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Preventing the Transmission of Murine Norovirus to Mice (*Mus musculus*) by Using Dry-heat Sterilization

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A critical component of an animal care biosecurity plan includes the sterilization of materials that come into direct contact with the animals. Dry-heat sterilization is gaining popularity in animal research facilities due to lower cost, less space utilization, no water usage, and the ability to sterilize water-sensitive materials. Currently, dry-heat sterilization ovens are validated against *Bacillus atropheus* spore strips with the assumption that a lack of sporulation is equivalent to successful sterilization. However, no published studies describe sterilization of rodent cages that contain relevant rodent pathogens by using this method. To determine if a dry-heat sterilizer can sterilize rodent cages and bedding against relevant rodent pathogens, we created murine norovirus (MNV)-contaminated cages by using mice with known MNV infection and shedding. The contaminated cages were either sterilized with the dry-heat sterilizer or not sterilized. Naïve, 4-wk-old, CD-1 mice were placed in the dry-heat–sterilized cages, contaminated unsterilized cages, or standard autoclaved cages for 2 wk. The mice were subsequently placed into clean, autoclaved cages for the remainder of the study. Fresh fecal pellets were collected at weeks 0, 12, and 16 and submitted for MNV PCR. Whole blood was collected for MNV serology at weeks 0, 8, 12, and 16. At week 16, all mice that had been in the unsterilized contaminated cages were positive for MNV by both fecal PCR and serology, whereas the mice in the dry-heat–sterilized and autoclaved cages were negative for MNV by both methods at all time points. Our study supports the use of dry heat sterilization as a viable sterilization method for rodent cages and bedding.

Abbreviations: MNV, murine norovirus

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Introduction

Biosecurity is defined as methods employed to detect, prevent, contain, and eradicate adventitious infectious agents. Biosecurity is an essential part of an animal care program to protect animal health and to control physiologic changes that could potentially affect research results.[1] One critical component of the biosecurity plan includes the sterilization of materials that contact the animal (for example, food, caging, bedding, and other research equipment). Sterilization options used in animal research facilities include the steam autoclave, dry-heat sterilization, ethylene oxide, and vaporized hydrogen peroxide.[17] Each method of sterilization has its own advantages and disadvantages depending on the application and the materials being sterilized.

Traditionally, the steam autoclave has been the most commonly used method for the sterilization of materials used in animal research facilities.[16,17] Steam autoclaves use moist heat and pressure to kill nearly all exposed microorganisms. Steam autoclave methods have the advantages of rapid, well-defined, easily controlled cycles, rapidly microbiocidal activity, and lack of toxicity to both animals and users.[15] Despite being the most common method, steam autoclaves have several disadvantages, such as use of water, high space needs, high initial cost, materials that cannot withstand autoclaving, and high maintenance costs.[16]

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Dry-heat sterilization is defined as thermal sterilization at humidity levels of less than 100%.[3,11] The 2 types of dry-heat sterilization applications use static or forced-air dry heat. The static dry-heat sterilizer has heating coils near the bottom of the chamber that allow heat to rise from the bottom to the top of the chamber.[12] Most modern dry-heat sterilizers use forced air, a nonpressurized oven chamber, electric coils as a heating element, and convection fans and dampers for even heat distribution.[11] The forced-air dry-heat sterilizer better maintains temperature and an even heat load compared with the static dry-heat sterilizer. Dry-heat sterilization has been gaining popularity because of several advantages such as no water usage, smaller space needs, less overall weight, lower initial and maintenance costs, and the ability to sterilize water-sensitive materials.[16] However, despite the advantages, dry-heat sterilization has never been validated against known pathogenic microorganisms other than *Bacillus atropheus* spores.

The objective of this study was to validate dry-heat sterilization against murine norovirus (MNV). MNV was selected for this study because it is one of the most prevalent pathogens detected in animal research facilities, it is easily transmissible in dirty bedding, it is resistant to disinfection, and it can disrupt normal physiology and research outcomes.[2,4-6,13,14] Noroviruses are nonenveloped, single-stranded, positive-sense RNA viruses in the family *Caliciviridae*; they can infect humans, mice, and many other animal species.[4,14] Immunocompetent mice infected with MNV are typically asymptomatic with persistent fecal shedding.[18] However, in some strains of immunodeficient mice, clinical signs such as weight loss, hunched posture, unkempt

hair coat, and death can occur.[8-10,14] This study evaluated the ability of dry-heat sterilization to prevent the transmission of MNV from contaminated bedding. Naïve, outbred sentinel mice were exposed for a 2-wk period to MNV-contaminated bedding that was unsterilized, dry-heat–sterilized, or autoclaved (control). After the exposure period, blood and feces were collected from the mice and tested for MNV antibodies and MNV infection, respectively, over a 16-wk period. We hypothesized that dry-heat sterilization would prevent the transmission of MNV to naïve female CD-1 mice.

