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Inactivation of porcine epidemic diarrhea virus with electron beam irradiation under cold chain conditions

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**Abstract**

The many instances of COVID-19 outbreaks suggest that cold chains are a possible route for the spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). However, owing to the low temperatures of cold chains, which are normally below 0°C, there are limited options for virus inactivation. Here, high-energy electron beam (E-beam) irradiation was used to inactivate porcine epidemic diarrhea virus (PEDV) under simulated cold chain conditions. This coronavirus was used as a surrogate for SARS-CoV-2. The possible mechanism by which high-energy E-beam irradiation inactivates PEDV was also explored. An irradiation dose of 10 kGy reduced the PEDV infectious viral titer by 1.68–1.76 log\(_{10}\) TCID\(_{50}\)/100 µL in the cold chain environment, suggesting that greater than 98.1% of PEDV was inactivated. E-beam irradiation at 5–30 kGy damaged the viral genomic RNA with an efficiency of 46.25%–92.11%. The integrity of the viral capsid was disrupted at 20 kGy. The rapid and effective inactivation of PEDV at temperatures below freezing indicates high-energy E-beam irradiation as a promising technology for disinfecting SARS-CoV-2 in cold chain logistics to limit the transmission of COVID-19.

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1. Introduction

Viruses are submicroscopic infectious agents that cause many serious diseases in humans. Inactivating viruses in the transmission route is an important way to prevent infectious diseases caused by viruses. The recent coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been a massive global health challenge. SARS-CoV-2 belongs to the Coronaviridae family of viruses and is an enveloped, single-stranded, positive-sense RNA virus. Many studies have demonstrated that temperature is the most influential factor for the survivability of coronaviruses in the external environment, and lower temperatures lead to slower viral inactivation (Aboubakr et al., 2021). Studies have demonstrated that coronaviruses can persist infectiously for several months or up...
to a year at 4 °C, and they lose little infectivity when kept at –60 °C for many years (Tennant et al., 1994; Casanova et al., 2009; Mullis et al., 2012). SARS-CoV-2 has been reported to remain viable for at least 14 days at 4 °C, while its inactivation time is 5 min at 70 °C (Chan et al., 2020; Chin et al., 2020). Riddell et al. recovered infectious SARS-CoV-2 after at least 28 days of incubation at 20 °C (Riddell et al., 2020). These observations suggest that the virus is stable in low-temperature environments.

Cold chain logistics generally refers to a system in which refrigerated and frozen products are maintained in a prescribed low-temperature environment. The standard temperature for frozen storage is –20 °C during production, storage, transportation, and sale. However, the low temperature in cold chain logistics favors the survival of SARS-CoV-2 (Shi et al., 2020; Dhakal et al., 2021). Thus, the cold chain could be a potential vehicle for coronavirus transmission, and object-to-human transmission through direct contact with contaminated cold chain products might play a role in SARS-CoV-2 transmission (Chen et al., 2022). The first known cases of COVID-19 identified in Wuhan, China, were mostly linked to the Huanan Seafood Market, where cold chain transportation was frequently used (Epidemiology Working Group for NCIP Epidemic Response, 2020). After controlling the COVID-19 outbreaks in Wuhan, several local outbreaks in Beijing, Kashgar, Tianjin, Dalian, and Qingdao, China showed strong connections to cold chain logistics (Liu et al., 2020; Chi et al., 2021; Ji et al., 2021). The index cases had no COVID-19 case contact history, but they had handled imported frozen products. Moreover, SARS-CoV-2 nucleic acid was detected on the surfaces of imported cold chain goods or containers related to these outbreaks (Council, 2020; Liu et al., 2020). Phylogenetic analyses suggested high genomic homology between the virus isolated from the infected cold chain workers and the virus detected on the packages' surfaces (Liu et al., 2020). Notably, viable SARS-CoV-2 was isolated from the outer package surface of imported cod in Qingdao (Chinese Center for Disease Control and Prevention, 2020; Yuan et al., 2020). Most recently, from January 15 to January 26, 2022, 75 new cases of COVID-19 were reported in Beijing, China, in which 43 patients were engaged in imported cold chain work (Jia, 2022). The evidence for SARS-CoV-2 transmission through the cold chain is suggestive but not conclusive. The World Health Organization (WHO)–China joint study listed the spread of SARS-CoV-2 from contaminated cold chain products to humans as a possible introduction and transmission route of the epidemic (Team, 2021). Accordingly, virus disinfection in the cold chain should be considered for controlling the spread of COVID-19 (Ji et al., 2021; Qian et al., 2022).

