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Gamma Irradiation of Animal Serum: Validation of Efficacy for Pathogen Reduction and Assessment of Impacts on Serum Performance

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Abstract

The treatment of animal serum by gamma irradiation, for the purpose of mitigating the risk of introducing a pathogen (virus, mollicute, or other microbe) into a cell culture, is a process that has been executed (and perhaps understood) primarily by irradiation contractors utilized by serum manufacturers. The selection of appropriate exposure conditions and irradiation doses is driven by a number of critical factors including: (1) the validation and control of the irradiation process itself; (2) the efficacy of the applied irradiation dose range for inactivating pathogens of interest; (3) determination and control of critical process attributes; (4) the potential impacts of these irradiation dose levels on the serum being irradiated; and finally, (5) the potential impact of irradiated serum on the medicinal product and the associated manufacturing process where serum is ultimately used. In order to increase awareness of these topics throughout the cell culture community, we have addressed these critical factors in the current review.

Introduction

This article is part of a series of papers that are being authored under the sponsorship of the International Serum Industry Association (ISIA) with the purpose of establishing best practices for processes employed in the gamma irradiation of animal serum. In the present article, we describe best practices for validating the efficacy of viral inactivation during gamma irradiation of serum. In general, these practices can be applied for validating pathogen reduction for other microbes. A survey of gamma irradiation efficacy for the inactivation of pathogens, especially viruses and mollicutes (mycoplasmas and acholeplasmas), in frozen serum will be presented. Finally, as will be explained further, a useful window of irradiation dosage must be established, not only in terms of fluency required for pathogen inactivation, but also keeping in mind that the desired performance characteristics of the material being irradiated must remain intact. The latter must be empirically evaluated both by serum manufacturers and end-users of the irradiated serum. Best practices for these evaluations have also been described. As will become apparent, there is a necessary trade-off between optimization of pathogen reduction by gamma irradiation and preservation of performance of the irradiated serum as a medium additive for cell culture applications.

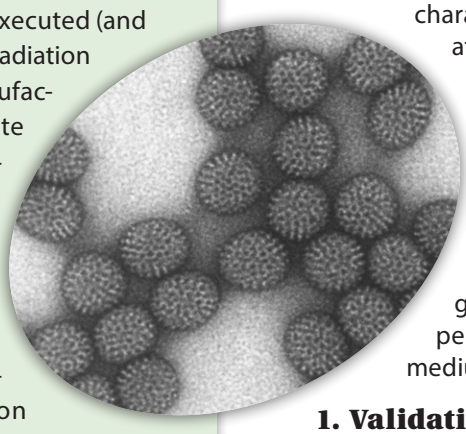

IMAGE: A negatively-stained transmission electron micrograph of rotavirus particles. Rotavirus is a member of the *Reoviridae* family. (Credit: CDC/Dr. Erskine Palmer, http://phil.cdc.gov/phil/)

1. Validation of the Efficacy of Gamma Irradiation for Viral Inactivation

The main objectives of a validation study involving gamma irradiation of animal serum (or other animal-derived liquid materials) are to: (1) establish the kinetics of virus inactivation in such matrices at various increasing irradiation doses; and (2) select a minimum irradiation dose at which adequate virus inactivation is achieved. An irradiation dose is expressed as fluency, in units of kiloGrays (kGy) or in units of megarads (MR), where 1 MR = 10 kGy. The term "fluency" incorporates both dose-rate and time, making this a very useful term for the comparison of efficacy in different exposure scenarios. Efficacy information obtained through validation is necessary for establishing the commercial product irradiation dose range. Serum performance at increasing irradiation doses must be taken into account in order to determine the highest irradiation dose at which physicochemical, biochemical, and biological performance properties of serum product are deemed acceptable.

Irradiation processes and the design of viral clearance validation studies are governed by several regulatory guidance documents including:

- International Conference on Harmonisation (ICH) Q5A(R1) guidelines[1]
- FDA 1993 Points to Consider document[2]
- US Pharmacopeia (USP) chapters <1050.1>[3] and <1024>[4]
- EMA/CVMP/743/00-Rev 2 document[5]
- EMA/CHMP/BWP/457902/2012 Rev 1 document[6]
- European Pharmacopoeia (Ph. Eur.) serum monograph[7]
- World Health Organization cell substrates guidance[8]

Specifically, these documents require that: (a) the kinetics of virus inactivation be established; (b) a minimum effective irradiation dose be selected for adequate $\log_{10}$ reduction of virus titer; (c) the packaging configuration for commercial-scale irradiation be determined; and (d) the reproducibility of the process be assured by appropriate dosimetry for each run (through measurement and documentation of minimum and maximum absorbed dose).