Materials and Methods

Dry-heat sterilizer equipment and cycle validation. To ensure that the dry-heat sterilizer had the appropriate cycle time and temperature to achieve sterilization, a validation cycle was run using empty mouse cages with thermocouples. The targeted parameters in the validation cycle were 260 °F (127 °C) for at least 45 min. Omega SRTC-TT-J-24-180 Thermocouples (Omega Engineering; Norwalk, CT) were connected to a Graphtec GL-840 midi Data Logger (Graphtech America; Irvine, CA) to measure the temperature within a select number of cages during the dry-heat sterilization cycle. Allentown Micro-Vent System model MBS75JRHMV "75 JAG" mouse cages (Allentown; Allentown, NJ) with 1/8-in. corncob bedding (Envigo; Indianapolis, IN) was used for the dry-heat cycle validation. The same cages and bedding were used in the modified soiled bedding sentinel study.

A Gruenberg Model CG45V24SS Dry Heat Sterilizer (Thermal Product Solutions; New Columbia, PA) was used to sterilize the cages in this study. The Gruenberg Model CG45V24SS Dry Heat Sterilizer uses circulated hot air for the sterilization of hard goods. Briefly, a high-volume, vertical up airflow system is used to ensure uniform heat distribution throughout the chamber. A circulation fan, located in the plenum chamber at the top of the sterilizer, directs air into a circulation duct that runs down the back of the chamber. The heated air exits the duct and enters the chamber through a fenestrated panel near the bottom of the chamber. The heated air flows vertically up through the chamber and is directed back to the fan for reheating and recirculation. Electric heat is supplied by a seamless-tubular incoloy metal type heater suspended in the plenum. A powered exhauster located on the sterilizer is turned on by the controller at the end of the cycle to provide cooling prior to unloading the chamber.

Four of the 42 total mouse cages were prepared with thermocouples to validate the dry-heat sterilization cycle. Empty cage bottoms had a thermocouple taped in the center of the cage. Once the thermocouple was taped securely, approximately 200 g of corncob bedding was spread evenly throughout the cage bottom. The cage bottom with the thermocouple was stacked in a nested fashion with 2 other cages without thermocouples. The cage with the thermocouple was placed in a stack of 3 and occupied the bottom, middle, or top position. The top cage of each stack of 3 had a wire bar and microisolation lid added prior to being placed into the dry-heat sterilizer. Six stacks (18 cages total) of the described nested cages were placed on the top rack of the dry-heat sterilizer. Cages with thermocouples were assigned locations based on where cold spots might be present in the dry-heat sterilizer.

To create a full load in the dry-heat sterilizer, stacks of nested cages with corncob bedding were prepared for the bottom rack of the sterilizer. Four cages without thermocouples were stacked in a nested configuration as described above. Six stacks (24 cages total) of the described nested cages were loaded onto the bottom shelf of the dry-heat sterilizer.

Once all cages were loaded, the thermocouples were attached to the Midi data logger for real-time temperature readings within the selected cages. The dry-heat sterilizer was set at a maximum chamber temperature of 285 °F (141 °C) for a duration of 90 min. Temperature was recorded every minute for the entire cycle, including the cool down period. Temperature data was transferred to an excel file and analyzed to determine the ramp up time (time that all cages reached 260 °F [127 °C]), the soak time (time that all cages maintained 260 °F [127 °C]), and the cool down time (time that all cages reduced to 200 °F [93 °C]). These data were used to program the appropriate cycle settings for the rest of the study.

Once the appropriate cycle was identified, the validated cycle was repeated with *Bacillus atrophaeus* spore biologic indicators. A Releasat Biologic Indicator Culturing Set (Mesa Labs; Mesa, AZ) was used to ensure the dry-heat sterilizer could kill this agent. Briefly, the cages were set up as described above except biologic indicator spore strips were placed inside the chosen cages. The cages were loaded into the dry-heat sterilizer as described above. The loaded dry-heat sterilizer was then run using the validated cycle. Temperature data in the test cages were recorded with thermocouples and a data logger as described above to ensure repeatability. Once the cycle was complete, the cages were unloaded and the biologic indicator spore strips were removed for culture.

