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Effects of Pelleting, Irradiation, and Autoclaving of Rodent Feed on MPV and MNV Infectivity

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Murine norovirus (MNV) and mouse parvovirus (MPV) are among the most common adventitial viruses seen in laboratory mice, and infections arise in barrier facilities despite rigorous biosecurity programs. Some authors have implicated nonsterilized feed as a source of MPV in rodent facilities, but none have conclusively documented viral particles in the feed. In this study, we hypothesized that both viruses can resist the pelleting process but not subsequent irradiation or autoclaving, thus revealing a potential source of outbreaks in rodent facilities. To test this hypothesis, we contaminated powdered feed with 10-fold concentrations of MNV and MPV and fed it to both Swiss Webster (SW) and C57BL/6NTac (B6) mice to determine a 'powdered ID_{50}' according to seroconversion over a 28-d period. We repeated the experiment by using powdered feed that we contaminated with increasing viral doses (as no. of powdered ID_{50}) and subsequently pelleted; from these results, we determined a 'pelleted ID_{50}.' Finally we assessed the effect of irradiation and autoclaving on contaminated pellets by using the same experimental design. The powdered ID_{50} was relatively low and identical in both mouse strains (2.51×10^2 pfu) for MNV but higher in B6 (copy number, 3.20×10^6) than SW (3.98×10^4 copies) for MPV. As hypothesized, mice were infected by contaminated rodent feed despite the pelleting process. Indeed, pelleting resulted in a 1- to 2-log increase in ID_{50} in both strains for MNV and MPV. Irradiation and autoclaving of infected pellets effectively prevented seroconversion of mice exposed to all doses of MNV, whereas a single mouse seroconverted at the highest dose of MPV (1.35×10^7 copies). These data suggest that both MNV and MPV remain infectious after conditions reproducing the rodent chow pelleting process and that nonsterilized rodent chow might be a source of viral outbreaks.

Abbreviations: MNV, murine norovirus; MPV, mouse parvovirus

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Rodent colonies enrolled in biomedical research are often housed in barrier facilities with the express purpose of preventing contamination by adventitial pathogens that may produce disease or interfere significantly with research. Nonetheless, research institutions continue to contend with unexpected epizootic pathogen outbreaks despite the implementation of rigorous biosecurity programs designed to exclude, contain, and eradicate these pathogens from their colonies. Laboratory veterinarians often dedicate considerable resources to find the sources of these infections. Efforts are rarely rewarded and blame often assigned unconvincingly to common causes such as unapproved import of infected mice, 'leaky' quarantine programs, fomites, or the inoculation of contaminated biologicals[9] as likely sources of infection. The continued presence of these pathogens in barrier facilities despite stringent biosecurity programs should, however, prompt us to evaluate alternative sources of contamination such as feed and bedding which come in direct contact with the animals and therefore are most likely to cause infection.

Murine norovirus (MNV) and mouse parvovirus (MPV) are among the most common adventitial viral pathogens in laboratory mice.[28,42] Both are nonenveloped viruses that are notoriously stable in the environment and resistant to inactivation from heat, desiccation, and many common disinfectants, all of which characteristics might facilitate their transmission to and within animal facilities.[9,28,51] Although MNV may cause lethal infection in severely immunodeficient mice,[31] clinical signs are generally absent in immune-competent mice.[24] Nonetheless, MNV has been shown to infect macrophages, dendritic cells, and B cells and is therefore likely to interfere with immunologic and inflammatory studies.[24] MPV likewise causes asymptomatic infection in mice but has a propensity to infect lymphoid tissues and may interfere with research through its effects on the host immune response.[35] As such, both viruses are often excluded from barrier facilities but yet remain prevalent in research colonies.[5,40,42]

Epidemiologic studies have strongly suggested that MPV outbreaks in barrier facilities could be due to contamination of the feed and that irradiation or autoclaving might help in the containment and eradication of the agent.[28,45,50] In one study,[45] irradiation of feed alone was sufficient to eradicate MPV in certain colonies, thus suggesting that the source of contamination was in the feed itself and not from the contamination of bag surfaces after irradiation and packaging. Unfortunately, despite compelling evidence, attempts to isolate or detect the virus in the feed have all failed. The main challenge to detection of pathogens in the feed is that food is produced in large lots and stocks are used and replaced very rapidly in rodent facilities. As such, any potentially contaminated feed typically is consumed weeks to months before the outbreak is identified

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in a colony, leaving no contaminated feed to assess. By the time an infection is detected, the former lot is consumed, the current lot is likely to be negative, and samples of the lot of feed used during the incubation period are unavailable for evaluation.[45] Another reason for not finding viruses may lie in the processing of raw materials in feed mills, which would result in unequal viral distribution within a given lot or even individual pellets. Likewise, no studies have evaluated feed as a potential source of MNV contamination, although a similar transmission cycle to MPV can be theorized given the presence of the virus in wild mice.[13,41] The high prevalence of MNV in research colonies might be best explained by the high environmental resistance of the virus, the persistent infection in certain strains, the relatively low infectious dose, the ease of detection in soiled bedding sentinels or inconsistent standards for prevention or control in certain institutions.[16,25,36] Nonetheless, the question remains regarding whether food contributes to the commonality of this pathogen.

