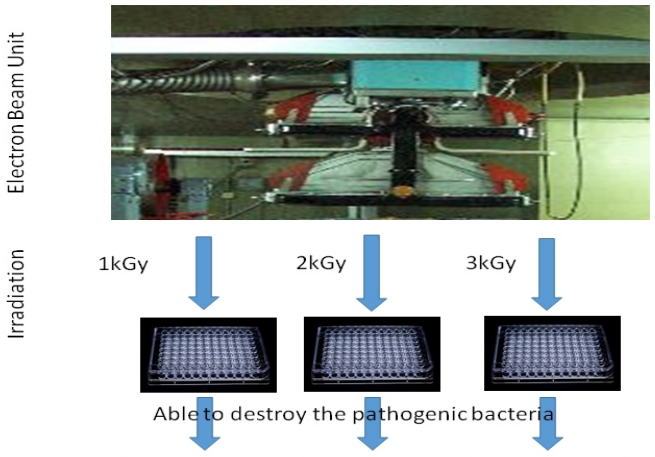
Aim: Determination of suitable dosage for eradication of different pathogenic bacteria in water will be useful for further detailed research in this regard.

Methodology: In the current investigation, known concentration of different actively growing bacterial culture were inoculated in 96-well-plate and exposed to 1, 2 and 3 kGy Electron Beam (EB) irradiation to understand the suitable destructive dosage.

Results: 1 kGy EB irradiation completely destroyed more than 2 × 10^7 number of *E. coli*, approximately 2 × 10^6 number of MRSA, 5 × 10^5 number of Salmonella, *V. cholerae* and *S. aureus* and 1 × 10^4 number of *P. aeruginosa*, respectively. Based on the study, the hierarchy of susceptible bacteria was as follows: *E. coli* < MRSA < *V. cholerae* < *S. aureus* < *Salmonella* < *P. aeruginosa* < *B. cereus* < *L. monocytogenes.*

Interpretation: The dose required for elimination of pathogens in 96 well plate was always lesser than that required for pathogens in the meat. 96-well-plate method is a simple and rapid method to assess different bacteria with different concentration in a single time and, hence, is useful and rapid technique to to determine the destructive irradiation dose for various bacteria and chemical pollutants in the substrate.

Keywords: Electron beam, Irradiation, MRSA, Pathogens, 96-well-plate



BACTERIA	1 kGy (cfu/100µl)	2kGy (cfu/100µl)	3kGy (cfu/100µl)
E. coli	>2.2X 10^6	-	-
MRSA	2.1 X 10^6	>8.4 X 10^6	>8.4×10^7
V. cholerae	8.7 X 10^5	>2.8 X 10^7	>2.8×10^7
S. aureus	6 X 10^5	>4.8 X 10^6	>4.8×10^6
Salmonella	5.3 X 10^5	4.3 X 10^6	>3.4 ×10^7
P. aeruginosa	4.8 X 10^4	3.1 X 10^6	>1.2×10^7
B. cereus	2.1 X 10^4	6.7 X 10^5	>2.1×10^7
L. monocytogenes	-	1.4 X 10^6	2.2×10^7

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Introduction

Control of various pathogens in water is considered important to avoid the spread of water borne infection. As per World Health Organization (WHO, 2012), around 1.5 million deaths have been reported due to water borne diseases. Various processes are being followed to control pathogenic bacteria in water. Recently, Electron Beam Irradiation (EBI), a non-thermal processing technique, is gaining much attention owing to its potential antibacterial activity. Electron Beam is the flow of electrons with energy, and the energy is obtained as kinetic energy when the electron moves in a high electric field. The commercial use of EBI began in 1950s, initially applied to crosslink the polymers, *i.e.,* polyethylene film and wire insulation. In the later decades, owing to its rapid sterilization process, it was widely practiced in most of the countries to sterilize medical devices (Waite *et al*., 1998). In 1980, international organizations related to food safety *viz*., FAO, WHO and IAEA recommended the use of irridation for food preservation with the upper limit of 10 kGy. EBI treated foods have shown dramatic reduction in food borne pathogenic bacteria, spoilage bacteria, viruses, pest and insects without any change in food quality (Jeyakumari *et al*., 2020).

