Gamma Irradiation as an Effective Method for Inactivation of Emerging Viral Pathogens

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Abstract. Gamma irradiation using a cobalt-60 source is a commonly used method for the inactivation of infectious specimens to be handled safely in subsequent laboratory procedures. Here, we determined irradiation doses to safely inactivate liquid proteinaceous specimens harboring different emerging/reemerging viral pathogens known to cause neglected tropical and other diseases of regional or global public health importance. By using a representative arenavirus, bunyavirus, coronavirus, filovirus, flavivirus, orthomyxovirus, and paramyxovirus, we found that these enveloped viruses differed in their susceptibility to irradiation treatment with adsorbed doses for inactivation of a target dose of 1×10^6 50% tissue culture infectious dose (TCID₅₀)/mL ranging from 1 to 5 MRads. This finding seemed generally inversely correlated with genome size. Our data may help to guide other facilities in testing and verifying safe inactivation procedures.

Efficient and reliable inactivation of specimens is an important component of many laboratory operations, but especially critical for biocontainment laboratories handling emerging/reemerging pathogens causing neglected tropical infectious diseases of epidemic as well as infectious disease of regional and global (pandemic) public health importance. Multiple methods can be applied following approved safety testing by institutional and national authorities including those based on chemical, heat, and radiation treatment.^{1–7} Gamma irradiation, mainly using a cobalt-60 source, is an established and commonly used method in the biocontainment field. This inactivation method is preferred for specimens that are used for, but not limited to, a variety of serological, broader immunological, and biochemical assays for which structural integrity of proteins and particles is of certain importance.

The purpose of this study was to evaluate the efficacy of gamma irradiation to inactivate emerging/reemerging pathogens of different virus families requiring biocontainment. In the United States, many of these viruses are select agents regulated by the Division of Select Agents and Toxins of the Centers for Disease Control and Prevention and the Animal and Plant Health Inspection Services of the Department of Agriculture.^{8,9} The data were used to determine irradiation doses for complete inactivation of specimens containing these viruses for safe removal from biocontainment.

As the starting material, we used viral seed stocks in storage medium (Dulbecco's modified Eagle's medium [DMEM] with 10% fetal bovine serum [FBS]). We chose one representative member of the families Arenviridae (genus *Mammarenavirus*, Lassa virus [LASV], strain Joshia), Phenuiridae (genus *Phlebovirus*, Rift Valley fever virus [RVFV], strain 2008 ZH501), Coronaviridae (genus *Betacoronavirus*, severe acute respiratory syndrome coronavirus [SARS-CoV], strain Tor 2), Flaviviridae (genus *Flavivirus*, tick-borne encephalitis virus [TBEV], far eastern subtype virus, strain Sofjin), and Orthomyxoviridae (genus *Influenzavirus A*, A/Vietnam/1203/2004 (H5N1), and two representative members of the families Filoviridae (genus *Ebolavirus*, Ebola virus [EBOV], strain Mayinga and genus *Marburgvirus*, Marburg virus [MARV], strain Ci67)

and Paramyxoviridae (genus *Henipavirus*, Hendra virus [HeV], Nipah virus [NiV], strain Malaysia). Samples were diluted to the target dose of 1×10^6 50% tissue culture infectious dose [TCID₅₀]/mL with DMEM, and 1 mL aliquots were added to 2 mL screw-cap vials (Sarstedt, Nümbrecht, Germany). Vials were sealed in a plastic bag and the bag was submerged in disinfectant (5% MICRO-CHEM-PLUS™; National Chemical Laboratories, Inc., Philadelphia, PA) to check for leaks and subsequently sealed in a second bag. The double-sealed samples were removed from containment through a dunk tank containing 5% MICRO-CHEM-PLUS according to standard operating procedures (SOPs) approved by the Institutional Biosafety Committee.

Gamma irradiation was performed with a JL Shepherd Model 484R irradiator (JL Shepherd and Associates, San Fernando, CA) using a cobalt-60 source. The irradiation chamber had a rotating platform to guarantee uniform exposure to gamma radiation. It comfortably accommodates a 2-L beaker that contains samples in either dry or wet ice. During irradiation, samples were colocated with lithium fluoride film dosimeters for verification of absorbed doses in megarads (Mrads; 1 rad = 0.01 Gy). The irradiator is not located in high containment, rather in a designated and access-controlled nearby location. Following removal from biocontainment, the samples were immediately irradiated on dry ice as described earlier, affirming “chain of custody” procedures. Each run of irradiation was documented with the appropriate parameters and actual absorbed dose according to SOPs.