Virus disinfection in the cold chain environment is more challenging than in conventional environmental virus disinfection because the temperature is below freezing. Heating, chemical disinfection, and ultraviolet (UV) irradiation are the three most commonly used virus disinfection methods (Anelich et al., 2020; Han et al., 2021b). Heating obviously cannot be applied in cold chain logistics. Commonly used chemical disinfectants are generally only effective at room temperature, and they tend to freeze when applied at temperatures below freezing, resulting in a decreased disinfection efficiency (He et al., 2021). Moreover, most chemical disinfectants are toxic and odorous, and large-scale application will cause secondary contamination problems for cold chain products (Lee et al., 2008; Benítez et al., 2021). UV is another widespread disinfection method, and UV irradiation can reportedly inactivate SARS-CoV-2 at room temperature (Inagaki et al., 2020; Simmons et al., 2021). The major disadvantage of UV irradiation is its shallow penetration depth (Kühn et al., 2003). UV light has a low power of penetration, and the ice cover typically found on cold chain products markedly hinders the effectiveness of UV disinfection (Cockell et al., 2002; Han et al., 2021b). UV irradiation normally inactivates viruses on the surface of an object via direct irradiation, but if the virus is located in cracks, crevices, or is on the inner surface of the package, UV will be unable to fully inactivate the virus (Hirneisen et al., 2010; Gayán et al., 2013; Guerrero-Beltrán and Barbosa-Canovas, 2004; Bosch et al., 2018). Furthermore, unlike ordinary packaging, cold chain packaging commonly uses insulation materials to maintain a low temperature. Insulation materials also decrease the ability of UV irradiation to sterilize the outer packaging. Ozone-based disinfection technology has been suggested for use in cold chain logistics to inactivate SARS-CoV-2. However, ozone is highly corrosive, and prolonged exposure to high concentrations is lethal to humans (Turner et al., 2016). The large-scale application of ozone raises safety concerns and can damage the cold chain equipment. Therefore, it is urgent to develop an efficient, environmentally friendly method for disinfecting viruses in the cold chain environment to remove the potential for viral transmission through the cold chain.

Ionizing radiation is a form of radiation that has sufficient energy to remove electrons from atoms, leading to the formation of ions (Brahmakshatriya et al., 2009). Ionizing radiation has been approved by the US Food and Drug Administration to inactivate pathogens and spoilage microorganisms in food. Typical ionizing radiation such as gamma ray, X-ray, and electron beam (E-beam) radiation are used in food and medical processing (Yang et al., 2013). In E-beam radiation, high-velocity electrons are concentrated into a narrow beam with a very high power density. Unlike gamma radiation, E-beam radiation does not require radioisotopes (cobalt 60 or cesium 137) to generate ionizing radiation. For E-beam irradiation, ionizing radiation is generated by an electronic device called a linear accelerator. When the accelerator is switched off, no radioactive protection is needed. Thus, compared with gamma ray radiation, E-beam radiation does not produce radioactive waste and is more environmentally friendly (Praveen et al., 2013; Deng et al., 2020). Additionally, E-beam radiation is more energy efficient than X-ray radiation because X-rays are created by directing electrons at metal targets (Tahergorabi et al., 2014). Owing to the merits of E-beam irradiation, this technique has been used to inactivate microbes (Praveen et al., 2013; Predmore et al., 2015; Cusinato et al., 2016). E-beam irradiation has been shown to inactivate pathogenic bacteria in sewage, sludge, and even in food (Farooq et al., 1993; Praveen et al., 2013; Tahergorabi et al., 2014). Studies have shown that E-beam irradiation can efficiently disinfect enteric viruses, poliovirus-1 (VR-1562), and rotavirus (SA-11) in sewage sludge; murine norovirus 1, poliovirus, and rotavirus in fresh produce; murine norovirus in low-salt “jogaejeotgal”; human adenovirus in water; human norovirus in cabbage kimchi; and avian influenza virus in...
poultry (Braithakshatriya et al., 2009; Sanglay et al., 2011; Espinosa et al., 2012; Praveen et al., 2013; Han et al., 2021a; Jeong et al., 2021; Roque et al., 2022). However, these studies of E-beam irradiation were carried out at room temperature. It is possible that at temperatures below freezing the disinfection efficiency would differ from that at room temperature. It is not yet clear whether E-beam irradiation can efficiently disinfect coronavirus at temperatures below freezing, which might hinder the application of E-beam irradiation for disinfecting SARS-CoV-2 in the cold chain. 

To examine whether E-beam irradiation can efficiently inactivate SARS-CoV-2 in cold chain conditions, we evaluated the susceptibility of a SARS-CoV-2 surrogate, porcine epidemic diarrhea virus (PEDV), to E-beam irradiation on different surfaces in frozen conditions. SARS-CoV-2 and PEDV both belong to the Coronaviridae family of viruses. They are enveloped, positive-sense, single-stranded RNA viruses with similar genome sizes. Previous studies have reported that the genome size and the envelope structure are two major factors that affect virus inactivation using ionizing radiation (Feng et al., 2011; Feldmann et al., 2019). Thus, we assumed that ionizing radiation would have a similar inactivation efficiency towards PEDV and SARS-CoV-2. Our data showed that an irradiation dose of 10 kGy effectively inactivated PEDV on the top and bottom surfaces of commonly used wrapping materials in freezing conditions. After the E-beam irradiation treatment, massive RNA breakage was observed in the PEDV genome. There was also considerable capsid damage under high-dose irradiation, suggesting a mechanism by which E-beam irradiation inactivates coronaviruses. These findings will help design more efficient E-beam disinfection equipment for inactivating SARS-CoV-2 in the cold chain environment.

2. Materials and methods

2.1. Virus propagation and cell culture

Vero E6 cell was a gift from Dr. George F. Gao in the Institute of Microbiology, Chinese Academy of Sciences. Cells were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 200 mg/mL streptomycin, 200 IU/mL penicillin and cultured at 37 °C with 5% CO₂. Porcine Epidemic Diarrhea virus (PEDV) strain PEDV-HB3 was a gift provided by Dr. Jinghua Yan in the Institute of Microbiology, Chinese Academy of Sciences. All the viral experiments were performed in a biosafety level-2 (BLS-2) laboratory.