Recently, most of the countries are interested in establishing Electron Beam Accelerator (EBA) for waste water treatment owing to its advanced oxidation processes to remove the pollutants from the waste. Wherever traditional technology is not worthy enough to remove the pollution, EBI will be a suitable alternative; hence, huge literature are available regarding degradation of pollutants from the water (Duarte *et al.*, 2002; Nickelsen *et al.*, 1992; Kurucz, *et al.*, 1995). In addition, the toxic compounds present in water can also be removed using EBI (Borrely and Sampa, 2000). On account of this, EBI treated water has shown a greater improvement in its quality. Since most of the studies carried out earlier have focused on the control of chemical pollutants or dyes or other organic materials, very limited literatures are available regarding the control of pathogenic bacteria in water using EBI. Hence, the present study aimed to assess the effect of EBI on different bacteria in different concentrations in water by employing 96 well assay plates.

Materials and Methods

ATCC bacterial cultures: Eight types of pathogenic cultures *viz., Escherichia coli, Salmonella, Vibrio cholerae, Pseudomonas aeruginosa, Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus* and Methicillin resistant *Staphylococcus aureus* were inoculated in Brain heart infusion (BHI) broth and incubated at 37°C till turbidity reached around 0.5 McFarland.

Enumeration of bacteria: Enumeration of bacterial pathogens were carried out as per Bacteriological Analytical Manual with slight modifications, *i.e.,* one ml of each 0.5 McFarland bacterial culture were transferred into 9 ml of test tube containing PBS and serially diluted (10 fold dilutions). Then, 100 µl of each dilution was taken and spread over the pre-set plate count agar (PCA) plate and incubated overnight for determining the exact number of bacteria present in 100 µl of culture.

Table 1 : Comparative MBC value (cfu $100\mu l^{-1}$) for different doses of electron beam irradiation

Bacteria	1 kGy	2kGy	3kGy
E. coli	$>2.2X\,10^6$	-	-
MRSA	$2.1\,X\,10^6$	$>8.4\,X\,10^6$	$>8.4\times10^7$
V. cholerae	$8.7\,X\,10^5$	$>2.8\,X\,10^7$	$>2.8\times10^7$
S. aureus	$6\,X\,10^5$	$>4.8\,X\,10^6$	$>4.8\times10^6$
Salmonella	$5.3\,X\,10^5$	$4.3\,X\,10^6$	$>3.4\times10^7$
P. aeruginosa	$4.8\,X\,10^4$	$3.1\,X\,10^6$	$>1.2\times10^7$
B. cereus	$2.1\,X\,10^4$	$6.7\,X\,10^5$	$>2.1\times10^7$
L. monocytogenes	-	$1.4\,X\,10^6$	2.2×10^7

96 well sterile cell culture plates: 96 well sterile cell culture plates were procured from Hi Media (Mumbai) and wells in all the rows were filled with 100 µl of phosphate buffer saline (PBS). A 100 µl of 0.5 McFarland known bacterial culture were inoculated in first well of first row and mixed properly and 100 µl transferred to subsequent wells in the same row to follow a two-fold dilution in subsequent wells till 12th well. Similarly, all the pathogens were added to each first row well and 2-fold dilutions were made.

Electron beam irradiation: Electron beam accelerator (5MeV Capacity) available at Board of Radiation & Isotope Technology (BRIT), Vashi, Navi Mumbai was utilised for the experiment. Different pathogenic bacteria were inoculated in each row with known concentration. The highest bacterial concentration in the first well and it was half quantity in subsequent well; hence, least bacterial count in the last well (12th well) (highest to lowest concentration in each column) (Table 1). The plate was exposed to the 1, 2 and 3 kGy of EBI and incubated overnight. Appearance of turbidity was considered as presence of bacteria in the wells.