The biologic indicator spore strips were taken to the University of Florida diagnostic lab where they were cultured to ensure deactivation of the spores. A lack of growth after dry-heat sterilization would indicate a successful cycle. All work was done in a Class II A/B3 biosafety cabinet (NuAire; Plymouth, MN). The biologic indicators were removed from the outer packaging aseptically and transferred to the Releasat Biologic Indicator Culturing Set culture tube. The culture media tube with the biologic indicator was loosely closed with a cap and transferred to a 37 °C incubator. The culture was checked once a day for 5 d for any signs of bacterial growth.

Animals and housing. All procedures were reviewed and approved by the IACUC of the University of Florida, an AAALAC-accredited facility. Mice used on this study were maintained according to the *Guide for the Care and Use of Laboratory Animals*.[7] A total of 44 female CD-1 Mice (*Crl:CD1(ICR);* strain code 022; age, 4 wk; weight, 21.2 ± 1.2 g) were purchased from Charles River Laboratories (Raleigh, NC) and used for this study. All animals were allowed a 1-wk acclimation period after arrival at the facility. Five mice were housed in each cage. Mice used in the modified sentinel study were housed 2 mice per cage. Mice were housed in Allentown Micro-Vent System model MBS75JRHMV "75 JAG" mouse cages (Allentown; Allentown, NJ) on an individually ventilated cage rack. Mice were fed a standard commercial rodent diet (2918, Envigo, Indianapolis, IN), provided reverse osmosis–bottled water ad libitum, and housed on autoclaved ⅛in. corncob bedding (7092, Envigo, Indianapolis, IN) until initiation of the study. All mice received a cotton square for enrichment. Mice were housed in a temperature-controlled room (70 to 77 °F [21.1 to 25 °C]) on a 12:12-h light:dark cycle (nonrecessed fluorescent lighting, average 20 foot-candles [217 lux]), ≈10 to 15 air changes hourly, and 30% to 70% relative humidity. Mice were weighed once a week until completion of the study. Sentinels were used to test mice for ectromelia, rotavirus, hanta (Hantaan) virus, lymphocytic choriomeningitis virus, minute virus of mice, mouse adenovirus 1, mouse adenovirus 2, mouse cytomegalovirus, mouse hepatitis virus, mouse parvovirus, pneumonia virus of mice, polyoma virus, reovirus 3,

Sendai virus, Theiller's murine encephalomyelitis virus, *Filobacterium rodentium*, *Citrobacter rodentium*, *Clostridium piliforme*, *Corynebacterium kutscheri*, *Mycoplasma pulmonis*, *Pasteurella pneumotropica*, *Salmonella* spp., *Streptobacillus moniliformis*, *Encephalitozoon cuniculi*, fur mites, and pinworms (*Syphacia* spp. and *Aspicularis* spp.) quarterly.

Identification of mice actively shedding MNV. To create MNV-positive soiled bedding for this study, mice known to be positive for MNV were first identified through the University of Florida's animal health monitoring sentinel program. On a quarterly basis, whole blood was collected from sentinel mice using a Hematip Microsampler (Charles River Laboratories; Wilmington, MA) and tested for antibodies to MNV using a Multiple Fluorometric Immunoassay (MFIA; Charles River Laboratories; Wilmington, MA). On an annual basis, exhaust air dust plenum swabs were collected and tested for MNV and other prevalent pathogens through the PCR Rodent Infectious Agent Panel (Charles River Laboratories, Mouse Surveillance Plus PRIA). Briefly, the exhaust air dust panel on the IVC cage rack is removed during a rack change and the exhaust air duct plenums were swabbed with a pink sticky swab provided by Charles River Laboratories.

Animal health monitoring reports were compiled into a searchable database. Sentinel (donor) mice and IVC racks that were positive for MNV were identified in this database. The database search was limited to reports between January 2018 and December 2018.

Because mice can clear MNV infections and no longer shed the virus, donor mice were tested for active MNV shedding. To confirm active shedding, identified donor mice had 1 to 2 fresh fecal pellets collected by gentle restraint and abdominal palpation. Fresh fecal pellets from each mouse were placed into a separate 1.5-mL microfuge tube (Fisher Scientific; Hampton, NH) and submitted for MNV PCR (Charles River Laboratories; Wilmington, MA). Donor mice that were confirmed to have active MNV shedding were included in the study.