Design of Efficacy Validation Studies

Validation studies for inactivation of viruses by gamma irradiation should be designed in accordance with the above regulatory requirements, in a manner that is well-documented and controlled. Several key points to consider in designing a viral inactivation study include:

(1) Selection of the challenge virus panel
(2) Test matrix considerations
(3) Appropriateness of the scaled-down model
(4) Matrix spiking and study controls
(5) Establishing and documenting dosimetry
(6) Determination of inactivation per kGy applied dose
(7) Virus quantitation considerations
(8) Data interpretation
(9) Establishing the irradiation dose range
(10) Selection of the irradiation facility
(11) Selection of the testing laboratory
(12) Shipment to and from the irradiation facility
(13) Shipment to and from the testing laboratory

This paper will focus on the first nine topics, ranging from choosing challenge virus to determining irradiation dosing. The other topics will be the subject of future publications in this series.

Selection of the Challenge Virus Panel

A variety of challenge viruses are required for use in irradiation validation studies (*e.g.*, **Table 1**). The selection

TABLE 1. Examples of challenge (spiking) viruses used in validating the efficacy of gamma irradiation.

Virus	Family	Genome	Envelope	Particle Size (nm)	Resistance to Inactivation
Adenovirus (*e.g.*, canine adenovirus [CAV])	*Adenoviridae*	ds-DNA	No	70–100	Moderate–high
Akabane or Aino virus	*Bunyaviridae*	ss-RNA, segmented	Yes	90–120	Low
Cache Valley virus (CVV)	*Bunyaviridae*	ss-RNA, segmented	Yes	90–120	Low
Calicivirus (*e.g.*, feline or canine)	*Caliciviridae*	ss-RNA	No	30–40	High
Porcine circovirus (PCV) (*e.g.*, PCV-2)	*Circoviridae*	ss-DNA, circular	No	15–22	Very high
Bovine viral diarrhea virus (BVDV)	*Flaviviridae*	ss-RNA	Yes	40–60	Moderate
Infectious bovine rhinotracheitis virus (IBRV) or bovine herpes virus (BHV) (*e.g.*, BHV-1)	*Herpetoviridae*	ds-DNA	Yes	120–200	Low
Pseudorabies virus (PRV)	*Herpetoviridae*	ds-DNA	Yes	120–200	Low
Parainfluenza virus (PIV) (*e.g.*, PIV-3)	*Paramyxoviridae*	ss-RNA	Yes	150–300	Low
Parvovirus (*e.g.*, porcine parvovirus [PPV], or mouse minute virus [MMV])	*Parvoviridae*	ss-DNA	No	18–25	Very high
Encephalomyocarditis virus (EMCV)	*Picornaviridae*	ss-RNA	No	27–35	Moderate
Swine vesicular disease virus (SVDV)	*Picornaviridae*	ss-RNA	No	27–35	Moderate
Polyomavirus (*e.g.*, simian virus 40 [SV40])	*Polyomaviridae*	ss-DNA, circular	No	40–45	Very high
Bluetongue virus (BTV)	*Reoviridae*	ds-RNA, segmented	No	60–80	Moderate
Reovirus (*e.g.*, respiratory-enteric orphan virus-3 [REO-3])	*Reoviridae*	ds-RNA, segmented	No	60–80	Moderate
Bovine ephemeral fever virus (BEFV)	*Rhabdoviridae*	ss-RNA	Yes	70×150	Low
Equine infectious anemia virus (EIAV)	*Retroviridae*	ss-RNA, diploid	Yes	80–120	Low
Feline leukemia virus (FeLV)	*Retroviridae*	ss-RNA, diploid	Yes	80–120	Low
Hog cholera virus (HCV)	*Togaviridae*	ss-RNA	Yes	60–70	Low

ABBREVIATIONS: Double strand (ds) and single strand (ss)

of appropriate viruses is based on several factors such as:

- a risk analysis for the serum product and the serum manufacturing process;
- the epizootic status (*i.e.*, animal disease epidemiology) of the serum's country of origin;
- applicable information that might be provided by the Office International des Epizooties (OIE);
- consideration of new and emerging pathogens;
- the potential risk to the serum end-user;
- the potential for adventitious agents in the final medicinal product manufactured using the serum; and
- the safety risk to patients—recipients of the medicinal product manufactured using the serum.

As a standard practice, the panel of challenge viruses selected should represent:

- different families including both RNA and DNA viruses possessing diverse genomic organizations (*e.g.*, single-stranded [ss] and double-stranded [ds], negative- and positive-sense, linear, circular, segmented, and non-segmented);
- viruses with and without envelopes;
- varied particle sizes ranging from small to large; and
- agents of differing levels of resistance to gamma irradiation.