Overall, no studies have specifically evaluated the infectivity of MNV or MPV in the feed, the resistance of infectious particles to the standard pelleting process or the effects of subsequent γ-irradiation on infectivity. Wild rodents indeed may contaminate corn or other raw products, especially at harvest time or before production while in storage.[2,50] During the pelleting process, the feed is subject to 65 to 80 °C (149 to 176 °F), pressure, steam, and drying cycles that likely kill many heat-labile microorganisms.[49] However, the process is not meant to sterilize feed or eliminate infectious viral particles. The highly resistant nature of MNV and MPV could allow them to remain infectious. In addition, the constituents and composition of the food itself, such as fat and proteins, may influence the thermal inactivation of viruses and protect particles from the effects of food processing.[22] Some authors advocate the use of ionizing radiation or autoclave sterilization of rodent chow to mitigate the risks of entry into barrier facilities.[8] Irradiation is a relatively efficient decontamination process but is costly and does not guarantee feed sterility, even at doses as high as 50 kGy, the maximum allowable dose established by the FDA.[7,27,49] In fact, no controlled study has evaluated the effect of irradiation on MNV, MPV, or other rodent viral pathogens in rodent feed. The autoclaving process uses a combination of steam at high pressure to sterilize the feed.[27,49] Autoclaving kills bacteria, viruses, fungi, and parasites but is time-consuming, costly, decreases nutritional value, and is not without failures, especially with dense material such as rodent chow.

The purpose of this study was to evaluate the effects of pelleting and irradiation on MNV and MPV infectivity in 2 common laboratory mouse strains and comparing it to the gold standard sterilization method, autoclaving. Considering the technical and ethical limitations of performing such a study in a factory feed mill, we partnered with food engineers to faithfully reproduce the various industrial processes in a controlled laboratory setting to assess the capability of these viruses to infect mice after these processes.

Materials and Methods

Mice. The infectious agents selected for this study were MNV4 and MPV1e. Host susceptibility, seroconversion, and shedding of MPV can vary significantly according to mouse age, strain, and sex,[3,17] but these differences have not been observed for MNV. Nonetheless, all studies were performed in relatively MPV-resistant (C57BL/6 [B6]) and -susceptible (Swiss Webster [SW]), young (age, 4 to 6 wk), female mice to account for eventual strain-associated differences and mitigate possible effects of sex and age on MPV and MNV infectivity. SW mice (Tac:SW) and B6 (C57BL/6NTac) were obtained from Taconic Biosciences (Germantown, NY). Vendor reports indicated that mice were seronegative for ectromelia virus, mouse rotavirus, lymphocytic choriomeningitis virus, mouse hepatitis virus, MPV, minute virus of mice, MNV, pneumonia virus of mice, reovirus, Sendai virus, mouse encephalomyelitis virus (GDVII), Hantaan virus, K virus, Mouse adenovirus types I and II, polyomavirus, thymic virus, lactate dehydrogenase elevating virus, and *Mycoplasma pulmonis*. Mice were individually housed in a dedicated animal room with a negative pressure differential relative to the corridor, room temperature of 70 ± 2 °F (21 °C), room humidity of 30% to 70%, 12:12-h light:dark cycle, and 10 to 15 air changes hourly. Mice were housed in sterilized static microisolation caging (Allentown Caging, Allentown, NJ) containing corncob bedding (Bed-o'Cobs 1/4 in., The Anderson's, Maumee, OH), enrichment (Crink-l'Nest, The Anderson's), irradiated rodent chow (diet 5053, Purina Mills International, St Louis, MO), and reverse-osmosis–purified water in water bottles. Cages were changed weekly in a class II biosafety cabinet within the animal room. Work space, instruments, gloves, and cage exteriors were disinfected (contact time, 5 min; 1% solution of Virkon-S, Lanxess, Pittsburg, PA) before and after handling of mice. All animal care and experimental procedures were approved by the Salk IACUC and performed in accordance with all federal policies and guidelines governing the use of vertebrate animals.

MNV propagation and quantification. MNV4 was propagated in RAW 264.7 cells (TIB71, American Type Culture Collection, Manassas, VA) cultivated in 1-L suspension spinner flasks (Wheaton Science Products, Millville, NJ) containing DMEM (catalog no. SH30243.02, HyClone, Logan, UT) supplemented with 10% FBS (Atlanta Biologicals, Flowery Branch, GA), and ciprofloxacin (10 µg/mL). RAW 264.7 cells were infected with MNV4 (multiplicity of infection, 0.1) when the cell count was approximately 10^6 cells/mL. Cellular supernatant was collected and clarified by centrifugation (2000 × *g* for 10 min at 4 °C) at 48 h after infection, when cells displayed 90% to 100% cytopathic effect. Clarified supernatant containing virus was cryopreserved at −80 °C. The concentration of MNV4 was determined by using a previously described plaque assay.[26]

MPV propagation and quantification. The MPV1e isolate was kindly provided by Craig L. Franklin (University of Missouri, Columbia, MO) and maintained by oral inoculation of naïve mice. Briefly, 4-wk-old ICR mice were inoculated by oral gavage of 0.2 mL MPV1e spleen homogenate suspended in DMEM. Spleens were collected at day 7 after infection, homogenized in DMEM, and the supernatant was clarified by centrifugation (2000 × *g* for 10 min at 4 °C). Clarified supernatant containing virus was cryopreserved at −80 °C. The concentration of MPV1e was determined by using a quantitative real-time PCR assay described below.

Serology. Dried blood-spot specimens for serologic testing were collected by submandibular bleeding in nonanesthetized mice as previously described.[15] MNV and MPV serology panels were performed by using validated assays (multiplex fluorescent immunoassay serology platform) at IDEXX BioAnalytics (Columbia, MO) using Opti-Spot (IDEXX BioAnalytics, Columbia, MO) dried blood samples, and equivocal results confirmed by immunofluorescent assay.