For the PEDV-HB3 virus propagation assay, 100 µL PEDV-HB3 virus solution was added to the overnight cultured Vero E6 cells and incubated at 37 °C with 5% CO₂ for 1 h to allow virus particle to attach to the Vero E6 cells. After removing the media (DMEM containing unabsorbed viruses) and washing the cells with PBS, 20 mL cell culture maintenance medium (DMEM containing 7 ng/µL Trypsin) was added to the cells and incubated for 24–48 h to propagate the virus. We performed three cycles (alternating cycles of −80 °C and 25 °C) of freezing and thawing to separate the virus from the Vero E6 cells (Trudeau et al., 2016). Cellular debris was removed by centrifugation at 800 g for 5 min and then filtered through a 0.22 µM filter. The harvested virus was aliquoted and stored at −80 °C until use. Then the virus titers were determined by TCID₅₀ (median tissue culture infective dose) assay described in Section 2.4.

2.2. Stability of PEDV on the surfaces at −20 °C

Surface stability was assessed on corrugated cardboard, polystyrene plastics and polystyrene foam representing a variety of cold-chain transport materials and was carried out as described by Neeltje van Doremalen et al. (2020) with minor modifications (Neeltje van Doremalen et al., 2020). In short, 50 µL of PEDV with a titer of 10^1.15 TCID₅₀/100 µL was deposited on the surface. After being placed at −20 °C for 7, 14, and 30 days, 1 mL of DMEM was added to recover the inoculated virus. Virus titer was immediately measure by TCID₅₀ assay described in Section 2.4. All experiments were performed in at least triple repeats.

2.3. Inactivation of PEDV by E-beam irradiation

PEDV-HB3 with a titer of 10^2 TCID₅₀/100 µL was deposited on the top or bottom surfaces of three cold chain packing materials (corrugated cardboard, polystyrene plastics and polystyrene foam) that had been pre-chilled at −20 °C for 3 h. The thickness of corrugated cardboard, polystyrene plastics and polystyrene foam used in the experiments was 4.9 mm, 0.1 mm, 18.2 mm respectively. The samples were set on dry ice to maintain the frozen condition during the whole experimental process. Meanwhile, the samples were covered by sterile Saran wrap to avoid contamination of other pathogens in the experimental process. The 10 MeV electron accelerator (2 mA beam current) used in this study was designed and manufactured by the Chinese Academy of Atomic Energy. The distance between scan box and samples was 50 cm, and the scanning width is 70 cm. The samples were irradiated with the predefined doses of 0, 2, 5, 10, 20, 25, and 30 kGy at the temperature below −20 °C. The irradiation dose was determined according to the manufacturer’s guide. After E-beam irradiation, the samples were stored in −80 °C until performing subsequent analysis. All experiments were performed in at least triple repeats.
2.4. Viral infectivity detection by TCID_{50} assay

The virus titer was determined using median tissue culture infective dose (TCID_{50}) assay (Tian et al., 2013). Briefly, one hundred microliters of tenfold serially diluted virus stock (each dilution had eight replicates) infected overnight cultured Vero E6 cells in a 96-well plate for 72 h. After incubation, the plate was examined for the virus-induced cell cytopathic effect (CPE) in each well via microscopy. Virus titers were calculated using the Karber method (Karber, 1931). Similarly, virus titer after E-beam irradiation were also determined by TCID_{50} assay. According to the requirements of the "National Drug Administration Note [2002] No. 160" of China, if the virus titer is reduced by less than 4 Log_{10} TCID_{50}/100 μL, the inactivated virus samples should be passaged at least twice in fresh cells to ensure that no infectious virus remains. Therefore, even if the post-E-beam irradiated viruses did not cause CPE, the virus was also harvested and inoculated into the cells for infection as described before. Three generations of serial passage were performed to check the CPE. Only when no CPE was observed during the three serial passages, the virus was considered to be inactivated.

2.5. Viral capsid damage was determined by RNase assay

We conducted a ribonuclease (RNase) assay that was modified from Oh et al. (2020) and Elbashir Araud et al. (2020) to evaluate the integrity of the PEDV-HB3 capsid (Elbashir Araud et al., 2020; Oh et al., 2020). For this assay, a 140-μL volume of unirradiated or irradiated PEDV-HB3 was added to a 1.5-mL RNase-free microcentrifuge tube. Next, 2.8 to 30 μL of RNase (1 mg/mL, Genstar) was added to each tube. After the mixture was incubated in a water bath at 37 °C for 30 min, 1 μL of RNase inhibitor (40 U/μL, Lablead) was added to the mixture and incubated at room temperature for another 30 min. Here, we used the RNase inhibitor to remove the remaining RNase from the reaction. Correspondingly, 140 μL of virus before RNase treatment was used as control. Viral capsid damage was determined by the reduction in viral RNA copy number caused by RNase degradation.

RNA was extracted from 140 μL of RNase-treated PEDV-HB3 solution using a QIAamp Viral RNA mini kit (QIAGEN), following the manufacturer's instructions. Accordingly, 140 μL of virus without RNase treatment was used as a control. The extracted RNA was quantified by a HiScript II U+ One Step qRT-PCR Probe Kit (Vazyme) according to the manufacturer's instructions. The primers and the TaqMan probe targeting N gene of PEDV-HB3 were listed in Table 1. Thermal cycling was carried out on the Applied Biosystems 7500 Fast Real-Time PCR system (ViiA 7) as follows: reverse transcription at 55 °C for 5 min, denaturation of the RT polymerase at 95 °C for 30 s, and 45 cycles of qPCR at 95 °C for 5 s and 60 °C for 20 s. Then, we used a PEDV-HB3 standard curve to quantify the RNA copy number. Briefly, the PCR fragment of N gene purified from agarose gel electrophoresis using E.Z.N.A. Gel Extraction kit (Omega) was used as the standards. After measuring the concentration of the standards with a spectrophotometer Nanodrop (Eppendorf), the copy number of the standards was calculated using the following formula:

\[
\text{copy number} = \frac{C \times NA}{MW}
\]

C: The concentration of the standard.
NA: 6.02 × 10^{23}
MW: The average molecular weight of a standard.

