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Abstract:	Researchers worldwide are working tirelessly to develop vaccines and therapeutics to battle the ongoing pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This virus is classified as Risk Group 3; work with this virus can therefore only be conducted in BSL-3 laboratories [1]. However, once inactivated, it can be handled safely in BSL-2 laboratories that are more available in comparison, where much needed viral countermeasure research can be done at an accelerated pace. Researchers use a variety of chemical, heat or irradiation treatments to inactivate viruses, which can then be brought out of high containment to conduct molecular and immunological analyses in lower containment laboratories. Gamma radiation is an ionizing radiation and has been commonly used by maximum containment laboratories to render high-risk group viruses inactive [2]. Gamma irradiation is often the preferred method as it is known to preserve viral morphology and viral protein integrity [3]. Here we sought to determine the radiation dose required for complete inactivation of SARS-CoV-2. The primary mechanism of virus inactivation by ionizing radiation is caused by breakage and crosslinking of genetic material [4-6]. Therefore, we also wanted to see if the gamma irradiation process damaged viral RNA leading to a change in RT-PCR sensitivity. A radiation dose of 1 Mrad was required to completely inactivate 106.5 TCID50/ml of SARS-CoV-2 with a calculated D10 value of 0.16 Mrad. The influence of gamma radiation on PCR sensitivity was inversely related and dose-dependent up to 0.5 Mrad, with no additional reduction thereafter

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Materials and Methods

SARS-CoV-2 (hCoV-19/Canada/ON-VIDO-01/2020, GISAID accession# EPI_ISL_425177) was cultured in a high containment laboratory on Vero cells, CCL-81 (ATCC, USA) grown in Minimum Essential Medium (Hyclone, USA) containing 1% fetal bovine serum and 1% L-glutamine. T150 tissue culture flasks with 80-90% confluent cells were infected with SARS-CoV-2 at 1:1000 dilution and incubated at 37°C with 5% CO_2 until 90% cytopathic effect (CPE) became evident (approximately 3 days). The flasks were then harvested and clarified by centrifugation at 6000 x *g* for 5 minutes. One ml aliquots of the clear supernatant were transferred to 2 ml cryovials tubes (Sarstedt, Germany) and stored at −80°C freezer until irradiation treatment.

Gammacell 220 Excel (MDS Nordion Inc. ON, Canada), a self-shielded irradiator with a cobalt-60 source was used for this study. The irradiator's drawer can accommodate a 2L beaker where virus-tubes were placed along with dry ice. The drawer moves down vertically to carry the samples to the sample chamber for irradiation. The irradiator's central absorbed dose rate was 0.114 Mrad/hr when these inactivation experiments were conducted in early April 2020. We used increasing radiation doses of 0, 0.25, 0.5, 1, 2, 3, 4, and 5 Mrads. After irradiation, samples were taken back to the high containment laboratory to determine the viable virus titer in median tissue culture infectious dose ($TCID_{50}$) assay on Vero cells as described previously [7]. Briefly, 100 µL of neat and ten fold dilutions of each treatment was transferred to 96 well plates and incubated as above and read 3 days later; the plates were read for CPE and the $TCID_{50}$ was calculated as per Reed and Muench [8]. Negative cultures were confirmed negative by a second passage on Vero cells and monitored for 3 additional days.
For the RT-qPCR assay, viral RNA from non-irradiated and irradiated samples was extracted with the Viral RNA Mini kit (Qiagen, Germany), serially diluted from 10^{-1} to 10^{-7} in 10 mM Tris EDTA and run on a LightCycler 96 (Roche, Germany). The EXPRESS One-Step Superscript RT-qPCR Universal Kit (Invitrogen, USA) was used with primers and probes targeting the envelope (E) and the nucleocapsid (NP) genes [9,

10]. Thermal cycling conditions were 50°C for 15 min for reverse transcription, followed by 95°C for 20 seconds and then 40 cycles of 95°C for 3 seconds, 60°C for 30 seconds.