The spiking viruses should be of known history and confirmed identity, high quality, adequate purity, and at sufficiently high titer (preferably $>10^6$ tissue culture infective $dose_{50}$ [$TCID_{50}$] or plaque-forming units [PFU] per mL) in order to be capable of determining the actual upper limits of virus titer reduction with reasonable certainty.

Care should be taken regarding the use of field isolates vs. laboratory-adapted virus strains. The field strains may exhibit different characteristics (more quasi-species, diverse populations, defective and/or empty particles) and behaviors (different growth profiles in cell culture and sensitivities to inactivation) than those of laboratory-adapted strains. It is advisable to use laboratory virus stocks that have as many similarities as possible to the field isolates, with a low number of passages from isolation, if possible.

Test Matrix Considerations

It has been well-established that serum products, as well as other types of animal-derived materials, display various degrees of lot-to-lot (inter-lot) variability. This variability is inherent to serum composition (matrix) which, in turn, may affect the efficacy of gamma irradiation for inactivation of viruses obtained in the study. For example, serum may contain antibodies or other non-antibody inhibitors that may specifically or non-specifically bind with or neutralize spiking viruses. The presence of such factors in the serum may mask the presence of the spiking viruses, leading to underestimates of their titers using infectivity assays. Such adverse impacts on the virus titration assays used prior to and following irradiation can impact the overall irradiation study interpretation. Therefore, careful selection of representative serum for validation of the gamma irradiation process is very important.

The serum should be tested for the presence of anti-viral antibodies, and only antibody-free serum should be used in the validation study. Preferably, three lots of serum should be used for virus spiking purposes in order to better assess the impact of inter-lot variability. The antibody-free serum matrix should be tested for potential interference and cytotoxicity prior to conducting the inactivation study itself in order to determine any virus-blocking effects (interference) or potential toxic effects of the matrix on the indicator cells (cytotoxicity). Serum determined to be free of such interference or cytotoxic effects may be used for the actual virus spiking and irradiation study. This approach to mitigating the adverse impacts of neutralizing antibodies is considered more appropriate than the use of proxy serum derived from other animal species (*e.g.*, the use of horse serum for spiking studies involving BVDV).

Where possible, a worst-case matrix should be determined. This may be defined as serum that is free of cross-reacting antibodies and other interfering molecules with a relatively high total protein, lipid, and/or hemoglobin content. Such a matrix could, under certain conditions, be protective of the spiking viruses, impact virus dispersion and homogeneity within the serum being irradiated, or increase viral aggregation. Each of these factors may limit, to some extent, the determined efficacy of viral inactivation by gamma irradiation.

Appropriateness of the Scaled-Down Model

Validation of viral inactivation is not typically performed at the commercial serum lot scale. Therefore, a scaled-down process is utilized for virus spiking purposes during the validation. The down-scale model should be carefully designed to best represent an actual large-scale irradiation process in terms of serum type, matrix, packaging features, shipping provisions, dose-mapping, and irradiation process.

Matrix Spiking and Study Controls

Once an acceptable serum matrix has been selected, the spiking virus stocks have been prepared, a scaled-down model is established, and the possibility of interference and cytotoxicity has been ruled out, the experiment itself may be conducted. Virus is spiked (<10% vol/vol) into serum bottles (containers) under aseptic conditions such that one bottle contains one spiking virus type. The serum-virus mixture should be mixed properly to ensure homogenous virus distribution and to minimize the occurrence of virus aggregates. The spiking is then repeated with each virus type until three different serum lots have been spiked with each of the panel of selected viruses.

The following controls are typically incorporated into the experimental design:

- negative (non-inoculated) serum;
- spiked serum shipped to the irradiation facility but not irradiated;
- spiked stability (hold), kept frozen in the laboratory;
- virus stability (hold), kept frozen in the laboratory; and
- other controls as appropriate.

Establishing and Documenting Dosimetry

In order to accurately monitor the applied irradiation dose (especially product-absorbed dose), multiple dosimeters may be placed at appropriate points within the packaging configuration. Dosimeters can be immersed inside bottles of serum before freezing, affixed to the exterior bottle surfaces at several locations, and/or attached to the outside of cases of bottles in various spots. The irradiation containers (*e.g.*, totes, pallets, carriers) should be representative of commercial applications in terms of size, type, and packing density. However, it is recognized that the bottles used in the down-scale inactivation studies may differ (*e.g.*, volume and style) from those used for commercial-scale serum bottling and irradiation. Validation of dosimetry for an irradiation process will be addressed in a subsequent article in this series.