These standard samples were serially diluted 10-fold and subjected to the real-time RT-qPCR analyses together with the PEDV-HB3 samples to construct standard curves for calculation. Copy numbers of each RNase-treated PEDV-HB3 sample was divided by that of control PEDV-HB3 (samples were not treated with RNase) to calculate the copy number ratio. Assays were performed in triplicate.

2.6. Virus genomic damage was determined by long-range RT-qPCR assay

We conducted a long-range RT-qPCR assay to analyze the effect of E-beam irradiation on the viral genome. RNA was extracted from 140 μL of virus samples irradiated with different doses. Then, we used SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) to perform reverse transcription on 3 μL volume of RNA according to the standard protocol of the manual. This long-range reverse transcription with specific reverse transcription primer located at 21,594 bp of the PEDV-HB3 genome was carried out to ensure at least 8 K cDNA products from viral genome (Table 2). The qPCR primers were designed with Primer3Plus (http://www.primer3plus.com/cgi-bin/dev/primer3plus.cgi). Three qPCR primer sets were designed at 746 bp, 3655 bp and 7674 bp upstream of the reverse transcription primer site, respectively (Table 2). Viral cDNA was then subjected to qPCR using Hieff™ qPCR SYBR® Green Master Mix (Yeasen), following the manufacturer's instructions. Thermal cycling was carried out on the Applied Biosystems 7500 Fast Real-Time PCR system (ViiA 7) as follows: initial denaturation at 95 °C for 5 min; 40 cycles of denaturation at 95 °C for 10 s, annealing and extension at 60 °C for 30 s; and temperature increase for melting curve from 65 °C to 95 °C. We created the standard curves as described in Section 2.5 with minor modification to quantify the copy number. The PMD18-T plasmid containing S gene fragment was constructed as the standard (Table 2). The copy number ratio was calculated as described in the subsection of Section 2.5. The viral genomic damage rate was calculated as follows:

\[
\left(1 - \frac{\text{Copy number of irradiated viral RNA}}{\text{Copy number of control viral RNA}}\right) \times 100\%
\]

All experiments were performed in at least triple repeats.
Table 1

| Step                  | Primer or standard | Sequence (5'-3')                  | Position in the genome | Fragment size (bp) |
|-----------------------|--------------------|-----------------------------------|------------------------|-------------------|
| Quantitative PCR for N gene of PEDV-HB3 | Forward primer | CGCAAAGACTGAAACCCACCTAA          | N (26684–26704)       |                   |
|                       | Reverse primer | TTGCCTCTGGTTTACTCGGGGAT           | N (26858–26881)       |                   |
|                       | TaqMan probe    | FAM-TGTGGCACATGGCCAGGACTC CTGC-TAMRA |                        |                   |
| Standard curve for N gene of PEDV-HB3 | Standard sequence for N gene of PEDV-HB3 | GGGTTTGCTGGTGTCTGAAGGC GCCAAGACTGAAACCCACCTAA |                        | 787               |

The part of the primer targeting sequence of the standard curve is listed in the table. Underlined sequence, the sequence to which primers attach. The linear range of the standard curve is 10^3 copies/µL to 10^9 copies/µL.

Table 2

| Step                  | Primer or standard | Sequence (5'-3')                  | Position in the genome | Fragment size (bp) |
|-----------------------|--------------------|-----------------------------------|------------------------|-------------------|
| Reverse transcription for PEDV-HB3 RNA | Reverse primer | ACCTCAGACGCTCTGTTG             | S (21578–21594)       | >8000             |
| Target site 1         | Forward primer 20k-F | CACCAGGTGCTCACTAACA             | S (20702–20721)       |                   |
|                       | Reverse primer 20k-R | GGATGTTGGCCAGCAGCA             | S (20829–20848)       |                   |
| Target site 2         | Forward primer 18k-F | GCCGTGCATACATCTGTTG             | ORF1a/b (17818–17837) |                   |
|                       | Reverse primer 18k-R | TGCTCATGGTGTTAAGGCT             | ORF1a/b (17920–17939) |                   |
| Target site 3         | Forward primer 14k-F | ACTTGCAGCGCTAGTTACAG             | ORF1a/b (13767–13786) |                   |
|                       | Reverse primer 14k-R | TGCATACCTCTGCTGCAA             | ORF1a/b (13901–13920) |                   |
| Standard curve        | Standard sequence  | GGTGTCTGGTGTCTGTTG             | S (20681–20880)       | 248               |

The part of the primer targeting sequence of the standard curve is listed in the table. Underlined sequence, the sequence to which primers attach. The linear range of the standard curve is 10^3 copies/µL to 10^9 copies/µL.

2.7. Statistical analysis

All assays were performed in at least triple replicates. Student's t-test was used to evaluate the significance between datasets of TCID_{50} assay. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used to determine the significance in RT-qPCR. P value less than 0.05 indicates a significant difference between the two datasets.