Results

Irradiation dose required for SARS-COV-2 inactivation

Complete inactivation of SARS-COV-2 was achieved with 1 Mrad of radiation (Figure 1), which is consistent with previously published data for SARS-CoV-1 [2]. The dose required to reduce the viral titre by one log (D_{10} value) was determined from the slope of the regression line best fitting the dose curve of the virus from the $TCID_{50}$ units versus radiation dose (in Mrads). GraphPad Prism (GraphPad Software Inc.) was used for plotting, calculations and statistical analysis. Since a dose of 1 Mrad completely inactivated the virus, data from 2 Mrad and higher were not used for the D_{10} value calculations. The calculated D_{10} value for SARS-COVID-2 was 0.16 Mrad.

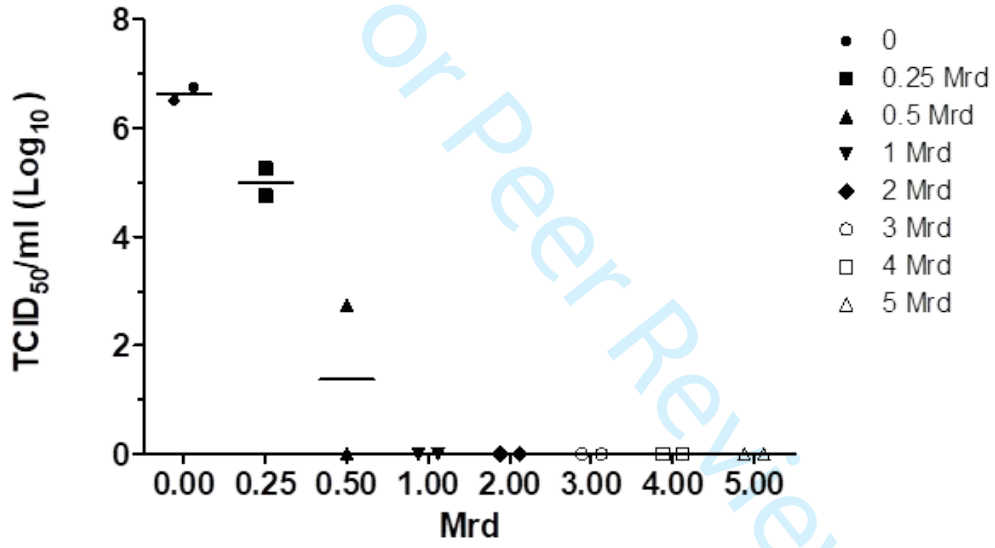

Figure 1. Inactivation of SARS-COV-2. One ml frozen samples containing $10^{6.5}$ $TCID_{50}$/ml of SARS-COV-2 virus were exposed to increasing doses of gamma radiation on dry ice.

Effect of high irradiation dose on PCR results

Researchers often expose their high-risk viral samples to many fold higher radiation dose than required for a “complete kill” for added safety. Such high doses of radiation can induce severe damage to the viral RNA leading to unexpected PCR results. Even though our virus sample was completely inactivated upon exposure to 1 Mrad, we subjected our samples to increasing doses of radiation up to 5 Mrad. Viral RNA preparations made from these samples were ten fold serially diluted and tested by RT-qPCR for NP and E genes and plotted against cycle threshold (Cq) (Figure 2).