Following irradiation, samples were returned to biocontainment for inactivation testing in tissue culture using an infectivity assay determining the TCID₅₀. Vero cells were infected with a 1:1 (v/v) dilution in DMEM of the irradiated sample. Following a 1-hour adsorption in a small volume (1 mL), an appropriate amount of maintenance medium (DMEM, 2% FBS) was added and cells were monitored daily for cytopathogenic effects over a period of 14 days. Negative cultures were passaged one time in freshly seeded Vero cells and monitored under the same conditions for another 14-day period. For EBOV, we used a recombinant virus expressing enhanced green fluorescence protein (EBOV-eGFP).¹⁰ This allowed additional monitoring using fluorescence microscopy. Similar recombinant viruses were not available at our laboratory for other pathogens tested here.

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TABLE 1
Infectivity testing in tissue culture following inactivation by gamma irradiation

	EBOV	MARV	LASV	RVFV	HeV	NiV	SARS-CoV	H5N1	TBEV
0 Mrad	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
0.5 Mrad	+/+/+	+/+/+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1 Mrad	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	-/-/-	+/+/+	+/+/+
2 Mrads	-/-/-	-/-/-	+/+/-	+/+/+	+/+/+	+/+/+	-/-/-	+/+/+	+/+/+
4 Mrads	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	+/+/+
5 Mrads	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-/-/-
6, 8, and 10 Mrads	-/-/-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

EBOV = Ebola virus (strain Mayinga); H5N1 = influenza virus (A/Vietnam/1203/2004); HeV = Hendra virus; LASV = Lassa virus (strain Josiah); MARV = Marburg virus (strain Ci67); n.d. = not done; NiV = Nipah virus (strain Malaysia); RVFV = Rift Valley fever virus (strain 2008 ZH501); SARS-CoV = severe acute respiratory syndrome coronavirus (strain Tor 2); TBEV = tick-borne encephalitis virus, far eastern subtype virus (strain Sofjin). Virus (1×10^6 50% tissue culture infectious dose/mL) was treated with increasing irradiation doses (Mrads, triplicates per dose). To determine remaining infectivity, irradiated samples were diluted with Dulbecco's modified Eagle's medium 1:1 (v/v) and used to inoculate Vero cells. Infectivity was determined by monitoring the cytopathogenic effect or green fluorescence (EBOV only) using light or fluorescence microscopy over a period of 14 days. Negative samples were passaged one time on Vero cells and monitored under the same conditions. Key: +/+/+, virus growth in one, two or three replicates (triplicates); -/-/-, no virus growth in one, two or three replicates (triplicates).

Initially, we determined the absorbed dose to inactivate EBOV and MARV, two representatives of the family Filoviridae. We started with triplicates of 1×10^6 TCID₅₀/mL of EBOV-eGFP and MARV that were treated with increasing irradiation doses of 0, 0.5, 1, 2, and 4 Mrads (for EBOV-eGFP also 6, 8, and 10 Mrads). We observed EBOV and MARV replication in Vero cells following irradiation with 0, 0.5, and 1 Mrad; no virus replication could be detected following a dose of 2 Mrads or higher (Table 1). Subsequently, we used 1 Mrad, the highest irradiation dose that did not completely inactivate the target dose of 1×10^6 TCID₅₀, to define the virus load that was inactivated by this suboptimal dose. For this, we irradiated 10-fold dilutions (1×10^7 – 10^0 TCID₅₀) of EBOV-eGFP and determined the remaining infectivity using a TCID₅₀ assay on Vero cells as described above. A dose of 1 Mrad still completely inactivated 1×10^3 TCID₅₀ of EBOV-eGFP (data not shown).

To confirm inactivation in a different and likely more sensitive readout system, we also treated mouse-adapted EBOV (ma-EBOV, 1×10^6 focus forming units [FFU]/mL) using irradiation doses of 0, 1, 2, 4, 6, and 8 Mrads (triplicates per dose).¹¹ Gamma-irradiated samples were diluted with DMEM 1:1 (v/v) and inoculated in a small volume (200 μ L) into BALB/c mice