3. Results

3.1. PEDV-HB3 remained infectious for at least 30 days at −20 °C

To assess the stability of coronavirus under cold chain conditions, PEDV-HB3 was inoculated onto the surfaces of three common cold chain packaging materials—corrugated cardboard, polystyrene plastic, and polystyrene foam—with
an inoculum of $10^{4.15}$ TCID$_{50}$/100 μL and kept at −20 °C. We collected samples at specified time points and analyzed the infectious activity of the virus through a TCID$_{50}$ assay. As shown in Fig. 1, PEDV remained viable on the package surfaces throughout 30 days of storage at −20 °C. The infectious PEDV titer was reduced by only 2 to 3 log$_{10}$ TCID$_{50}$/100 μL after 30 days (from $10^{4.15}$ to $10^{1.58}$ TCID$_{50}$/100 μL). After 30 days on polystyrene plastic, and from $10^{4.15}$ to $10^{1.54}$ TCID$_{50}$/100 μL after 30 days on polystyrene foam). After the same inoculum amount of PEDV was stored in a cryovial at −20 °C for 30 days, the virus titer was decreased by 1–2 log$_{10}$ TCID$_{50}$/100 μL (supplementary S1). Virus on the various surfaces showed differences in stability. After 30 days, the infectious viral titer on the porous materials (corrugated cardboard and polystyrene foam) showed a larger reduction than that on the non-porous material (polystyrene plastic) after 30 days (Fig. 1). The chemical and physical characteristics of the packaging materials affected virus viability. However, the observed differences might have partially resulted from the recovery efficiency of virus from the different surfaces because we observed a discrepancy in recovery efficiencies at 10 min after inoculation with PEDV (supplementary S2).

### 3.2. E-beam irradiation inactivated PEDV-HB3 at doses higher than 10 kGy

To test the coronavirus inactivation efficiency of E-beam irradiation under cold chain conditions, PEDV-HB3 deposited on the three common cold chain packaging materials on both the top and bottom surfaces were irradiated with predefined E-beam doses at −20 °C. TCID$_{50}$ assays were then carried out to quantify the infectious viral titer. A marked cytopathic effect was produced in cell cultures by PEDV that did not undergo E-beam irradiation (Fig. 2A). The infectious titers are shown in Table 3. In contrast, no cytopathic effects were observed in the cell cultures infected by PEDV samples that underwent 10, 20, 25, and 30 kGy irradiation. These cell cultures showed normal morphology that was the same as the cells that did not receive viral treatment (Fig. 2A). As shown in Table 3, the infectious titers of these irradiated virus were ≤ −0.5 log$_{10}$ TCID$_{50}$/100 μL. After treating with PEDV irradiated with 2 kGy or 5 kGy, a small number of cells displayed a marked cytopathic effect (Fig. 2A). The corresponding infectious viral titers are shown in Table 3. In PEDV that received a 2 kGy radiation dose, the infectious titer was reduced by 96.3%, while the titer was reduced by 96.9% with a dose of 5 kGy (Table 3). No significant differences in the reduction ratios of infectious titer were observed for PEDV inoculated on the top surfaces of the different packaging materials (Fig. 2C, p > 0.05). Similarly, no significant differences in infectious titer reduction were found between PEDV deposited on the top and bottom surfaces (Fig. 2C, p > 0.05). These data suggest that a rather low dose of E-beam irradiation (10 kGy) largely inactivates PEDV-HB3. The E-beam irradiation penetrated least 18.2 mm into the polystyrene foam, 4.9 mm into corrugated cardboard, and 0.1 mm into polystyrene plastic under the cold chain conditions. The 10 kGy dose reduced the virus titer by 1.68–1.76 log$_{10}$ TCID$_{50}$/100 μL (Table 3). The kinetics curve of PEDV-HB3 inactivation showed that the level of PEDV-HB3 inactivation corresponded to an increasing irradiation dose. At an irradiation dose of 10 kGy or higher, no cytopathic effect was observed in the cell culture plates (Fig. 2A). According to the requirements of the “National Drug Administration Note [2002] No. 160” of China, if the virus titer is reduced by less than 4 log$_{10}$ TCID$_{50}$/100 μL, the inactivated virus samples should be passaged at least twice in fresh cells to ensure that no infectious virus remains. Therefore, three serial passages were carried out for the virus samples irradiated with 10, 20, 25 and 30 kGy. As shown in Table 4, cytopathicity was not observed in any of the cell cultures in any passage. Taken together, these data indicate that a 10 kGy irradiation dose effectively inactivated the PEDV-HB3 on both the top and bottom surfaces of commonly used packaging materials in a simulated cold chain condition of −20 °C.