Determination of Inactivation per kGy Applied Dose

It is a regulatory requirement that the kinetics of inactivation be evaluated during the validation of inactivation steps.[1,3] Since gamma irradiation renders adventitious agents non-infectious by inactivating the genomic material, it is expected that the kinetics of inactivation will be evaluated. This is accomplished by irradiating the spiked serum with a series of increasing irradiation doses (*e.g.*, 0–60 kGy in 5 or 10 kGy increments) using at least one bottle per serum lot, per virus, and per test dose.

The spiking virus titers are determined before and after irradiation for each dose, and the reduction of virus titer resulting from the irradiation is plotted vs. dose for each virus to establish the kinetics of virus inactivation for that virus. The resulting information is often expressed in terms of a *D* value (*i.e.*, the dose in kGy required to cause a 1 $\log_{10}$ reduction in titer), although we find it more convenient to express the result in terms of the $\log_{10}$ inactivation obtained per kGy irradiation dose.[9]

Virus Quantitation Considerations

The methods to be used for determining virus titer pre- and post-irradiation are of critical importance in assessing the efficacy of gamma irradiation. Considering that gamma irradiation targets the viral genome, potentially causing strand breaks and other types of damage to the nucleic acids, it is essential *not* to use nucleic acid-based analytical tools (*e.g.*, polymerase chain reaction [PCR]) for determining virus titer. Although the virus may be completely inactivated (rendered non-infectious), nucleic acid testing methods might still be able to detect fragments of the viral nucleic acids, making it impossible to interpret the study results. Therefore, cell-based infectivity methods are used for virus titrations and virus reduction titers are analyzed using assays such as $TCID_{50}$, PFU, fluorescent focus assay (FFA), *etc*. The methods should be validated at least as to specificity and sensitivity (limit of detection), and any method limitations should be well understood. Finally, the testing facility and its staff should be experienced and knowledgeable in the fields of virology, method execution, and data interpretation.

Data Interpretation

Data analysis and interpretation are typically performed as described in ICH Q5A(R1)[1] and USP <1050.1>.[3]

Establishing the Irradiation Dose Range

The applied dose (fluency) range to be used for irradiating serum typically represents a window between the lower and higher irradiation dose, where sufficient virus inactivation is attained while the serum quality attributes remain minimally affected. An example of an irradiation dose range for treatment of serum products is 30–50 kGy. Although the lower value of the range (30 kGy) may represent a minimum due to EMA[5] and Ph. Eur.[7] requirements, it can be set higher to assure greater virus reduction. The upper value of the range can also vary depending on the potential impact of higher doses on serum quality and performance.

The actual dose range applied during irradiation is process application-specific and various end-users may require slightly different ranges, considering empirically determined impacts on serum performance in their own process applications (detailed in section 3). In general, irradiation dose ranges that are overly narrow (*e.g.*, <15 kGy difference between lower and upper values) can be difficult to deliver by irradiators in a consistent manner, likely resulting in periodic deviations and potential product rejection. This topic will be addressed in greater detail in a subsequent article in this series.

The inactivation efficacy results that have been reported in the literature to date are described next.

2. Efficacy of Gamma Irradiation for Pathogen Reduction in Serum

As there have been no additions to the literature addressing efficacy of gamma irradiation for inactivating potential viral contaminants in animal serum since our previous review was published[9], we provide a summary of the most important findings of that review in this paper. In order to set the stage for the high-level discussion on efficacy presented below, it is necessary to discuss some of the mechanistic aspects of viral inactivation by gamma irradiation.

Efficacy for Viral Inactivation

The inactivation of viruses by gamma irradiation is highly matrix- and temperature-dependent, and the mechanism of inactivation depends on the conditions present during inactivation. To help minimize unintended damage to critical animal serum components, for instance, the direct mechanism involving attack of energetic photons on nucleic acid bases is favored over the indirect mechanism involving the generation of oxygen radicals derived from radiolysis of water molecules. The direct mechanism is favored by irradiating serum in the sealed product containers at low temperature (typically –60°C or lower). Under the latter conditions, the inactivation of microorganisms such as viruses and mollicutes by gamma irradiation is typically first-order with respect to radiation dose, resulting in linear kGy dose vs. $\log_{10}$ inactivation curves.

The literature describing the inactivation of mollicutes and viruses in frozen animal serum consists of the following: (1) validation studies conducted by serum vendors in which fairly complete radiation dose/inactivation response results have been described; and (2) data from a variety of sources addressing the reduction in titer of specific adventitious agents in frozen serum that was irradiated at a typical dose window (*e.g.*, 30–50 kGy). The irradiation dose/inactivation response data typically have spanned gamma radiation doses low enough to be only marginally effective as well as doses resulting in maximal inactivation. Maximal inactivation is usually expressed as "≥ some $\log_{10}$ reduction value", since the amount of agent that may be spiked into the serum is limited. In practice, spiking levels are determined by: (1) the titer of the stock used for spiking; and (2) the maximum dilution of the serum allowed during spiking (typically 10%). For this reason, the upper limits of inactivation for susceptible adventitious agents (*e.g.*, mollicutes and large enveloped viruses) can only be approximated. As mentioned already, the other limiting factor for adventitious agent risk mitigation by gamma irradiation is the potential for adverse impacts on serum quality and performance.