(female, 6–8 weeks old; Charles River Laboratories, Wilmington, MA) by the intraperitoneal route ($n = 5$ per group; three groups per irradiation dose, $n = 15$). Animals were monitored for signs of clinical disease (i.e., weight loss, ruffled fur, hunched posture, paralysis, and hemorrhages) over a period of 21 days. For humane endpoint determination, we used the criteria approved by the Institutional Animal Care and Use Committee of the Rocky Mountain Laboratories (i.e., weight loss > 25%, ataxia, extreme lethargy, bloody discharge, tachypnea, dyspnea, or limb paralysis). All mice inoculated with untreated ma-EBOV or ma-EBOV treated with 1 Mrad became ill and had to be euthanized within 5 to 6 days. Mice inoculated with ma-EBOV treated with 2 Mrads or higher did not show any clinical signs of disease and survived (Figure 1). This confirmed the tissue culture data and shows that at least for filoviruses, in vitro testing seems as sensitive as animal testing using a highly susceptible mouse model (50% lethal dose, of 0.01 FFU).¹²

We next determined the gamma irradiation doses needed to inactivate LASV, RVFV, SARS-CoV, TBEV, H5N1, HeV, and NiV. We treated a virus load of 1×10^6 TCID₅₀/mL (triplicates per virus) with increasing irradiation doses of 0, 1, 2, 4, and 5 Mrads. SARS-CoV, harboring the largest genome of all

FIGURE 1. Infectivity testing in Ebola mouse model following inactivation by gamma irradiation. Mouse-adapted Ebola virus (1×10^6 focus forming units/mL) was treated using irradiation doses of 0, 1, 2, 4, 6, and 8 Mrads (triplicates per dose). To determine remaining infectivity, irradiated samples were diluted with Dulbecco's modified Eagle's medium (DMEM) 1:1 (v/v) and inoculated into BALB/c mice (female, 6–8 weeks old) by intraperitoneal injection ($n = 5$ per group; three groups per irradiation dose, $n = 15$). Animals were monitored for signs of clinical disease over a period of 21 days.

studied viruses here, was already completely inactivated by a dose of 1 Mrad. By contrast, a dose of 4 Mrads was needed to inactivate LASV, RVFV, HeV, NiV, and H5N1; the target viral load of TBEV needed 5 Mrads for complete inactivation by gamma irradiation (Table 1). As for EBOV-eGFP, we determined the NiV load that got completely inactivated by a dose of 2 Mrads, the highest absorbed dose that did not inactivate the target dose of 1×10^6 TCID₅₀/mL. We irradiated a series of ten-fold dilutions (1×10^7 – 10^0 TCID₅₀) of NiV and determined the remaining infectivity in Vero cells. A dose of 2 Mrads completely inactivated 1×10^4 TCID₅₀ of NiV (data not shown).

The absorbed dose of gamma irradiation is affected by multiple factors such as sample origin, sample composition, sample volume, irradiation temperature, distance to irradiation source, and others.⁶ The main viral inactivation mechanism is thought to be the destruction of replication-competent nucleic acid either directly by radiolytic cleavage or cross-linking of genetic material or indirectly by the action of radicals on nucleic acids and, to a lesser degree, proteins.^{6,13,14} According to regulations, process validation for a select agent can be performed with a representative virus family member as a surrogate. Here, we tested inactivation through gamma irradiation of selected representative emerging/reemerging viral pathogens in a liquid medium containing protein, one of the most common sample sources for irradiation inactivation in biocontainment operation. Future studies may focus on confirmation using additional members of virus families and infectious agents in different matrixes. We determined the minimum absorbed doses required to fully inactivate a defined peak virus load of 1×10^6 TCID₅₀ in this sample type for representative members of seven virus families (Table 1). Our results showed that these enveloped viruses differed in their susceptibility to gamma irradiation. We confirmed that, in general, inactivation seemed to be inversely correlated with genome size even though other pathogen characteristics may also influence efficacy of gamma irradiation.^{15,16} For safety and precautionary measures, our new SOPs now specify doubling the minimum dose needed to fully inactivate a certain target dose of the different viruses in a volume of 1 mL. Based on this study with inclusion of the “2× safety factor,” the recommended radiation doses for safe inactivation of 1×10^6 TCID₅₀ are as follows: coronaviruses, 2 Mrads; filoviruses, 4 Mrads; arenaviruses, bunyaviruses, orthomyxoviruses and paramyxoviruses, 8 Mrads; and flaviviruses, 10 Mrads. As inactivation doses are high for some viruses, proper integrity of specimens may be a concern for certain downstream analyses. These irradiation doses may be used as guidance for process validation at other facilities. Quality assurance panels may be helpful here similar to what has been done by the European Network for Diagnostics of Imported Viral Diseases for diagnostics of emerging pathogens.¹⁷

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