Real-time PCR testing of MPV. Total nucleic acids were extracted by using a commercially available platform (NucleoMag VET, Macherey-Nagel, Bethlehem, PA). The MPV PCR tests were based on the IDEXX BioAnalytics proprietary service platform (IDEXX Laboratories, Westbrook, ME). Briefly, the

MPV real-time PCR assay targets a region of the VP2 gene that is conserved among all of the MPV genomic sequences deposited in GenBank and uses a FAM/TAMRA-labeled hydrolysis probe. Hydrolysis-probe–based real-time PCR assays targeting a mammalian gene (18S rRNA) or bacterial gene (16S rRNA) were used to ensure DNA recovery and the absence of PCR inhibitors in nucleic acid extracted from mesenteric lymph node and antibody test samples or fecal samples, respectively. Real-time PCR analysis was performed by using standard primer and probe concentrations and a commercially available mastermix (LC480 ProbesMaster, Roche Applied Science, Indianapolis, IN) on a commercially available real-time PCR platform (LightCycler 480, Roche). The estimated yield of MPV DNA (as copy number) was calculated by plotting the real-time crossing point (Cp) values from the MPV PCR assay of the working virus stock on a standard curve of log-fold dilutions of a synthetic positive control of known concentration.

Statistical analysis. ID_{50} was calculated as previously described in singly housed mice.[17,20,43] Groups of 6 mice were inoculated with increasing serial concentrations that were estimated to flank the numbers of MNV and MPV particles required to infect 50% of the mice in a single group. At least 5 doses were used to allow accurate calculation. Using seroconversion as determined through the multiplex fluorescent immunoassay as a surrogate of positive infection, the ID_{50} for each mouse strain was calculated according to the Reed–Muench method.[44]

Experimental design. All studies followed the same basic experimental design. Irradiated powdered chow (5053 LabDiet) was infected in a BSL2 cabinet by using increasing serial concentrations of MPV or MNV and, depending on the study, further processed (pelleting alone or pelleting followed by irradiation or autoclaving). Mice were individually housed in autoclaved static microisolation caging under strict biocontainment practices. Each group of 6 mice of both strains was provided contaminated powdered or pelleted feed, which was placed in a mason jar with a perforated lid to allow mice to climb and eat without spillage. Contaminated feed was the only food source and was prepared to provide a cumulative dose over a 3-d period, given a standard daily consumption of 6 g of feed per mouse. Jars were removed once empty, and irradiated chow (LabDiet 5053) then was provided without restriction until termination of the experiment at day 28. For each experiment, positive-control groups of 6 mice from each strain received 10 times the highest concentration of powdered or pelleted feed in PBS by oral gavage, whereas negative-control groups received standard irradiated chow (LabDiet 5053) without restriction. Mice were bled for MNV or MPV serology before infection and then on days 7, 14, 21, and 28 after infection. ID_{50} for each study were then calculated according to the Reed–Muench method[44] and used as a basis for subsequent studies.

Part 1: infectivity of MPV and MNV in feed. The goal of this first study was to determine the ID_{50} of MNV and MPV in powdered feed in SW and B6 mice as determined through robust seroconversion. Irradiated chow (LabDiet 5053) in a powdered form was contaminated with 10-fold serial concentrations of MNV and MPV. Each group of SW mice ($n = 6$) received 1×10^1 to 1×10^5 pfu of MNV, whereas each group of B6 mice ($n = 6$) received 1×10^2 to 1×10^6 pfu. In a separate experiment, each group of SW mice ($n = 6$) received 1×10^1 to 1×10^6 copies of MPV, whereas each group of B6 mice ($n = 6$) received 1×10^3 to 1×10^7 copies. Feed was prepared under strict biocontainment in a BSL2 cabinet. Briefly, viral stock was diluted to the specified concentration in 200 µL of PBS and homogenized with the feed by using a closed and sealed portable food processor (Pro900 Series, Magic Nutribullet, Pacoima, CA). Contaminated feed was prepared in order of the lowest to highest dose, and the processor was cleaned and disinfected (1% Virkon-S [contact time, 5 min] and 70% ethanol) between doses. Infected feed was then provided as previously described, and seroconversion was monitored weekly for 28 d. A powdered ID_{50} was then calculated according to the Reed–Muench method.[44]

Part 2: effect of pelleting on infectivity. The goal of this second study was to evaluate the effect of pelleting conditions on the infectivity of MNV and MPV in chow and to determine a pelleted ID_{50}. The experiment could not be performed by using MPV in B6 mice due to the high powdered ID_{50} obtained (3.20×10^6 copies), which therefore prevented the production of sufficiently high-titer solutions. The experimental conditions and testing procedures were identical to the first experiment. Autoclaved food (LabDiet 5053) in a powdered form was contaminated by using increasing serial concentrations (in increments of powdered ID_{50}) for MNV (1 to 10,000 powdered ID_{50}) and MPV (0.63 to 630 powdered ID_{50}) and then pelleted. Each group of 6 SW or B6 mice received 2.51×10^2 to 2.51×10^6 pfu of MNV. In a separate experiment, each group of 6 SW mice received 2.51×10^4 to 2.51×10^7 copies of MPV. Contaminated feed was provided as previously described, and seroconversion was followed weekly for 28 d. A pelleted ID_{50} was then calculated according to the Reed–Muench method.[44]