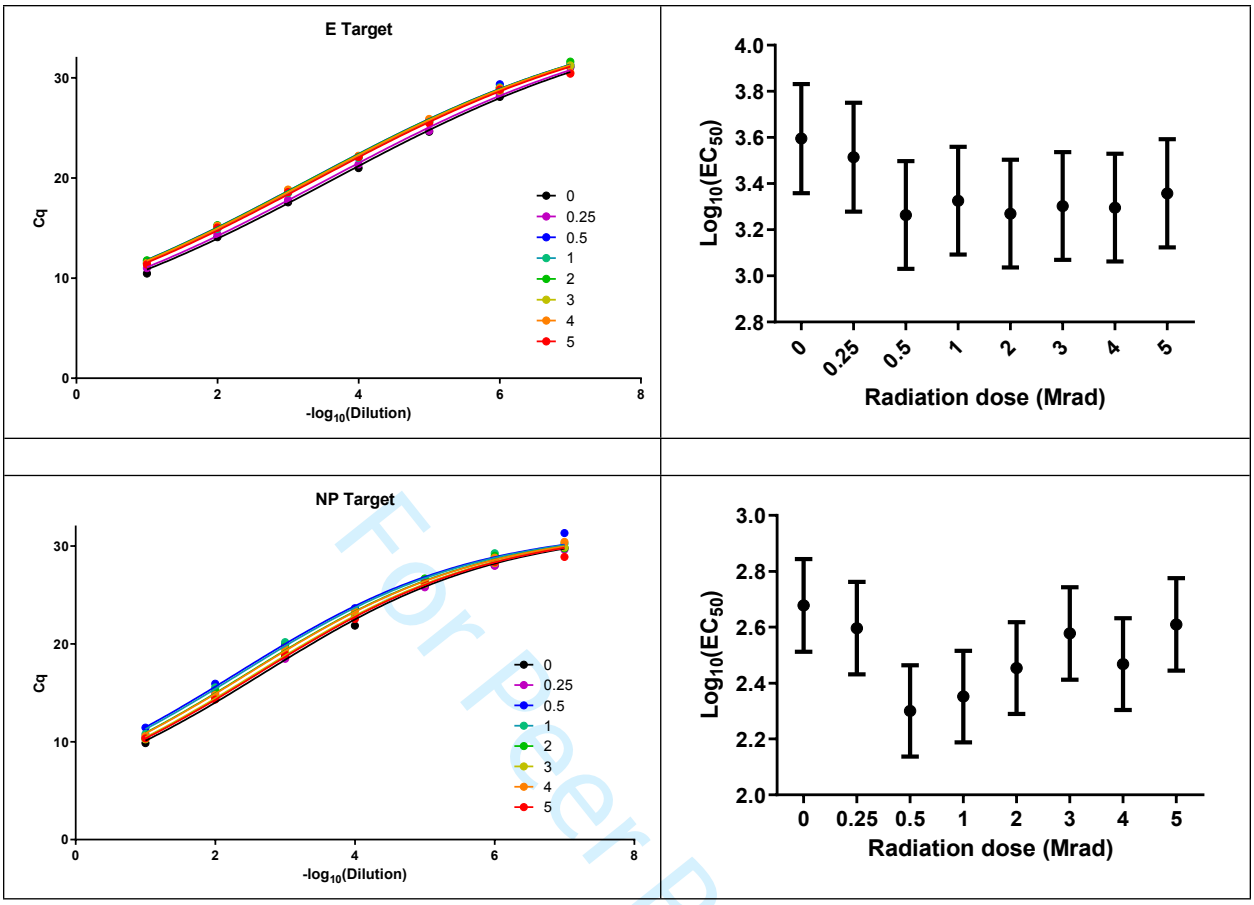

Figure 2. Influence of increasing doses of gamma radiation on PCR sensitivity. SARS-COV-2 viral samples were exposed to increasing doses of gamma radiation, RNA extracted from the irradiated samples were serially diluted (10 fold) and subjected to PCR testing. Dilution vs. cq for E and NP genes on the left panel; radiation dose vs. mid-point on the right panel.

As the plots followed a sigmoidal shape (s-shaped curve) that can be defined with 4 parameters (top and bottom asymptotes, slope, and mid-point (EC_{50}), we used GraphPad Prism to fit a 4-parameter curve to the Cq vs. $-\log_{10}$ Dilution. The three parameters Top, Bottom, and Slope are dependent on the RT-qPCR assay itself, not on the radiation dose; therefore, those parameters were shared across radiation doses within each assay. The EC_{50} was then the parameter that allowed us to evaluate the effect of radiation on the sensitivity of the RT-qPCR assay. If the EC_{50} decreased, more RNA would be required to reach the same Cq, due to damaged RNA targets being undetected in the assay. If the EC_{50} increased, then less RNA would be required to reach the same Cq, this could happen if radiation eliminated some secondary structures that would otherwise reduce reverse transcription efficiency. A slight dose dependent inhibition of PCR up to 0.5 Mrad of radiation dose was obvious (Figure 2, right panel).