### 3.3. High-dose E-beam irradiation disrupted PEDV-HB3 capsid integrity

To elucidate the mechanism by which E-beam irradiation inactivates PEDV, we used an RNase assay to test whether this radiation directly disrupted the integrity of the viral capsid. In the assay, an intact capsid prevents RNase from
accessing the viral RNA, but the RNase is able to degrade the viral RNA if the viral capsid is disrupted (Elbashir Araud et al., 2020; Oh et al., 2020). Damage of the viral capsid was determined based on the reduction in viral RNA copy number caused by RNase degradation. Briefly, PEDV-HB3 was treated with E-beam irradiation (0, 5, 10, 20, 25, and 30 kGy). After treatment, RNase was used to degrade RNA in the disrupted virions, followed by reverse transcription quantitative PCR (RT-qPCR) to quantify the viral RNA copy number. PEDV was deposited onto the top surface of polystyrene plastic and treated with predefined doses of E-beam irradiation (0, 5, 10, 20, 25, and 30 kGy) at −20 °C. The reduction in viral RNA copy number was not significantly different between the control group (0 kGy) and the low-dose irradiated groups (5 kGy and 10 kGy) \((p > 0.05)\) (Fig. 3). However, at 20, 25, and 30 kGy, the reduction in viral RNA copy number was significantly higher than that of the control group (0 kGy) \((p < 0.05)\). These results suggest that disruption of the PEDV capsid required a high dose of E-beam irradiation. E-beam irradiation of 10 kGy did not obviously damage the PEDV-HB3 integrity; however, we did observe that the 10 kGy dose effectively inactivated PEDV. These data imply that viral capsid damage was not the major mechanism by which the 10 kGy E-beam induced PEDV inactivation. It is possible that minor damage to the integrity of the PEDV-HB3 capsid by low-dose radiation was beyond the detection limits of the RNase assay.

Fig. 2. Deactivating effects of E-beam-irradiation on PEDV-HB3. (A) Cytopathic changes in Vero E6 cells inoculated with the PEDV-HB3 without E-beam irradiation (0 kGy), or with E-beam irradiation with 2, 5, 10, 20, 25 or 30 kGy. The representative images of cell cultures are shown. (B) Kinesics curve of inactivation of PEDV-HB3 by E-beam irradiation. The mean of infectious titers was plotted. Error bars represent the standard deviation (SD) of triple replicate experiments. (C) Analysis of the significant difference of viral titer reduction on various materials or on the top and bottom surfaces after predefined dose E-beam irradiation. Student’s t-test was used to evaluate the significance between datasets of TCID₅₀ assay. P value less than 0.05 indicates a significant difference between the two datasets.
Table 3
PEDV-HB3 virus titer (Log10 TCID50/100 µL) after irradiation at predefined dose.

| Materials          | Surface | Irradiation dose (kGy) | 0   | 2   | 5   | 10  | 20  | 25  | 30  |
|--------------------|---------|-------------------------|-----|-----|-----|-----|-----|-----|-----|
| Polystyrene Foam   | Top     | 1.26 ± 0.246            | −0.22 ± 0.121 | −0.35 ± 0.121 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 |
|                    | Bottom  | 1.18 ± 0.538            | −0.17 ± 0.088 | −0.26 ± 0.146 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 |
| Corrugated Paper   | Top     | 1.25 ± 0.313            | −0.24 ± 0.042 | −0.25 ± 0.088 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 |
|                    | Bottom  | 1.18 ± 0.300            | −0.15 ± 0.055 | −0.24 ± 0.075 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 |
| Polyethylene Plastic| Top     | 1.22 ± 0.214            | −0.23 ± 0.116 | −0.32 ± 0.066 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 |
|                    | Bottom  | 1.22 ± 0.285            | −0.26 ± 0.116 | −0.28 ± 0.104 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 |
| Mean               | −       | 1.22 ± 0.318            | −0.21 ± 0.099 | −0.28 ± 0.106 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 | ≤−0.5 |
| Virus titer reduction ratio (%)<sup>c</sup> | −   | 96.3 | 96.9 | ≥98.1 | ≥98.1 | ≥98.1 | ≥98.1 | ≥98.1 |

<sup>a</sup> The lowest detecting threshold (≤−0.5 Log10 TCID50/100 µL).
<sup>b</sup> Data in the table represent the mean ± standard deviation (SD).
<sup>c</sup> (1–10<sup>−Nt</sup> × 100 (%) where N<sub>t</sub> is the Log10 TCID50/100 µL value of the E-beam-irradiated virus and N<sub>0</sub> is the Log10 TCID50/100 µL value of the virus without E-beam irradiation.

Table 4
The induced cytopathiafter three passages following the inoculation of treated PEDV.

| Materials          | Surface | Irradiation dose (kGy) | 0 | 10 | 20 | 25 | 30 |
|--------------------|---------|-------------------------|---|----|----|----|----|
| Polystyrene Foam   | Top     | +                       | − | −  | −  | −  | −  |
|                    | Bottom  | +                       | − | −  | −  | −  | −  |
| Corrugated Paper   | Top     | +                       | − | −  | −  | −  | −  |
|                    | Bottom  | +                       | − | −  | −  | −  | −  |
| Polyethylene Plastic| Top     | +                       | − | −  | −  | −  | −  |
|                    | Bottom  | +                       | − | −  | −  | −  | −  |

Cytopathogenic effect, CPE: +.

Fig. 3. The effect of E-beam irradiation on the PEDV-HB3 capsid. The integrity of viral capsid was determined by the reduction of viral RNA copy number which was caused by RNase treatment. The reduction ratio of viral RNA copy number was defined as the ratio of viral RNA copy number before and after RNase treatment. Experiments of indicated dose (0, 5, 10, 20, 25 and 30 kGy) were repeated at least three times as described in Methods and Materials. Each solid point represents the result from an independent experiment. Significance was determined using a one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisonstest (p < 0.05). Error bars represent the standard deviation (SD) of replicate experiments.