Results and Discussion

In the present study, 96-well- plate was selected owing to its uniform size and thickness with universal standard; more over it is less than 2.5 cm and available readily with sterile condition, generally used for the assessment of virus potency (Visnuvinayagam *et al.,* 2015a). So, other variables (errors) such as size, shape and density are kept constant; and hence the effect of EBI on different concentration of bacterial culture can be estimated with accuracy. The study is to determine the destructive dose for all water borne, food borne and multidrug resistant bacterial pathogens susceptible in water medium. All the referred bacterial cultures of known quantity was plated in the 96 well plates and exposed to three different doses. Based on the earlier reports, most of the bacterias are destroyed by 3 kGy (Hossain *et al.*, 2018). Hence, in the present study all the bacteria were

exposed to three different dosage of EBI, *i.e.,* 1 kGy, 2 kGy and 3 kGy.

Pathogenic bacteria *viz., Salmonella, E. coli, V. cholerae* food borne diseases *L. monocytogenes*, *B. cereus, S. aureus* (responsible for water and food borne illness) MRSA and *P. aeruginosa* (multi drug resistant) were tested to assess the resistance and susceptible nature of pathogens towards EBI.

Known concentration of each bacteria were inoculated in different wells of 96 well plate and exposed to 1, 2 and 3 kGy of EBI and incubated overnight. Appearance of turbidity was considered as presence of bacteria in the wells. In 1 kGy treated plates, complete absence of turbidity was noticed on 1st, 6th, 5th, 8th, 10th, 3rd and 2nd well for *E. coli, Salmonella, V. cholerae, P. aeruginosa, B. cereus, S. aureus and* MRSA, respectively. In the case of *L. monocytogenes,* all the rows (even in 12th well) exhibited turbidity. Similarly, in case of 2 kGy treated plates, absence of turbidity was observed in 1st, 3rd, 1st, 2nd, 5th, 5th, 1st and 1st wells onwards for *E. coli, Salmonella, V. cholerae, P. aeruginosa, B. cereus, L. monocytogenes, S. aureus and* MRSA, respectively. In 3 kGy treated plates, all the wells were free of turbidity, except 1st well of *L. monocytogenes* (Table 1).

E. coli is most frequently encountered pathogen in retail fish markets of India. Recent report indicated that higher levels of multi drug resistant (MDR) *E. coli* was observed in the fish as well as water used in the retail fish markets (Visnuvinayagam *et al.,* 2015b; 2015c; 2016, 2018;). Extended spectrum of beta lactamase producing bacteria was also reported in the retail fish markets (Sivaraman *et al*., 2018). In the present study, initially the first row, first well had an *E. coli* concentration of 2.2×10^{7} CFU $100\mu l^{-1}$. However, no growth of *E. coli* was observed after subjecting it to 1kGy suggesting that this dose of EBI was effective in destroying all the bacteria in the first well itself.

Previous literature indicates scarce information regarding irradiation of *E. coli* in water medium. In 2013, Kundu *et al*. (2013) tried resistance pattern of *E. coli* by inoculating the culture in phosphate buffer saline in polypropylene tube and exposed it to 0.5 – 0.7 kGy irradiation. It was found that among the 27 strains of *E. coli,* only 11 strains were able to resist 0.7 kGy. In the indicated study, all the cultures were prepared in pellet form and further diluted and kept in 2 ml polypropylene vials for the experiment. However, the present study was made simple by testing in 96 well culture plates with more accurate estimation technique. Similar work done by Arthur *et al.* (2005) in substrate viz., beef inoculated with 10^{3}CFU cm^{-2} (low concentration) and 10^{6}CFU cm^{-2} (high concentration) of *E. coli* and exposure to 1 kGy EBI found complete degradation of *E. coli* for low concentration treated meat and 5.7 log reduction in high concentration treated meat.