MNV-infected Mice. Due to the limited number of donor mice that were confirmed positive for MNV, additional mice were inoculated with MNV to provide an adequate amount of MNV positive bedding for this study. Four-week-old (n = 20), female, CD-1 Mice (*Crl:CD1(ICR);* strain code 022) were gavaged with a fecal slurry from the positive mice to create a stock of MNV-positive mice. To create the fecal slurry, infected mice were individually housed in an empty cage without bedding or enrichment for 4 h. Fresh feces were collected and weighed. Feces were added to a 50-mL conical tube (Fisher Scientific; Hampton, NH) and mixed with sterile 0.9% normal saline (ICU Medical; Lake Forest, IL) at a ratio of 1 g of feces to 10 mL of saline. The fecal slurry was manually homogenized in the saline diluent and then strained of larger particulate matter with sterile 4 × 4 gauze. Once filtered, 0.2 mL of the fecal slurry was orally gavaged to each stock mouse. At 4 and 20 wk after gavage, fecal pellets were collected from each mouse to confirm MNV shedding through MNV PCR.

Dry-heat sterilization of bedding from cages of infected sentinels. To create MNV-contaminated cages, MNV-positive mice were housed 5 mice per cage (total of 4 cages). Soiled bedding was allowed to accumulate in their cages for 2 wk. After 2 wk, soiled bedding was collected at the regularly scheduled bimonthly cage change. Soiled bedding from each of these cages was weighed and pooled. Eight clean, empty cages were filled with 100 g of pooled, contaminated bedding and 100 g of fresh, corncob bedding. These 8 cages were used as either positive control cages (contaminated cages, n = 4) or dry-heat–sterilized cages (n = 4). Autoclaved bedded cages (n = 4) were included as negative controls.

The 4 dry-heat–sterilized cages and 38 empty cages with bedding were assigned a number and assigned a cage position in the dry heat sterilizer through an online random number generator (random.org). Once cage positions were assigned, the cages were stacked and loaded into the dry-heat sterilizer as described above. Empty cages were included to create a full sterilizer load. All cages in the sterilized cage group received a biologic indicator as described above. Cages also received a Round Mini 4-Position Temp-Plate 140, 180, 220, and 260 °F (60, 82, 104 and 127 °C; Palmer-Wahl Instruments; Ashville, NC) temperature indicator. Temp-plates were added to ensure that cages reached the targeted temperature of 260 °F. The cages were run in the dry-heat sterilizer using the validated cycle described above. Biologic indicators were processed as described above. Freshly autoclaved cages were used for the study. Temp-plates were checked to confirm that the cage had achieved 260 °F during the dry heat sterilization cycle.

To show that the cage was not a source of MNV contamination, the door, chamber walls, ceiling, floor, and racks of the dry-heat–sterilized cages were sampled with a sticky swab before and after the cycle. The swab was then submitted for MNV PCR.

Modified contaminated bedding sentinel study. To test our hypothesis, a modified study was performed using the dry-heat–sterilized bedding cages, contaminated bedding cages, and standard autoclaved cages. Naïve, 4-wk-old, female, CD-1 mice (n = 24) were used in the study. Prior to enrollment on study, blood and feces were collected from all mice to test for MNV infection. Mice that were negative for MNV were included on study.

Dry-heat–sterilized cages, contaminated cages, and autoclaved cages were used to house 2 mice per cage (4 cages per group, 8 mice per group). Mice were housed in these cages for a 2-wk period. After this 2-wk exposure period, cages were changed and all mice were placed on standard, autoclaved, corncob bedding for the remainder of the study. No further soiled bedding exposure was performed. Blood was collected from each mouse for MNV MFIA on weeks 8, 12, and 16. Fresh fecal samples were collected and pooled for MNV PCR at weeks 12 and 16 after exposure.

Results

Dry-heat sterilizer cycle validation. Dry-heat sterilization is typically achieved when the cage temperature reaches 260 °F (127 °C) for longer than 45 min. The average time needed for the cages to reach 260 °F was 39 ± 3 min (Figure 1). The average time that cages remained at or above 260 °F was 73 ± 5 min (Figure 1). The average time to cool down from 260 °F to 200 °F (93 °C) or less was 23 ± 1 min (Figure 1). The biologic indicators were negative for sporulation and growth at 5 d after sterilization.