Part 3: effect of irradiation and autoclaving on infectivity. The goal of the third study was to evaluate the effects of irradiation and autoclaving on the infectivity of MNV and MPV after pelleting. The experiment could not be performed with MPV in B6 mice due to the high powdered ID_{50} (3.20×10^6 copies), which therefore prevented the production of sufficiently high-titer solutions to account for all groups. The experimental conditions and diagnostic procedures were identical to the first 2 experiments. For the irradiation study, irradiated food (LabDiet 5053) in a powdered form was contaminated by using increasing serial concentrations (in increments of pelleted ID_{50}) for MNV (1, 10, 50, and 100 pelleted ID_{50}) and MPV (0.4, 3.8, 18.9, and 37.8 pelleted ID_{50}) and then pelleted as described earlier. Each group of 6 SW mice received 6.28×10^4, 6.28×10^5, 3.14×10^6, or 6.28×10^6 pfu of MNV, whereas mice in each group of 6 B6 mice received 6.28×10^3, 6.28×10^4, 3.14×10^5, or 6.28×10^5 pfu of MNV. In a separate experiment, each group of 6 SW mice received 1.35×10^5, 1.35×10^6, 6.75×10^6, or 1.35×10^7 copies of MPV. Given that autoclaving is the 'gold standard' for sterilizing food, the autoclaving study was performed by using only doses of 100 and 37.8 pelleted ID_{50} for MNV and MPV, respectively. As such, each group of 6 SW or B6 mice received 6.28×10^6 pfu or 6.28×10^5 pfu of MNV, respectively, whereas another group of 6 SW mice received 1.35×10^7 copies of MPV in a separate experiment. The feed was pelleted as described earlier. Pellets intended for the irradiation study were aliquoted, vacuum-sealed, and γ-irradiated (25 kGy, electron beam) at a commercial irradiator. Dose mapping was carefully evaluated before the study by using mock loads, to ensure uniform dose distribution throughout the load, and Radiation Sterilization Indicator Labels (Crosstex, Englewood, CO) were included in each feed aliquot to confirm the radiation dose that was delivered. Pellets intended for the autoclaving control were aliquoted into sterilization pouches (Fisherbrand Instant Sealing Sterilization Pouches, Fisher Scientific, Pittsburgh, PA) and autoclaved (prevac; minimal sterilization temperature, 121 °C; chamber pressure, 10 to 15 psi; sterilization time, 20 min; dry time, 5 min) at the Salk Institute. Indicator strips (Comply 1250 Steam Sterilization Indicator Strip, 3M, St Paul, MN) and heat-

sensitive autoclave tape (Fisherbrand White Autoclave Tape, Fisher Scientific) were included in each load to assure correct sterilization. Inoculated feed was then provided as described previously, and seroconversion was followed weekly for 28 d.

Pelleting. In the nutrition industry, the pelleting process begins with mixing and blending base ingredients. This mix is then ground to produce a fine meal that is steadily fed into a horizontal metal cylinder called a conditioner. During this process, the meal is injected with dry steam. Conditioning raises the temperature of the meal to 65 to 80 °C (149 to 176 °F) for 5 to 10 s. The high temperature from the steam gelatinizes the starches, binds the diet ingredients together, and reduces the microbiologic load.[49] The conditioned meal is then steadily fed into a die that produces a 'stick' of chow of a given diameter; a knife is used to cut the stick into pieces of the desired size. The pellets are dried to approximately 10% moisture (for a standard rodent diet);[49] at this moisture level, little free water is available to support microorganismal growth.[49,50]

Conditioners are bulk machines that require the production of a minimum of 3 to 6 tons of feed per load. As such, it was not technically feasible nor ethically responsible to reproduce this experimental design involving infectious agents in a pelleting factory that is used to meet commercial needs. Therefore, we worked with LabDiet to reverse-engineer and deconstruct the pelleting process to allow smaller-scale production that faithfully reproduced the manufacturing conditions found in the industry. The irradiated powdered chow was inoculated with the incremental doses of MNV and MPV as described in part 1. Inoculated meal was injected with steam to reach 80 °C (176 °F) for 5 to 10 s and then pressed for 60 s into a stainless-steel die by using an industrial 20-ton press (Figure 1). The pellet was removed from the die and placed on a metal grid within an aluminum tube. The pellet was dried to the desired humidity level (10%) by using forced heated air from a heat gun (model KX1650 740l/min 1750 Red Power Heat Gun, Black and Decker, New Britain, CT) placed at a standard distance to provide a constant temperature of 135 °C (275 °F) for 90 s. The water contents added and removed through these steps were consistent with a final moisture level of 10%, according to the weight changes at each step. Pellets were then cooled to room temperature and given to mice. Pelleting was performed in order of the lowest to highest concentration, and equipment was disinfected (1% Virkon-S [contact time, 5 min] followed by 70% ethanol) between groups to prevent cross-contamination. In addition, each aliquot of pellets was vacuum-sealed, bagged, sprayed with 1% Virkon-S, and stored in a sealed container before use.

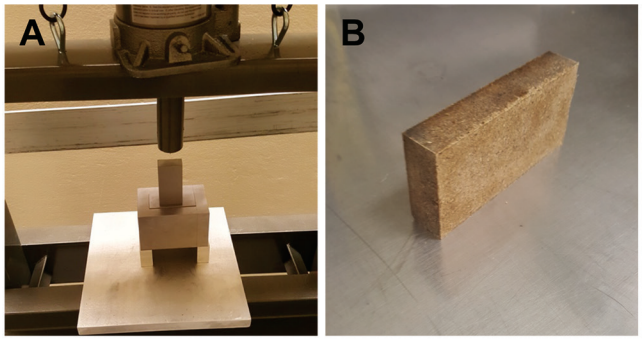

Figure 1. Deconstructed pelleting process. (A) Pressing of steamed meal in heavy-duty die by using a 20-ton press. (B) Final pellet product.

Table 1. Determination of powdered ID_{50} for MNV in Tac:SW and C57BL/6NTac mice

	No. of seropositive mice	
Dose (pfu)	SW	B6
1×10^1	0	not analyzed
1×10^2	1	1
1×10^3	6	6
1×10^4	6	6
1×10^5	6	6
1×10^6	not analyzed	6

Each group comprised 6 mice. The powdered ID_{50} value calculated according to seroconversion was 2.51×10^2 pfu MNV for infected Tac:SW and C57BL/6NTac mice both.