Table 2 displays the efficacy of gamma irradiation for inactivation of seven viruses from a variety of families spiked into fetal bovine serum (FBS).[10-13] It can be seen that gamma irradiation is quite effective, even at the lower end of the dose window (30 kGy) for inactivation of larger enveloped and non-enveloped viruses such as PIV-3, IBRV, REO-3, BVDV, and CAV. On the other hand, the efficacy for the much smaller PPV and MMV (*i.e.*, the two parvoviruses) is substantially lower. Even at the higher end of the irradiation dosage window (50 kGy), only 2.8–3.7 $\log_{10}$ inactivation of these parvoviruses would be expected, based on these results. The presence or absence of a lipid envelope *per se* does not appear to be a major determinant of gamma irradiation inactivation susceptibility within this series of viruses.

House *et al*.[14] reported inactivation efficacy results for viruses from different families spiked into FBS and subjected to gamma irradiation (**Table 3**). The $\log_{10}$ reduction per kGy for each of the viruses examined was obtained from the D_{10} values reported in the paper as follows:

$$\frac{\log_{10}\text{ reduction}}{\text{in titer per kGy}} = 1 \div \left(D_{10}\,\frac{\text{MR}}{1\log_{10}\text{ reduction}} * 10\,\frac{\text{kGy}}{\text{MR}} \right)$$

TABLE 2. Efficacy of gamma irradiation from dose/inactivation response studies with seven viruses spiked into FBS.*

Virus	Family	Genome	Envelope	Particle Size (nm)	$\log_{10}$ Reduction in Titer per kGy	$\log_{10}$ Reduction per 30 kGy
CAV	*Adenoviridae*	ds-DNA	No	70–100	0.203	6.1
BVDV	*Flaviviridae*	ss-RNA	Yes	40–60	0.198	5.9
IBRV	*Herpetoviridae*	ds-DNA	Yes	120–200	0.310	9.3
PIV-3	*Paramyxoviridae*	ss-RNA	Yes	150–300	0.209	6.3
MMV	*Parvoviridae*	ss-DNA	No	18–25	0.071	2.1
PPV	*Parvoviridae*	ss-DNA	No	18–25	0.055	1.7
REO-3	*Reoviridae*	ds-RNA, segmented	No	60–80	0.194	5.8

*Table modified from reference[9]

TABLE 3. Efficacy of gamma irradiation on frozen FBS.*

Virus	Family	Genome	Envelope	Particle Size (nm)	$\log_{10}$ Reduction in Titer per kGy	$\log_{10}$ Reduction per 30 kGy
Akabane	*Bunyaviridae*	ss-RNA, segmented	Yes	90–120	0.40	12
Aino	*Bunyaviridae*	ss-RNA, segmented	Yes	90–120	0.29	8.7
MMV	*Parvoviridae*	ss-DNA	No	18–25	0.093	2.8
FMDV**	*Picornaviridae*	ss-RNA	No	27–35	0.19	5.7
SVDV	*Picornaviridae*	ss-RNA	No	27–35	0.20	6.0
BTV	*Reoviridae*	ds-RNA, segmented	No	60–80	0.12	3.6
BEFV	*Rhabdoviridae*	ss-RNA	Yes	70×150	0.34	10
HCV	*Togaviridae*	ss-RNA	Yes	60–70	0.18	5.4

*From studies by House *et al*.[14] **Foot-and-Mouth disease virus (FMDV)

TABLE 4. Efficacy of gamma irradiation at typical dose levels for viral activation.*