We found no evidence of MNV contamination in the dry-heat sterilization validation. Biologic indicators and Temp-plates indicated that cages had been sterilized. All 4 of 4 biologic indicators from the dry-heat–sterilized cages were negative for sporulation, and all 4 of 4 Temp-plates reached 260 °F (126.7 °C). Dry-heat sterilizer swabs were negative for MNV both before and after sterilization.

MNV inoculation of Stock Mice. All stock mice (20 of 20) that were inoculated with the infected fecal slurry tested positive for MNV by fecal PCR. PCR of pooled feces from each cage confirmed persistent MNV shedding in all cages that housed stock mice at 20 wk after inoculation.

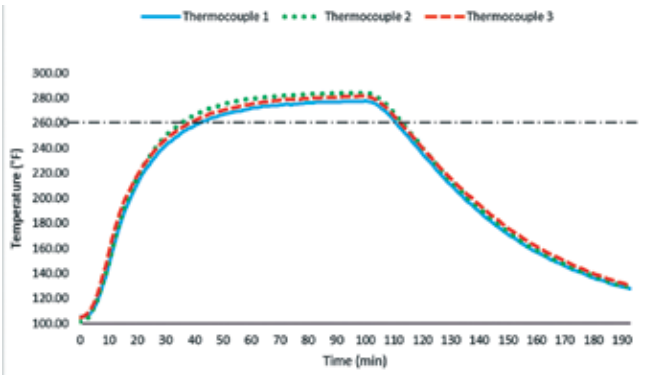

Figure 1. Dry-heat sterilizer cycle validation results. Thermocouple temperature data collected during the dry heat sterilizer during the validation cycle. Ramp up time is defined as the time needed for the intracage temperature to reach 260 °F (127 °C). Soak time is defined as the time period that cages were at or above 260 °F. Cool down time is defined as the time needed for cage temperatures to decrease from 260 °F to 200 °F (93 °C). A dashed line shows 260 °F, the temperature at which sterilization is achieved with a minimum soak time of 45 min.

Table 1. MNV fecal PCR results.

	Week 0	Week 12	Week 16
Dry-heat–sterilized bedding	0 of 4	0 of 4	0 of 4
Standard, autoclaved bedding	0 of 4	0 of 4	0 of 4
MNV-contaminated bedding	0 of 4	0 of 4	4 of 4

Fresh feces were collected from each mouse and pooled by cage at weeks 0, 1,2, and 16. Results are reported in positive cages per group at each time point.

Table 2. MNV serology results.

	Week 0	Week 8	Week 12	Week 16
Dry-heat–sterilized bedding	0 of 8	0 of 8	0 of 8	0 of 8
Standard, autoclaved bedding	0 of 8	0 of 8	0 of 8	0 of 8
MNV-contaminated bedding	0 of 8	0 of 8	0 of 8	7 of 7

Whole blood was collected from each individual mouse by using a hematip blood sample collector at weeks 0, 8, 12, and 16 and was submitted for serology. Results are reported as individual mice that were positive per group at each time point.

Transmission of MNV by contaminated bedding. Mice housed in contaminated cages that had first been subjected to dry-heat sterilization did not become infected with MNV. Feces collected from mice in the dry-heat–sterilized cages (0 of 4), the autoclaved cages (0 of 4), and contaminated cages (0 of 4) were negative for MNV by PCR at weeks 0 and 12 (Table 1). However, at week 16, 4 of 4 cages in the contaminated group tested positive for MNV on fecal PCR, whereas all cages in the dry-heat–sterilized (0 of 4) and standard autoclaved groups (0 of 4) were negative for MNV by fecal PCR at wk 16 (Table 1).

Mice in cages that were sterilized by dry heat did not seroconvert to MNV-positive status. All mice in the dry-heat–sterilized (0 of 8), autoclaved (0 of 8), and contaminated cages (0 of 8) were negative for MNV serology at weeks 0, 8, and 12 (Table 2). At 12 wk after exposure, 1 of the 8 mice in the contaminated cages showed general signs of illness (weight loss, hunched, unkempt hair coat) and was euthanized. A gross postmortem examination failed to identify a definitive cause of death; histologic examination was not performed. The remaining 23 mice appeared healthy and free of clinical disease throughout the study. At week 16, the remaining 7 of 7 mice in the contaminated cages were positive for MNV on serology, whereas all mice in the dry heat sterilized (0 of 8) and autoclaved (0 of 8) cages were negative for MNV on serology (Table 2).