Conclusions

Ionizing radiations interact with matter to generate free electrons and unstable ions, which in turn break the structures of the nucleic acids. In addition, ionizing radiation of biological material results in the formation of reactive hydroxyl radicals, which cause further damage to RNA, DNA and other cellular macromolecules resulting in cell death [11]. Microbial agents with larger genome are more sensitive to

ionising radiation compared to those with smaller ones. Since viruses have smaller genome in comparison to other infectious agents such as bacteria, they generally require higher doses of irradiation [12]. A measure called D_{10} value or D value (decimal reduction value) is used to describe the radiation dose required to inactivate 90 per cent (i.e. one log or one decimal) of the microbial agents in a given sample. The D_{10} value that was calculated for SARS-COV-2 was 0.16 Mrad, with complete inactivation achieved with 1 Mrad of absorbed radiation dose. Coronaviruses in general have a D_{10} value of <0.2 Mrads [13].

Sterilisation is often described as the inactivation of all microorganisms; however, it is in fact a probability. The effectiveness of the sterilisation process is measured in terms of the reduction of microbial population in a given sample over a set period, or dose in case of radiation. One Mrad of radiation was sufficient to inactivate $10^{6.5}$ $TCID_{50}$/ml SARS-COV-2 virus with a calculated D_{10} value of 0.16 Mrad. This calculated D_{10} value can be used to determine the dose required to achieve a predetermined sterility assurance level (SAL) in a sample with known microbial concentration. SAL is frequently used to describe the likelihood of a single microbial agent present in an inactivated sample [14]. For example, if a sample that contained 10^6 viruses is exposed to 6 times the D_{10} radiation dose, i.e., a dose required to inactivate 10^6 viruses, there is a chance that one viable virus remain in that sample. A sample is considered sterile if it is treated to achieve a sterility assurance level of 10^{-6}, where the chances of finding a viable microbial agent would be one in a million. In the case of a sample containing 10^6 $TCID_{50}$/ml SARS-COV-2, a gamma radiation dose of 1.92 (0.16*12) Mrad would achieve a SAL of 10^{-6}. There are several factors that could influence D_{10} values; frozen samples on dry ice require more radiation dose than samples on wet ice or at room temperature. Since our radiation treatments were done using frozen samples on dry ice, the D_{10} value determined here would represent the worst-case scenario for sample temperature during irradiation.

Researchers often expose high-risk virus samples from maximum containment laboratories to excessive amounts of radiation to achieve much higher SALs to be safe; an excessive radiation dose has the potential to damage the integrity of viral RNA or DNA. In this case, we wanted to test what effect, if any, such excessive radiation doses would have on PCR sensitivity. We found excessive radiation, up to 5 Mrads or >31 D_{10} radiation doses did not affect PCR results. However, there was discernable incremental reduction in PCR sensitivity up to 0.5 Mrad of radiation. Interestingly though, a dose of 0.5 Mrad or less would not have been sufficient to inactivate all the viruses in the sample; 1 Mrad was required for complete inactivation. Once all the viruses were inactivated at a required minimum dose of 1 Mrad, there was no further reduction in PCR sensitivity observed up to a dose of 5 Mrad. Gamma radiation is often the preferred choice for viral inactivation, as it is known to preserve the integrity of viral morphology and protein structures; whether this holds true for SARS-CoV-2 requires further research.

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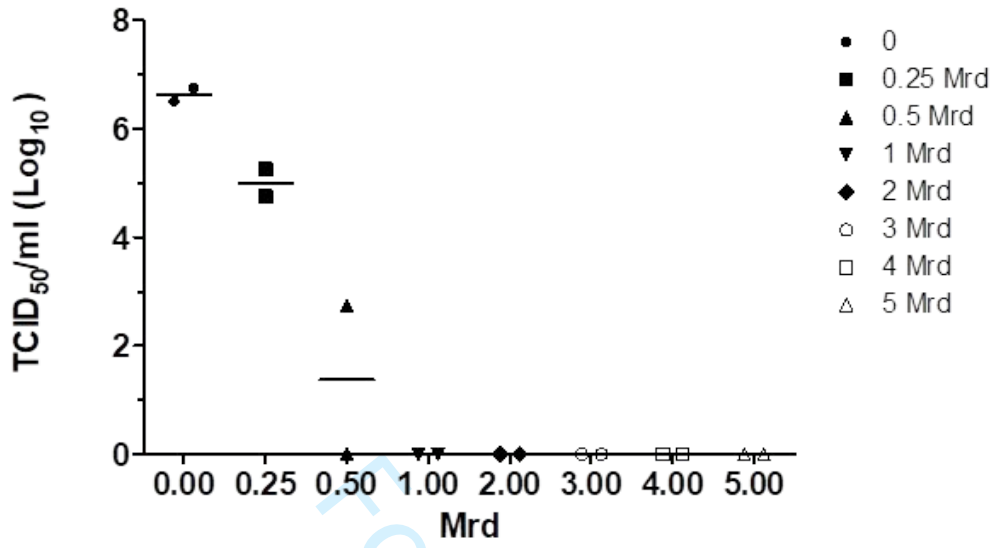