Virus	Family	Genome	Envelope	Particle Size (nm)	Irradiation Dose Used (kGy)	Container Volume	Log_{10} Reduction in Titer Achieved	Reference
CVV	*Bunyaviridae*	ss-RNA, segmented	Yes	90–120	26–34	500 mL	≥ 5.4**	[16]
PCV-2	*Circoviridae*	ss-DNA, circular	No	15–22	30	500 mL	1.0**	[15]
					45	500 mL	1.0**	[15]
BVDV	*Flaviviridae*	ss-RNA	Yes	40–60	25–35	500 mL	≥ 4.3	[11]
					20	2 L	4	[17]
IBRV	*Herpetoviridae*	ds-DNA	Yes	120–200	25–35	500 mL	≥ 4.7	[11]
PIV-3	*Paramyxoviridae*	ss-RNA	Yes	150–300	25–35	500 mL	≥ 7.1	[11]
MMV	*Parvoviridae*	ss-DNA	No	18–25	25–35	500 mL	3.8	[11]
SV40	*Polyomaviridae*	ss-DNA, circular	No	40–45	26–34	500 mL	1.4**	[16]
BTV	*Reoviridae*	ds-RNA, segmented	No	60–80	25–35	500 mL	3.3	[11]
					25	500 mL	3.5–4.0	[17]
REO-3	*Reoviridae*	ds-RNA, segmented	No	60–80	26-34	500 mL	≥ 7.1**	[16]
FeLV	*Retroviridae*	ss-RNA, diploid	Yes	80–120	25–35	500 mL	3.3	[11]
					20	500 mL	3.0–4.0	[17]
					20	2 L	2.0–3.0	[17]

*Table modified from reference[9] **Average of three replicate spiked containers

As found for the viruses in **Table 3**, the largest viruses in this series (Aino, Akabane, and BEFV) were more effectively inactivated by gamma irradiation than the medium-sized or smaller viruses. The smallest virus of the series (MMV) displayed the lowest inactivation efficacy (the highest radio-resistance). Within this series, BTV was inactivated to a lesser extent than might have been predicted on the basis of its particle size and the results shown in **Table 2** for REO-3 (another member of the *Reoviridae* family).

The efficacy of viral inactivation at the dose levels typically employed commercially for gamma irradiation has been reported by a variety of investigators[11, 15-17] (**Table 4**). These results are indicative of industry experience with inactivation of viruses in frozen serum in 500 mL or 2 L containers. The results for PIV-3, REO-3, and BVDV are consistent with results from the dose-response studies (**Tables 2** and **3**). On the other hand, the inactivation results obtained for MMV were approximately an order of magnitude higher than might be expected from the dose-response data presented in **Tables 2** and **3**. The results obtained with FeLV, BTV, and SV40 indicated a lower inactivation efficacy than might be expected based solely upon the particle sizes for these viruses. PCV-2 exhibited minimal inactivation even at the relatively high gamma irradiation dose of 45 kGy.

A comparison of gamma irradiation efficacy for different virus families of concern is shown in **Figure 1**. The log_{10} reduction values shown are based on the consensus inactivation constants (log_{10} reduction in titer per kGy fluency; **Tables 2** and **3**) multiplied by the gamma irradiation doses modeled.

The most susceptible of the virus families depicted in **Figure 1** is the *Bunyaviridae*. These are relatively large (90–120 nm) enveloped viruses. The remaining virus families included in **Figure 1** are the relatively small, non-enveloped

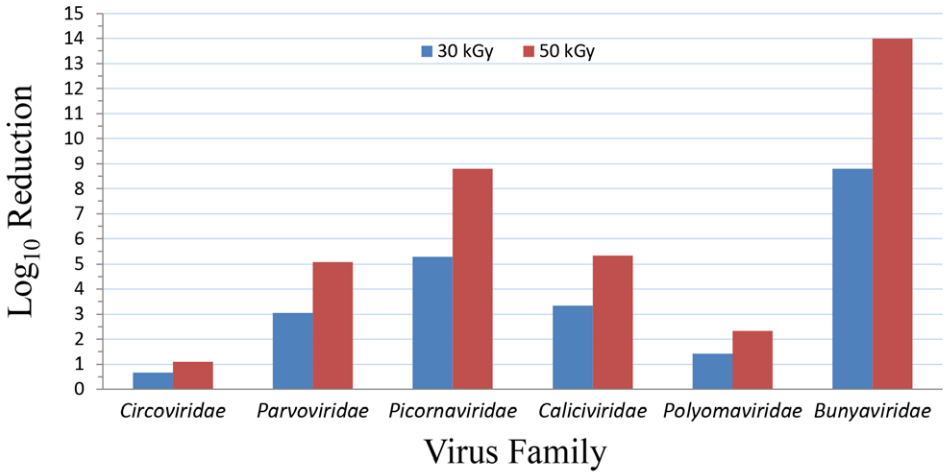

FIGURE 1. Comparison of the resistance of viruses to the direct effects of gamma irradiation in a frozen bovine serum matrix. The bars indicate the expected log_{10} reduction in titer resulting from irradiation doses of 30 and 50 kGy (which were modeled as they represent the typical fluency range used). The lower the bar, the more resistant the virus is to inactivation.