Results

For all experiments, mice were seronegative for MNV and MPV prior to infection. All positive controls seroconverted for MPV or MNV, and all negative controls that received irradiated diet remained seronegative throughout the 28-d experimental period.

Part 1: infectivity of MPV and MNV in feed. The goal of the first study was to establish the ID_{50} of MNV and MPV in powdered feed in SW and B6 mice, as determined through robust seroconversion.

For MNV, none of the 6 SW mice infected with 1×10^1 pfu seroconverted, 16.6% (1 of 6) of mice infected with 1×10^2 pfu seroconverted, and all mice infected with larger doses seroconverted (Table 1). As expected, seroconversion occurred rapidly, with the detection of 38% seropositive mice after 1 wk and 100% seropositivity after 2 wk. The pattern of infection for MNV was similar in B6 mice, with only 16.6% (1 of 6) of mice seroconverting to the lowest dose (1×10^2 pfu), whereas all mice infected by using larger doses seroconverted (Table 1). In addition, seroconversion occurred rapidly, with 60% of mice detected as seropositive after 1 wk and 97% detected after 2 wk. The powdered ID_{50} for MNV in both SW and B6 was 2.51×10^2 pfu.

For MPV, none of the SW mice infected with 1×10^2 to 1×10^4 copies seroconverted, whereas 83% (5 of 6) of mice infected with 1×10^5 copies seroconverted and 100% of the 6 mice infected with 1×10^6 copies seroconverted (Table 2). Compared with MNV, seroconversion to MPV occurred later, with 100% of mice detected as seropositive at 2 wk. As expected, B6 were more resistant to infection with MPV compared with MNV, with seroconversion to MPV only in mice infected with 1×10^7 copies (100%, all 6 mice; Table 2). In addition, seroconversion to MPV was delayed relative to MNV, with no seropositive mice detected at 1 or 2 wk after inoculation, 92% seropositivity detected at 3 wk, and 100% at 4 wk. The powdered ID_{50} for MPV was 3.98×10^4 copies in SW mice and 3.20×10^6 copies in B6 mice.

Part 2: effect of pelleting on infectivity. In SW mice, the pelleted ID_{50} for MNV was 6.28×10^4 pfu, which represents a greater than 2-log increase in ID_{50} as compared with the powder. None of the mice infected with 1 powdered ID_{50} (2.51×10^2 pfu) or 10 powdered ID_{50} (2.51×10^3 pfu) seroconverted, 16% (1 of 6) mice infected with 100 powdered ID_{50} (2.51×10^4 pfu) seroconverted, and all 12 mice infected with 1000 powdered ID_{50} (2.51×10^5 pfu) or 10,000 powdered ID_{50} (2.51×10^6 pfu) seroconverted (Table 3). The timeline for seroconversion to pelleted MNV was similar to that for infection with powdered feed, with 8% of mice detected as seropositive after 1 wk and 100% after 2 wk. In B6 mice, the MNV pelleted ID_{50} was 6.3×10^3 pfu, which represents a greater than 1-log increase in ID_{50} as compared with powdered feed. None of the mice infected with 1 powdered ID_{50} (2.51×10^2

Table 2. Determination of powdered ID_{50} for MPV in Tac:SW and C57BL/6NTac mice

	No. of seropositive mice	
Dose (copies)	SW	B6
1×10^2	0	not analyzed
1×10^3	0	0
1×10^4	0	0
1×10^5	5	0
1×10^6	6	0
1×10^7	not analyzed	6

Each group comprised 6 mice. The powdered ID_{50} values calculated according to seroconversion were 3.98×10^4 copies MPV in infected Tac:SW mice and 3.20×10^6 copies in infected C57BL/6NTac mice.

Table 3. Determination of pelleted ID_{50} for MNV in Tac:SW and C57BL/6NTac mice

Dose		No. of seropositive mice	
No. of powdered ID_{50}	No. of pfu	SW	B6
1	2.51×10^2	0	0
10	2.51×10^3	0	1
100	2.51×10^4	1	6
1000	2.51×10^5	6	6
10,000	2.51×10^6	6	6

Each group comprised 6 mice. The pelleted ID_{50} values calculated according to seroconversion were 6.28×10^4 pfu MNV in infected Tac:SW mice and 6.28×10^3 pfu MNV in infected C57BL/6NTac mice.

pfu) seroconverted, where 16% (1 of 6) of mice infected with 10 powdered ID_{50} (2.51×10^3 pfu) seroconverted, and all 18 mice infected with 100 (2.51×10^4 pfu), 1000 (2.51×10^5 pfu), or 10,000 powdered ID_{50} (2.51×10^7 pfu) seroconverted (Table 3). All mice were detected as seropositive at 2 wk but none at 1 wk.

In SW mice, the MPV pelleted ID_{50} was 3.57×10^5 copies, which represents approximately a 1-log increase in ID_{50} as compared with powdered feed. None of the mice infected with 0.63 powdered ID_{50} (2.51×10^4 copies) seroconverted, 50% (3 of 6) of mice infected with 6.3 powdered ID_{50} (2.51×10^5 copies) seroconverted, 83% (5 of 6) of mice infected with 63 powdered ID_{50} (2.51×10^6 copies) seroconverted, and all 12 mice infected with 315 (1.26×10^7 copies) or 630 powdered ID_{50} (2.51×10^7 copies) seroconverted (Table 4). The timeline for seroconversion to MPV in pelleted feed was similar to the infection due to powdered feed, with 96% of mice detected as seropositive at 2 wk and 100% at 3 wk.