3.4. E-beam irradiation damaged the PEDV genome

Previous studies have shown that damage to the viral RNA genome is strongly correlated with virus infectivity (Elbashir Araud et al., 2020). E-beam radiation results from machine-accelerated electrons, which are capable of breaking viral RNA strands, thus inactivating viruses. Therefore, the mechanism of virus disinfection by E-beam irradiation could be through direct breakage of the viral RNA material. To prove this hypothesis, we conducted a long-range RT-qPCR assay to investigate the integrity of the PEDV-HB3 genome after exposure to E-beam irradiation. Given that photons and electrons can cause random breaks or other damage to the viral RNA strands during E-beam irradiation, we designed three sets of qPCR primers located 746 bp, 3655 bp, and 7674 bp upstream of the reverse transcription (RT) priming site to evaluate the integrity of the viral genome (Fig. 4A, Table 2). The target site of primer set 1 was located in the S gene, which encodes 

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The target sites of primer set 2 and primer set 3 were located in ORF1a/b, which encodes the replicase (Fig. 4A). As shown in Fig. 4B–D, all three target sites showed that the E-beam irradiation damaged the viral RNA in a dose-dependent manner. For the long-range RT-qPCR target site that was 746 bp from the RT priming site, the 5 kGy dose did not significantly decrease the abundance of intact RNA compared with the non-treatment control group (Fig. 4B). This might have been because the target region was too close to the RT priming site, resulting in a low chance of viral RNA genome damage in the 746 bp-long region. The target region located further from the RT priming site would better reflect the accuracy of the evaluation because the RT product is greater than 8000 bp. For the target site 3655 bp from the RT priming site, the 5 kGy irradiation dose significantly decreased the abundance of intact RNA (Fig. 4C). As shown in Fig. 4D, E-beam irradiation with 5, 10, 20, 25, and 30 kGy doses decreased the intact RNA abundances by 46.25%, 62.92%, 86.63%, 92.11%, and 90.00%, respectively. These results indicate that genome damage might be why the E-beam irradiation induced PEDV inactivation.

4. Discussion

Despite some uncertainty, the COVID-19 pandemic has highlighted cold chain logistics as a possible route for viral transmission. Reports have shown that SARS-CoV-2 nucleic acid has been isolated from cold chain frozen food and packaging surfaces, and viable SARS-CoV-2 has been detected on the outer surfaces of cold chain packages (Liu et al., 2020). These findings have increased public attention for the potential for viral spread through cold chain logistics (Han et al., 2020). Low temperatures have been reported to favor virus stability (Casanova et al., 2010; Chan et al., 2011; Biryukov et al., 2020; Matson et al., 2020; Riddell et al., 2020; Morris et al., 2021; Sharma et al., 2021). SARS-CoV-2 has been shown to survive at −20 °C for 21–30 days (Feng et al., 2021; Huang et al., 2021; Qian et al., 2022). Our study also demonstrated that PEDV, a surrogate for SARS-CoV-2, remained viable on three types of cold chain packaging material for at least 30 days at −20 °C. Most cold chains are required to maintain a temperature below −20 °C. Thus, it is not surprising that SARS-CoV-2 can survive for a long time on the surfaces of these packages and could infect stevedores or other workers through direct contact with cargo. Therefore, virus disinfection in the cold chain is necessary to protect public health. In this study, we demonstrated that E-beam irradiation, also known as cold pasteurization, is an effective method for inactivating coronavirus under cold chain conditions.

It is much harder to disinfect viruses under cold chain conditions than at room temperature. Heating cannot be used to inactivate viruses in cold chain logistics, and the effectiveness of chemical disinfectants is considerably reduced by the low temperatures needed for cold chains (He et al., 2021). In addition, the shallow penetration of UV light limits its ability to disinfect viruses in frozen conditions (Cockell et al., 2002; Kühn et al., 2003). Our study showed that a 10 kGy dose of E-beam irradiation effectively inactivated PEDV-HB3 on both sides of commonly used packaging materials at −20 °C.
Moreover, E-beam irradiation is produced by a linear electron accelerator rather than by radioactive isotopes; therefore, it is more environmentally friendly than gamma ray radiation (Tahergorabiet al., 2014). Studies have also shown that the time needed for disinfection by E-beam irradiation is generally shorter than for gamma irradiation because of the higher doses of E-beams ($10^2-10^6$ Grays$^{-1}$) than gamma rays (0.1–1 Grays$^{-1}$) (Barbosa-Cánovas et al., 1998; Fellows, 2000; Hansen and Shaffer, 2001; Sanglay et al., 2011; Tahergorabiet al., 2014). The precise dosage of E-beam irradiation is also easier to control than gamma rays, making this method a good fit for disinfecting sensitive products such as food in cold chain logistics.

Below freezing, the 10 kGy E-beam irradiation dose inactivated PEDV on two surfaces of corrugated cardboard, polystyrene plastic, and polystyrene foam. The 10 kGy E-beam indiscriminately inactivated PEDV on both the top and bottom surfaces of an 18 mm-thick piece of polystyrene foam. The density of a material is a key factor that affects the electron penetration of E-beam irradiation (Siwek and Edgecock, 2020). A higher material density leads to a shallower penetration depth (Laurell et al., 2006; Siwek and Edgecock, 2020). Several studies have shown that 10 kGy irradiation from a 10 MeV E-beam accelerator can penetrate at least 3 cm into a material with a density of 1 g/cm$^3$ without energy loss (Venugopal et al., 1999; Jaczynski and Park, 2003). The densities of corrugated cardboard, polystyrene plastic, and polystyrene foam are 0.45–0.70 g/cm$^3$, $\sim$0.90 g/cm$^3$, and 0.015–0.04 g/cm$^3$, respectively. Our findings and those of the previous studies indicate that E-beam radiation can penetrate the commonly used cold chain packaging materials used in this study without loss of energy. The Food and Agriculture Organization of the United Nations, the WHO, and the International Atomic Energy Agency stated in the 1980s that “A food presents no toxicological hazard when its overall average absorbed dose does not exceed 10 kGy. It no longer is required to undergo toxicology testing, and it is safe in terms of nutrition and microbiology”. The current study showed that a radiation dose of 5 kGy caused a 96.9% reduction in infectious viral titer, and a 10 kGy dose caused a 98.1% reduction in infectious viral titer. This result means that no infectious virus was detected at the irradiation dose of 10 kGy. Thus, we infer that a 10 kGy E-beam would inactivate coronaviruses on the package surfaces without causing food safety side effects and could be used in the cold chain industry. In conclusion, E-beam irradiation is a preferable method for inactivating viruses in the cold chain because it is effective, safe, and environmentally friendly.