TABLE 5. Efficacy of gamma irradiation for mollicute inactivation in frozen bovine serum.*

Mollicute	Type of Bovine Serum Spiked	Irradiation Dose Used (kGy)	Log_{10} Reduction in Titer Achieved	Reference
Acholeplasma laidlawii	fetal	12.5–13.4	9.6	[12]
	fetal	17.0–17.8	>10	[12]
	fetal	7	6.0	[11]
	fetal	15	>8.0	[11]
Mycoplasma hyorhinis	calf	26–34	≥6.3**	[16]
Mycoplasma orale	calf	26–34	≥6.6**	[16]
Mycoplasma pneumoniae	calf	26–34	≥6.9**	[16]

*Table modified from reference[9] **Average of three replicate spiked containers

viruses that represent a high concern to users due to their overall resistance to inactivation. Of the various families evaluated, the *Circoviridae* and *Polyomaviridae* appear to be the most gamma irradiation-resistant. Due to the relative scarcity of empirical inactivation data for these two virus families, the modeled inactivation values depicted for them are associated with a higher degree of uncertainty than, for instance, those shown for the other families. For this reason, the *Circoviridae* and *Polyomaviridae* families may each be considered as worst-case challenge viruses for gamma irradiation in a frozen serum matrix.

Efficacy for Inactivation of Larger Microbes

Table 5 provides a summary of the limited efficacy data reported for inactivation of mollicutes by gamma irradiation. These studies have employed conditions, matrices, and packaging containers typical of those used for routine irradiation of serum batches. The overwhelming consensus conclusion from these experiments is that large organisms such as mollicutes (and, by implication, even larger organisms such as bacteria and fungi) may be completely inactivated at the lower end of the typical irradiation dose window used for serum treatment (30–50 kGy).

One cautionary note may be useful at this point. Pathogen inactivation methods (including gamma irradiation) are validated in terms of log_{10} reduction factors. By definition, efficacy defined in this manner can be thought of only as approaching (but not attaining) completeness. When investigators use the term "complete inactivation", it is not correct to interpret this as signifying 100% inactivation. The meaning of "complete" usually depends on the individual investigator but is often interpreted as ≥4 log_{10} reduction in infectious titer, or the inability to detect infectious virus after treatment (*i.e.*, below the assay limit of detection). Because of this, pathogen inactivation approaches such as gamma irradiation should be viewed as approaches for risk mitigation, not risk elimination.

As mentioned previously, the establishment of a useful irradiation dose window for serum treatment is not established solely upon inactivation efficacy results such as those described above. If this were the case, simply increasing the irradiation dose to, for instance, 100 kGy would enable the treatment to inactivate all potential contaminants, including even those representing the worst-case for this risk mitigation approach (*i.e.*, the members of the *Circoviridae* and *Polyomaviridae* virus families). However, the irradiation dose window to be used for routine serum treatment must take into account the potential damaging impacts on serum performance. Such impacts are typically investigated both by serum manufacturers and the end-users themselves, and will be described in the following sections.

3. Assessment of Serum Performance Following Gamma Irradiation

A limited set of performance characteristics are typically examined by serum manufacturers to determine if serum treated at proposed irradiation doses retain all required properties, from a cell culture performance point of view. End-users typically must evaluate the irradiated serum in their own cell culture applications to assure that equivalent responses are obtained in cultures maintained on the irradiated vs. non-irradiated serum. Examples of these types of evaluations are described below.

Analysis of Irradiated Serum by Serum Providers

It is expected that serum manufacturers operate with a high degree of understanding about product quality and safety along with confidence that the irradiated serum and irradiation process itself perform consistently based on predetermined standards. The key question that the serum manufacturer should address concerns serum comparability: *What is the impact of irradiation on the serum quality, safety, and efficacy (performance) in comparison to the same serum prior to irradiation?*

A list of analytical approaches to be considered by serum producers, when characterizing the quality, safety, and efficacy of irradiated serum, is provided below.

- Irradiation batch record review, especially the certificate of irradiation (CoI), and records of any in-process controls monitored during gamma irradiation such as:
  - Review of cold chain (temperature) monitoring records for product while in transit to and from the gamma irradiation facility, and shipping duration
  - Irradiation process duration

  - Revalidation of dosimetry after each replenishment of the source ^{60}cobalt
  - Dose-monitoring
- Product appearance, both in the frozen and liquid (thawed) state, looking particularly for:
  - Color changes
  - The presence of observable particular matter
  - Stratification and homogeneity after bottle mixing
- Serum filterability post-irradiation
- Full biochemical profiling to include pH, osmolality, and electrophoretic pattern (EPP) in order to understand specific changes in serum biochemistry (*e.g.*, references[17, 18])
- Cell culture performance (*e.g.*, references[10, 11, 17-20]) evaluations using a variety of cell types and culture systems that are most often used by biopharmaceutical and vaccine manufacturers
  - Cells should be maintained for at least 3–5 passages in medium supplemented with the irradiated serum.
  - Cells should be monitored for performance alongside the same cells maintained on medium supplemented with non-irradiated serum of the same lot.
  - Key performance indices should include, but not be limited to:
    - Cell morphology
    - Cell density
    - Doubling time
    - Metabolic profile
    - Cell viability
    - Genetic toxicology
- Stability of the irradiated serum, employing appropriate stability-indicating assays
- Extractable and leachable studies on the final serum packaging container (used for commercial lots) exposed to irradiation