Part 3: effect of irradiation on infectivity. The goal of the third study was to evaluate the effects of irradiation on the infectivity of MNV and MPV and to compare them with the gold standard, autoclaving, after the pelleting process. None of the mice seroconverted after receiving feed that had been inoculated with MNV or MPV, pelleted, and autoclaved, thus confirming that autoclaving sterilized the feed efficiently. Similarly, none of the mice seroconverted that were provided feed inoculated with MNV, pelleted, and irradiated (Table 5), thus suggesting that irradiation was sufficient to decrease the number of functional MNV particles below the threshold of infection. A single SW mouse fed with pelleted and irradiated diet that had been inoculated with 1.35×10^7 copies of MPV seroconverted strongly after 2 wk (Table 6). The serologic result was atypical, yielding very high reactivity for NS1 on multiplex fluorescent immunoassay (MFI); this result was confirmed as a positive reaction through immunofluorescent assay (IFA). However, samples from this

Table 4. Determination of pelleted ID_{50} for MPV in Tac:SW mice

Dose		No. of seropositive mice
No. of powdered ID_{50}	No. of copies	
0.6	2.51×10^4	0
6.3	2.51×10^5	3
63	2.51×10^6	5
315	1.26×10^7	6
630	2.51×10^7	6

Each group comprised 6 mice. The pelleted ID_{50} value calculated according to seroconversion was 3.57×10^5 copies in MPV-infected Tac:SW mice.

animal showed no reactivity for VP2 on multiplex fluorescent (MFI) or immunofluorescent (IFA) assays. To confirm the positive result, we retrospectively tested feces of the positive animal by PCR analysis of stored samples collected on days 7, 14, and 21. All results were positive, thus suggesting the presence of parvoviral nucleic acid in the feces. We also PCR-analyzed the spleen and MLN of the infected mouse; both assays were positive, thus confirming a true infection in this mouse despite irradiation of feed.

Discussion

This study is the first to empirically evaluate the respective effects of pelleting, γ-irradiation, and autoclaving on rodent feed contaminated with MNV or MPV. To conduct a systematic comparison, we evaluated the effects of each process on ID_{50} by using a standardized methodology.

We first determined the effect of pelleting on viral infectivity. Pelleting mostly relies on thermal (high-temperature) and nonthermal (high-pressure) processes to inactivate infectious particles. Thermal processing in the food industry has been optimized to reduce the number of or destroy microorganisms, especially bacteria. Unfortunately, enteric viruses have a great capacity to survive extreme conditions, thus allowing persistence in the environment and host infection.[22] Parvoviruses are notoriously resistant to inhospitable environmental parameters, when compared with other viral families,[39] and MNV, as a naked virus that lacks an envelope, would be expected to survive similarly.[12] Among murine parvoviruses, minute virus of mice has been evaluated the most extensively for its heat resistant properties. Despite being described as one of the most thermally sensitive viruses within *Parvoviridae*,[19] minute virus of mice is incompletely inactivated when exposed to 80 °C (176 °F) for as long as 1 h[4] or at 115 °C (239 °F) for 30 s.[46] In our experiment, the ID_{50} of MPV after pelleting of contaminated powdered chow was 3.57×10^5 copies, thus suggesting that MPV admixed to rodent feed can withstand consecutive heat exposures to 80 °C (176 °F) for 5 to 10 s and 135 °C (275 °F) for 90 s to remain infective after pelleting. Murine noroviruses are more sensitive to thermal inactivation than parvoviruses:[6] 4-log reductions of minute virus of mice were attained after exposure to 90 °C (194 °F) for 60 s,[4] whereas MNV required only 71 °C (160 °F) for 30 s to achieve similar reduction.[6] In our experiment, the ID_{50} of MNV after pelleting of contaminated powdered chow was 6.28×10^4 pfu in SW mice and 6.28×10^3 pfu in B6 mice, suggesting that MNV in rodent feed can remain infective after pelleting. We did not see evidence to suggest that the thermal resistance of MPV and MNV differed greatly in our current study. Indeed, the ID_{50} were increased by 1 to 2 logs after pelleting of feed contaminated with MNV and MPV. The similar heat sensitivity that we observed in our study might be explained

Table 5. Seroconversion of Tac:SW and C57BL/6NTac mice after irradiation of MNV-contaminated pelleted feed

	Tac:SW		C57BL/6NTac	
Dose (no. of pelleted ID_{50})	Dose (no. of pfu)	No. of seroconverted mice	Dose (no. of pfu)	No. of seroconverted mice
1	6.28×10^4	0	6.28×10^3	0
10	6.28×10^5	0	6.28×10^4	0
50	3.14×10^6	0	3.14×10^5	0
100	6.28×10^6	0	6.28×10^5	0

Each group comprised 6 mice.

Table 6. Seroconversion of Tac:SW mice after irradiation of MPV-contaminated pelleted feed.

Dose		No. of seroconverted mice
No. of pelleted ID_{50}	No. of copies	
0.4	1.35×10^5	0
3.8	1.35×10^6	0
18.9	6.75×10^6	0
37.8	1.35×10^7	1

Each group comprised 6 mice.

by properties of the suspending media. Most studies comparing thermal resistance between MVM, MNV, or other viruses were performed in liquids. However, the media in which the virus is suspended plays a large role in thermal sensitivity.[39] In particular, the fat and protein compositions of food influence the thermal inactivation of viruses and protect particles from the effects of food processing.[22] As such, viral susceptibility to pelleting might have been affected by feed composition and have played a considerable role in our current results.