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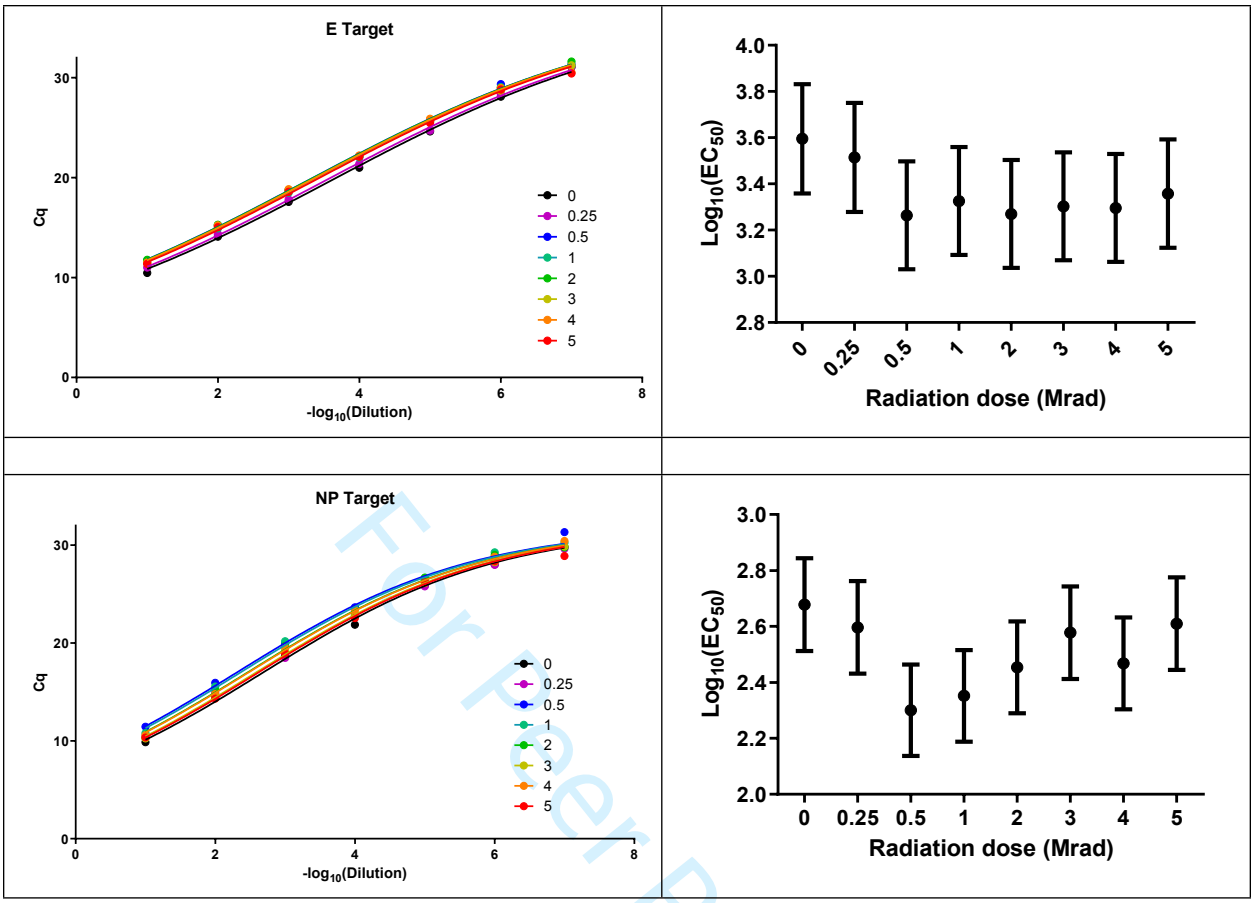

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