A technical report summarizing this knowledge of the gamma irradiation process and the comparability in serum characteristics should be prepared. This may then be freely shared with potential end-users of the irradiated serum. Although serum manufacturers are not responsible for the development of a comparability strategy to be employed by serum end-users, the serum provider's report is typically requested by end-users to supplement their own comparability evaluations (see the next section).

Analysis of Irradiated Serum by Serum End-Users

Typically, end-users rely to some extent on the knowledge and technical information provided to them by the serum producers. However, serum users following good manufacturing practices (GMP) are required (*e.g.*, reference[21]) to conduct additional testing and characterization when switching from non-irradiated to irradiated serum. The key question GMP serum users need to address also concerns comparability: *What is the impact of the irradiated serum on the manufacturing process (cell culture and purification) and the drug substance/drug product in comparison with the same process employing non-irradiated serum?*

In order to address such a question, end-users of irradiated serum are expected to develop their own product-specific comparability strategies to satisfy regulatory requirements extant across various world regions. In general, the following testing and characterization of more than one serum lot are typically considered:

- Cell culture and purification performance testing using representative small-scale models and, if appropriate, larger engineering-scale models employing various in-process controls
- Biologic drug substance (active pharmaceutical ingredient), and where applicable, product testing, and characterization
- Process validation at appropriate scale (*e.g.*, GMP or other representative scale)
- Agency interactions and submission of comparability data for use of irradiated vs. non-irradiated serum
- Implementation in commercial manufacturing

When irradiated serum is used in the early phase of process development, the comparability testing exercise is not required. In this case, the analytical testing outlined above will be modified and built into the biologic drug substance process development. Therefore, the process development file should contain all relevant details on raw materials, cell culture, purification, and drug substance.

For end-users who are not manufacturing GMP products, some form of comparability testing may still need to be done simply to confirm that responses obtained in their particular cell culture applications are not substantially different when using irradiated vs. non-irradiated serum. This possibility should be explored before switching to irradiated serum.

Conclusions

This paper is part of a series of articles intended to provide more transparency around the processes involved in the gamma irradiation of serum. The topical series has been introduced in a separate paper in this journal.[22]

Gamma irradiation of animal serum products is a mature barrier (risk mitigation) technology that has been utilized for several years. Numerous serum manufacturers now offer irradiated serum commercially after having evaluated and/or validated the efficacy of gamma irradiation and the potential impacts on serum performance. Many serum end-users have been relying on irradiated serum in their specific product manufacturing processes as a means of mitigating the risks of encountering adventitious microbial contaminants. This treatment modality has been proven to effectively inactivate mollicutes as well as a range of common animal viruses (*e.g.*, bunyaviruses, retroviruses, herpesviruses, rhabdoviruses, flaviviruses, picornaviruses, and reoviruses). It has also been firmly established in various well-controlled studies that several virus groups

(*e.g.*, parvoviruses, polyomaviruses, and circoviruses) are more radio-resistant and are inactivated less effectively. For these reasons, gamma irradiation has been viewed as a means of "reducing" but not "eliminating" the viral risk associated with the use of serum in cell culture media.

While the serum industry evaluates biochemical, physicochemical, and performance variations post-irradiation, it is expected that end-users will develop their own product-specific compatibility and comparability strategies.

Validation of gamma irradiation is of paramount importance as it ensures a consistent and well-controlled serum treatment process while providing consistent quality, safety, and performance for various end-user applications. Historically, gamma irradiation process development and validation has consisted of:

- Viral inactivation
- Irradiated product evaluation
- Optimal irradiation dose-range determination
- Dose-mapping
- Product packaging
- Product temperature maintenance
- Product shipment conditions
- The irradiation process itself

While many of these aspects should be performed for any new irradiation process, a case can be made that not all need to be repeated for changes to existing processes. Under certain circumstances (*i.e.*, when the same types of viruses are spiked into the same types of matrices), and in agreement with end-users, the accumulated knowledge of virus susceptibility can be used to justify the evaluation of a more limited set of irradiation doses (*e.g.*, 30–50 kGy). Such an irradiation window can then be used for conducting irradiation process validation followed by thorough product characterization and performance evaluation in relevant application systems by the end-user.

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Note

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