In addition, the nutrition industry has evaluated high-pressure processing as an inactivating step for viruses. This type of processing is typically applied through specialized equipment such as high-hydrostatic–pressure devices, which may not be a suitable comparison for the pressure applied to powdered feed processed in a press. Nevertheless, previous studies on high hydrostatic pressure and enteric viruses may help to explain the limited effects of pelleting on MPV and MNV infectivity that we observed in our current study. High hydrostatic pressure is believed to inactivate nonenveloped viruses through the disruption of virion structure and the viral capsid.[33,34] The effect of high hydrostatic pressure varies greatly depending on virus species and size, processing time, pressure magnitude, and the nature of the suspending media or food,[22] including sugar, protein, fat, and salt contents. No studies have evaluated the effect of high hydrostatic pressure on MPV, but 552 MPa (approximately 40 tons per $in.^2$) is required to inactivate porcine parvovirus and greatly exceeds the pressure we applied in our current study.[52] Furthermore, similar studies indicate that 300 MPa (approximately 22 tons per $in.^2$) for 5 min was necessary to inactivate MNV,[34] again greatly exceeding the levels that we applied here. Therefore, 20-ton pressures alone would not likely cause significant inactivation of either MNV or MPV. Temperature and high hydrostatic pressure may have synergistic or opposite effects on viral infectivity. Low temperatures can enhance pressure-induced inactivation of MNV,[22] but no studies have evaluated the synergistic effects of high temperature and pressure on the infectivity of MPV and MNV. Nonetheless, our findings are consistent with other studies suggesting that very harsh temperature and pressure conditions are required to inactivate MPV and MNV and that the pelleting conditions used to produce rodent feed are insufficient to inactivate either virus.

Our data indicate that pelleting can increase the ID_{50} of MNV and MPV by 1 to 2 logs or, in other words, can reduce the infectious titer by 1 to 2 logs. To understand the biologic significance of this effect, one must consider: 1) the infectious dose of the virus in different mouse strains, 2) the source of the contamination, and 3) the distribution of viruses within the feed. Our initial study involving powdered feed suggested that the ID_{50} of both MNV (2.51×10^2 pfu) and MPV (3.98×10^4 to 3.20×10^6 copies) are relatively low as compared with the quantities shed by infected mice. In other words, very few infectious particles are necessary to cause a productive infection in both SW and B6 mice. In contrast, the number of particles shed in by a single infected mouse in the field—and that could contaminate raw material—is very high. Indeed, BALB/c and B6 mice can shed more than 10^6 MPV particles per milligram of feces at 3 d after infection,[20] whereas B6 mice can shed persistently 10^4 to 10^6 genome equivalents of MNV per milligram of feces, depending on the viral strain.[37] The premise of our study is that raw materials, such as corn, get contaminated with the feces or carcasses of infected mice before chows are mixed and pelleted. Rodent feed typically is made in large conditioners, requiring the production of 3 to 6 tons of feed per load. As such, even if the raw material was infected with several fecal pellets or a contaminated carcass before pelleting, the dilution effect would make infection from a viral contaminant extremely improbable if viruses were distributed homogeneously in the feed.

However, previous experience in the human and animal food industry[10,30] suggests that homogeneous distribution of microorganisms in food batches is rare. The structural heterogeneity of the food matrix and the food production process often result in heterogenous distribution and clustering of contaminants, especially in solid, semisolid, or powdered foods.[29] Such clustering explains why testing of food products for contamination is often uninformative unless several batches are evaluated. Similarly, studies that have looked at MPV contamination of rodent feed through testing have all failed to find the virus in the feed,[45] thus suggesting heterogenous distribution. Therefore, viral contamination of raw material in the field would probably be clustered at very high concentration in pockets of feed and a 1- to 2-log pelleting-associated decrease in a cluster of contamination would probably be insufficient to mitigate the risks of infection, especially considering the low infectious dose of both viruses. It is worth mentioning that this hypothesis is relevant for both MPV and MNV, given that both agents have been found in wild mice,[2,47] as well as other rodent viruses that show a similar pattern of environmental resistance.

As previously mentioned, it was not technically feasible nor ethically responsible to reproduce our experimental design involving infectious agents in a pelleting factory that is used commercially. We worked with LabDiet engineers to reverse-engineer and deconstruct the pelleting process to allow smaller-scale production yet faithfully reproduce the manufacturing conditions found in the industry. This artificial process

posed various challenges and might have influenced our results and the overall significance of our findings. Nonetheless, each process was repeated and validated on several trial runs to ensure homogenous distribution of the viruses, application of consistent temperature and pressure conditions, and minimal variation between each trial. To match commercial standards for moisture content, we weighed the feed before and after the application of steam and drying, to ensure that pellets were maximally laden with 10% (± 5%) moisture content. Each quality-assurance step was carefully enacted to minimize variability between pellets, and the results derived from this study suggest that uniform distribution of virus was achieved. Indeed, results from the pelleted experiments for both MNV and MPV (Tables 3 and 4) suggest that mice infected with doses below the ID_{50} remained negative rather than becoming false-positives, as would occur after receiving spiked large doses from nonuniform distributions of the virus. Conversely, high-dose groups were consistently positive when doses exceeded the ID_{50}.