Regarding the mechanism of E-beam viral inactivation, studies have shown that E-beams destroy viral nucleic acids to inactivate Tulane virus, murine norovirus 1, and Influenza A (H3N8) (Predmore et al., 2015; Fertey et al., 2016). E-beam irradiation damages viral nucleic acids directly and indirectly. In direct damage, the electron beam directly breaks the viral nucleic acid sugar–phosphate backbone, resulting in the deletion, substitution, or insertion of viral nucleic acid bases (Gates, 2009; Lomax et al., 2013). In indirect damage, the electrons first react with H$_2$O to form various active particles such as hydroxyl radicals, hydrated electrons, and hydrogen. These active particles then react with viral nucleic acids to cause nucleic acid fragmentation and cross-linking (Costa et al., 2012; Sobotta et al., 2015). Breaks in the nucleic acid strand are generally sufficient to inactivate a virus (Durante et al., 2020). Consistent with this, the copy number of the integrated PEDV genome decreased with increasing radiation dose. Because the 5 kGy and 10 kGy E-beam irradiation decreased the intact RNA to 53.75% and 37.08%, respectively, our data imply that viral genomic RNA damage would greatly contribute to E-beam-induced PEDV inactivation in cold chain conditions.

Previous studies have reported that low-energy electron beams and gamma ray irradiation do not affect the structure of influenza virus proteins (Samuel and Goldblith, 1975; Alsharifi and Mullbacher, 2010; Fertey et al., 2016). In our study, we found that the integrity of the protein capsid was disrupted by E-beam irradiation at doses of 20 kGy or higher. This discrepancy might be owing to the different structures of capsids and envelopes in different virus types. However, we cannot rule out that the low temperature could have caused this discrepancy. Predmore et al. reported that a high dose of a high-energy electron beam damaged the murine norovirus 1 capsid (Predmore et al., 2015). Zhang et al. used Monte Carlo methods to simulate energy deposition and electron radiation ionization of high-energy electrons in SARS-CoV-2; these are important characteristic quantities related to the induction of biological damage (Zhang et al., 2020). The theoretical calculation provided basic physical information for SARS-CoV-2 inactivation by E-beam irradiation. Because the destruction of the protein capsid and lipid bilayer envelope can lead to further reductions in virus pathogenicity (De Roda Husman et al., 2004; Zhu et al., 2020; Sommer et al., 2001), a slightly higher E-beam irradiation dose should be applied in cold chain logistics to ensure SARS-CoV-2 inactivation.

At present, E-beam irradiation is used to inactivate microorganisms in plastic products, medications, medical equipment, and food (Tahergorabiet al., 2014). Globally, there are more than 1700 E-beam devices in commercial use for many products, providing an estimated added value of more than 100 billion USD (Chmielewski and Han, 2016). This study provides useful baseline data about a potential efficient and green technique to inactivate SARS-CoV-2 in the cold chain environment. Further investigations are needed to carefully consider how this process would be implemented in practice for cold chain logistics. For example, E-beam irradiation might be conveniently used for large-scale disinfection in cold chain warehouses or refrigerated trucks. Future investigations for improving E-beam technology could focus on reducing the high power consumption and implementing miniaturization and robotic designs for specific aims. It also would be helpful to develop more practical E-beam instruments for SARS-CoV-2 disinfection in the cold chain industry to prevent the cold chain from being a vector for COVID-19 transmission.
5. Conclusions

Owing to the possible transmission of SARS-CoV-2 via cold chains, there is an urgent need for effective, feasible, and green disinfection methods for use in cold chain logistics. Using PEDV as a SARS-CoV-2 surrogate, we evaluated the PEDV disinfection efficiency of a high-energy E-beam under cold chain conditions and elucidated the inactivation mechanism. PEDV remained infectious for at least 30 days on three common cold chain packaging materials at −20 °C, which further confirms the possibility of coronavirus transmission via cold chain logistics. After treatment with 10 kGy or higher E-beam irradiation, more than 98.1% of the PEDV on both the top and bottom surfaces of the packaging material was inactivated. E-beam irradiation damaged the viral genome at a dose of 5 kGy, whereas the irradiation disrupted the integrity of the viral capsid at 20 kGy. In summary, our study shows efficient disinfection of PEDV by E-beam under cold chain conditions. This study provides a basis for the application of E-beam irradiation for SARS-CoV-2 disinfection in cold chain logistics.

CRediT authorship contribution statement

Yan Liu: Investigation, Formal analysis, Writing – original draft, Writing – review & editing. Yang Shao: Resources. Lu Wang: Investigation. Weilai Lu: Resources. Shihua Li: Resources. Diandou Xu: Conceptualization. Yu Vincent Fu: Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.eti.2022.102715.

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