Similar to *E. coli*, another susceptible seafood borne bacteria is Methicillin resistant *Staphylococcus aureus* (MRSA), an emerging multi drug resistant (MDR) pathogen. Various researches are being carried out to control the MRSA infection in human (Visnuvinayagam *et al.*, 2019a, b). MRSA is frequently isolated from clinical samples from the hospitals and a few also reported in seafood markets of India (Murugadas *et al*., 2017; Sivaraman *et al*., 2016). But, there is no report with reference to the effect of EBI on MRSA. In the present study, 1KGy EBI was able to destroy 4.2×10^{6} CFU $100\mu l^{-1}$. In 2kGy, even in the first well absence of growth was noticed indicating 2 kGy effective in destroying even more than 8.4×10^{6} CFU $100\mu l^{-1}$ of MRSA. As previous data on resistance of MRSA to EBI dosage is unavailable, the present work can be used as a baseline information for further research.

A dosage of 1KGy and 2KGy of EBI was able to destroy 1.75×10^{6} CFU $100\mu l^{-1}$ and 2.8×10^{6} CFU $100\mu l^{-1}$ of *V. cholerae,* respectively. Even though *V. cholerae* is well known as a water borne disease causing pathogen, there is no proper data available on its complete elimination in water, though few data are available on the effect of gamma irradiation against this pathogen. Reports indicate a D_{10} value for *V. cholerae* as 1kGy, which can effectively eliminate this pathogen from shellfish (Mallett *et al.,* 1991).

S. aureus is the most frequently encountered pathogen in seafoods and also considered as one of the important seafood borne pathogen causing diarrhoea within short period of incubation (3 - 4 hrs). *S. aureus* is more susceptible to EBI next to *V. cholerae*. 1 kGy and 2 KGy of EBI was able to destroy 1.2×10^{6} CFU $100\mu l^{-1}$ and $>2.4\times10^{6}$ CFU $100\mu l^{-1}$ of *S. aureus, respectively*. No previous reports of EBI effect on the elimination of *S. aureus* in water medium is reported. In the present study, 1 kGy could destroy more that 1×10^{6} *S. aureus* cells in the water.

Among the food borne pathogens, *Salmonella* is one of the highly studied pathogens in meat, especially in poultry. Occurrence of *Salmonella* in retail market is extensively reported (Murugadas *et al*., 2015) and plays a major role in trade related issues due to high rejection by overseas. In the present study, 1 KGy and 2 KGy of EBI could eliminate 2.15×10^{6} and 8.6×10^{6} CFU $100\mu l^{-1}$ of *Salmonella,* respectively. Complete elimination of *Salmonella* is influenced by various parameters leading to contradictory reports on its elimination dose. As per Abu-Tarboush *et al*. (1997), 2.5 kGy was needed for complete elimination of *Salmonella*, however, Lewis *et al*. (2002) reported that 60% of inoculated chicken breast samples were negative after 1.8 kGy irradiation. Sarjeant *et al*. (2005) reported that 2.0 kGy and 3.0 kGy was able to reduce *Salmonella* by 3 log and 5 logs in meat. In the present study, it has been observed that 1 kGy was sufficient to kill 10^{5} *Salmonella* present in the water.

P. aeruginosa is one of the multi drug resistant (MDR) bacteria causing multiple infections in human and animals. In the present study, 1 kGy and 2 kGy of EBI was sufficient to destroy

9.68×10^4 and 6.2×10^6 CFU $100\mu l^{-1}$ of *P. aeruginosa,* respectively. Reports on the effect of EBI on *P. aeruginosa* are not available.