Discussion

The main objective of this study was to determine whether dry-heat sterilization would eliminate MNV from contaminated cages and prevent transmission to naive mice. Our study showed that none of the mice housed in dry-heat–sterilized cages developed infection or seroconversion of MNV up to 16-wk after exposure. Mice housed on standard autoclaved bedding also did not develop an infection to MNV at any time point. In contrast, all mice housed in contaminated cages developed an infection and seroconversion to MNV at 16 wk after exposure. These results indicate that dry-heat sterilization was able to prevent transmission of MNV to naïve mice.

Mice exposed to MNV-contaminated bedding were infected with MNV at 16 wk after exposure based on both fecal PCR and serology. This is in contrast to previous studies that reported robust transmission of MNV through contaminated bedding between 2 and 8 wk after exposure on both serology and fecal PCR.[10,18] We do not know why our data conflicts with the previous studies. Several factors may have contributed to the observed results. One potential factor may have been intermittent shedding of virus from the sentinel study mice. Previous studies have demonstrated an inconsistent ability to detect MNV through fecal PCR for both oral inoculation and soiled bedding transmission studies.[2,5,10,18,19] The inconsistent ability to detect MNV through fecal PCR may be due to intermittent shedding of virus.[5,10,19]

Another factor that may have contributed to our MNV transmission results was a short duration of exposure to MNV-contaminated bedding. Our study used a modified soiled bedding schedule in which soiled bedding was added to cages only during the initial 2-wk exposure period. We chose this paradigm instead of a conventional sentinel model because a previous study showed high rates of MNV transmission at 2 wk after exposure.[10] However, other previous studies showed variability in detection of MNV, despite adding fresh contaminated bedding weekly for 12 wk.[2,18,19] Increasing the frequency of exposure to contaminated bedding may have increased the probability of exposure and transmission to the mice in our study.

During the validation of our dry-heat sterilizer, we observed the melting of some of our filter top lids for the cages. Dry-heat sterilizers use high temperatures that may damage certain materials, such as rubber and non-heat-resistant plastics, such as polycarbonate, during the dry-heat sterilization process. During the validation process of our dry-heat sterilizer, several cage tops that were near the top of the chamber were melted or warped at cycle completion. Our study used polysulfone Allentown Jag75 cages that are rated up to 150 °C. Our dry-heat sterilizer had a high temperature set point of 285 °F (140 °C). The dry-heat sterilizer used in our study was a small, older model in which the air flow moved from the bottom to the top of the chamber. We speculate that this air flow pattern may have allowed for the heat to accumulate at the top of the chamber, thereby exceeding 150 °C. We did not place a thermocouple at the top of our chamber to confirm this finding. Newer dry-heat sterilizers have fans and dampers that allow better air flow and a more even distribution of heat to prevent hot and cold spots in the chamber. Therefore, even when using newer dry-heat sterilizer technology, users should be cognizant for the potential of heat accumulation to damage the cages.

A potential limitation of our study was that we only evaluated MNV. Our original study design included several other

pathogenic agents such as mouse parvovirus and fur mites. Because our institution excludes these agents, including them would have required isolator housing. This would have limited our study due to cost and logistical challenges based on the need for isolators. We used MNV because it is not an excluded pathogen, it is highly prevalent in many mouse colonies, it is endemic in our mouse colonies, it transfers easily in soiled bedding, and it is highly relevant as many researchers are starting to exclude MNV from their rodent populations as a potential research variable.[4,8-10,13,14,18] A future study should include other agents of interest to see if the dry-heat sterilizer has the same effect and results as our current study.

Another potential limitation of our study was that statistical analysis was not performed due to low animal numbers and the nature of the data. Because of this, we chose to present the data in a qualitative form. A larger future study should be performed to confirm the findings of this study.

Our study showed that dry-heat sterilization successfully prevented the transmission of MNV to naïve mice. Our study used cages containing contaminated bedding from mice that were positive for MNV and had been sterilized using dry heat before placing naïve mice in those cages. Our study design is not practical in a mouse research setting because cages are normally sterilized after being washed and supplied with clean bedding. Our findings indicate that properly validated equipment for dry-heat sterilization can sterilize clean cages and bedding and can eliminate and prevent transmission of pathogens of MNV and to mice.

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