The nutrition industry does not claim to sterilize rodent feed by pelleting, which is why autoclaving and irradiating feed have emerged as sensible precautions against potential microbiologic contamination of rodent feed. Several studies have suggested that MPV outbreaks in barrier facilities might be due to contamination of the feed and that irradiation or autoclaving could help in the containment and eradication of the agent.[28,45,50] However, no studies have systematically evaluated the effect of irradiation on potential viral contaminants in rodent feed. Irradiation has traditionally been used in industry for food preservation and decontaminating fresh produce from pathogens.[12,38] During irradiation, high-frequency γ-rays are released, thus creating high electromagnetic waves that penetrate food products and inactivate internalized pathogens by damaging critical cellular elements or the genetic material in the cell nucleus.[11] The overall result is a decrease in the number (log-fold) of infective particles below the infectious dose. Naked viruses, which lack an envelope derived from cells, are generally more resistant to γ-irradiation than bacterial and parasitic pathogens.[11,14] For these viruses, the irradiation process directly damages genetic material or indirectly reacts with nucleic acid through the production of free radicals generated when rays strike water molecules, thus resulting in single- or double strand breaks, cross-linkage breaks or nucleotide degradation.[14,48] In addition, γ-irradiation may damage virion structure, the capsid, and viral proteins, thus affecting infectivity.[11,14] Regarding other microorganisms, irradiation decreases the number of infectious viral particles as the dose of γ-irradiation increases. The effectiveness of irradiation is highly dependent on the environmental factors under which the organism is irradiated as well as on the medium and composition in which the organisms are contained.[11,48] MNV is often used in nutritional research as a surrogate for human norovirus to test the efficacy of irradiation on various food products. Although the FDA typically recommends doses as high as 4 kGy to inactivate bacterial pathogens such as *E. coli* and *Salmonella* spp., doses exceeding 10 kGy are generally required to inactivate MNV and prevent the spread of viruses from food.[14,23,38,48] Our study shows that irradiation at a dose of 25 kGy effectively mitigated the infective risks associated with MNV contamination in rodent feed. Although a similar effect was evidenced for MPV, a single mouse exposed to the highest dose 'escaped' and seroconverted. This outcome was confirmed as a true positive result, through fecal, MLN, and spleen PCR analysis. Several safeguards were taken to ensure the validity of our irradiation assessment. First, as discussed earlier, we took great care to minimize heterogeneity of viral distribution during pelleting and to avoid clustering. Second, we performed careful dose mapping before and during irradiation and used irradiation markers to ensure that all batches of contaminated feed received the standard dose of irradiation. We are therefore confident that the observed escape from MPV biocontainment was not due to experimental methodology and that 25 kGy of irradiation was insufficient to inactivate MPV at this highest titer for 1 of 6 animals.

We assumed that both MNV and MPV would be similarly inactivated by 25 kGy irradiation. This dose is commonly used in the United States for the irradiation of laboratory animal diets. Resistance to γ-irradiation may be due to a variety of extrinsic factors associated with an irradiated sample, including atmospheric content, temperature, protectors (the media or solids that are being irradiated [in our case, pelleted feed]), and the water content of the media or solid.[18] These extrinsic factors were essentially identical between the MNV and MPV groups, leaving intrinsic factors as possible explanations for differences in irradiation sensitivity. Several studies have shown that parvoviruses are relatively more radioresistant as compared with caliciviruses.[14,23,38] Radiosensitivity depends on viral particle size and nucleic acid composition (DNA compared with RNA and single- compared with double-stranded). MNV and MPV are both single-stranded and naked viruses, but they differ in genomic makeup and size (the MNV genome is approximately 7.4 kb of RNA, whereas MPV is approximately 5 kb of DNA) as well as in viral particle size (diameter: MNV, 28 to 35 nm; MPV, 20 nm).[1,31] Given these observations, it seems likely that the smaller genomic and viral particle sizes of MPV may explain the relative differences in the radiosensitivity of MNV and MPV. Further studies are needed to validate this assertion.

Overall, we questioned the biologic relevance of the single MPV escape in view of the original dose of viruses fed to these mice. The positive mouse was infected with 37.8 pelleted ID_{50} or 1.35×10^7 copies. To answer this question, we assumed that the number of genome copies (1.35×10^7 copies) was representative of the number of infectious virions. Considering an average shedding of 10^5 MPV per milligram from a wild mouse, this concentration of viral particles would correspond to the contamination of a single bag of feed with more than 2440 fecal pellets to achieve a similar seroconversion rate as seen for the group given 37.8 pelleted ID_{50} (that is, 16%). Under these circumstances, 25 kGy of irradiation would be insufficient to mitigate the risks associated with MPV contamination of the feed. However, the probability that a natural contamination would result in such high titers is fairly small, even if clustering of contaminated materials did occur. Nonetheless, the increased radioresistance of MPV may suggest the need to carefully evaluate the γ-irradiation doses necessary to fully mitigate the risk of radioresistant viruses. If higher doses were adopted, the industry would also have to address various regulatory requirements and potential downsides of high-dose irradiation, such as alteration of nutritional composition and increased costs. Indeed, the Food Additives Amendment to the Federal Food, Drug and Cosmetic Act (21 cfr 579) limits the irradiation dose for laboratory animal diets to less than 50 kGy. Exemptions for irradiation at 35 kGy must be and have been granted by the FDA in feed destined for gnotobiotic and germ-free mice, given that processing at those levels often results in irradiation of feed above the 50-kGy limit set by the act. However, higher doses may affect the nutritional composition of feed. Macronutrients such as carbohydrates, proteins, and fats are generally insensitive to irradiation, but some vitamins including A, K, B1, B6 and B12 are fairly sensitive.[32] Irradiation levels nearing the

legal maximum may significantly reduce the contents of these vitamins and cause clinical signs[7,21] unless the feed is fortified after irradiation, further adding to the cost of the feed.

In summary, our current study suggests that the heat and pressure conditions associated with the pelleting of rodent feeding are insufficient to mitigate the risks of infection from MPV and MNV in the case of feed contamination. Subsequent autoclaving of feed effectively sterilizes feed and prevents the infection of mice even after we spiked the feed with very high doses of MPV and MNV. Irradiation is efficient at decreasing viral load below infectious doses for both MNV and MPV, but risk of an MPV contamination of feed remains at exceptionally high viral doses. Future studies looking at the effect of various doses of γ-irradiation on the infectivity MPV and MNV are warranted to establish better industry standards.

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