B. cereus is a common seafood pathogen and current study indicated that application of 1 kGy and 2 kGy of EBI was effective in destroying 4.21×10^4 CFU $100\mu l^{-1}$ and 1.35×10^6 CFU $100\mu l^{-1}$ of this pathogen, respectively. No data is available regarding destruction of *B. cereus* in meat. However, Sarrias *et al.* (2003) reported a D_{10} value of around 2.0 – 2.8 kGy for EBI with regard to *B. cereus in rice.* De Lara *et al.* (2002) had a comparative evaluation of EBI resistance of *Bacillus* spores and vegetative cells and reported that the vegetative form of *B. cereus* required less EBI for the elimination with a D_{10} value of less than 1 kGy. However, the spore of *B. cereus* was much resistant to EBI indicating even a dose of 10 kGy to be insufficient. In the present study also, it was observed that among the tested bacteria, *Listeria monocytogenes* and *B. cereus* were more resistant to EBI.

L. monocytogenes is the second most studied pathogens next to *Salmonella* with regard to EBI application due to its high pathogenicity in both human and animals. Application of 1kGy EBI was insufficient to destroy 2.22×10^4 number of *L. monocytogenes* in 100µl of broth. However, 2.85×10^6 CFU $100\mu l^{-1}$ of *L. monocytogenes* were completely destroyed by 2kGy EBI. 3 kGy was sufficient to destroy 2.2×10^7CFU $100\mu l^{-1}$. Zhu *et al*. (2009) spiked different strains of *L. monocytogenes* and irradiated with 0, 1.0, 1.5, 2.0, or 2.5 kGy of EBI and reported a D_{10} value ranging from 0.56 to 0.58 kGy for *L. monocytogenes*. Similarly Su *et al.* (2004) observed that cold smoked salmon fish meat spiked with 10^8 CFU *Listeria* indicated 2.5 log reduction in 1.0 kGy treated samples and complete elimination of *L. monocytogenes* on 2.0 kGy exposure. Rodrigo *et al.* (1996) reported that most of the *L. monocytogenes* were destroyed by 2.5 kGy; but, few of the strains of *L. monocytogenes* were stable to EBI at lower dose. They recommended 4.5 kGy to be effective in completely eliminating *L. monocytogenes*. Based on the previous reports, it was observed that most of the *L. monocytogenes* strains were destroyed in meat after exposure to 2 – 2.5 kGy, however few strains required around 4.5 kGy for complete elimination from the meat.

Various researches conducted on the effect of irradiation on waste water treatment improved the water quality occurred on treatment with 40 to 120 kGy irradiation (Gu *et al*., 2017). Based on the previous work, it has been observed that, high doses of EBI are needed to degrade chemical or organic pollutants from the waste. However, to destroy pathogenic bacteria comparatively very low doses are sufficient. Production of three types of transient species is the cause for the antimicrobial property of EBI. These transient species are formed in water while hitting water with high energy electron beam, viz. aqueous electron (e-aq), hydrogen radical (H.) and hydroxyl radical (.OH) (Waite *et al*., 1998).

Monk *et al.* (1995) clearly described that the death of bacteria by irradiation is influenced by many external factors such as pH, temperature, chemical composition of food in which the bacterial cells are suspended. In addition, the concentration of bacteria and surface distribution of meat also plays a significant role. Since, damage to bacterial DNA is the main factor for destruction; bacteria with poor DNA repair mechanism are highly sensitive to irradiation (Monk *et al*., 1995). In Gamma Irradiation (GI) even the lower doses can cause adverse effects in the sensory characteristics of meat substrate, while it is negligible as far as EBI is concerned. The present study indicated the hierarchy of susceptibility of different bacteria to EBI is the following order: *E. coli* < MRSA < *V. cholerae* < *S. aureus* < *Salmonella* < *P. aeruginosa* < *B. cereus* < *L. monocytogenes.*

The current investigation concludes that 96 well plate study was proved to be an effective, simple and rapid technique to determine the destructive dose of EBI on different bacteria.

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