

# Contaminated Shipping Materials Identified as the Source of Rotaviral Infection of Exported Mice

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Over a 4-wk period in 2017, we received notification from 7 different institutions that mice exported from our SPF barrier facilities had tested positive for mouse rotavirus (MRV). The exports originated from several different buildings across multiple campuses. Our institution excludes MRV in all of our barrier facilities and has historically been free of this virus. Extensive testing of our rooms from which the exported mice originated did not detect the presence of rotavirus. The single commonality among the 7 shipments was the use of shipping boxes acquired from one vendor. These shipping boxes arrived at our institution prepackaged with unsterilized feed and bedding, which we hypothesized was the source of the rotavirus. To test this hypothesis, we housed naïve sentinel mice in clean cages with feed and bedding transferred from 29 unopened, unused shipping boxes. Sentinel mice were exposed to this bedding and feed for 14 d and then evaluated through MRV serology and PCR assay. Of the 29 sentinels, 24 were seropositive for MRV, and 14 of the 29 were PCR positive. These results provided direct evidence that MRV detected by recipient institutions originated from the contaminated feed or bedding within the shipping boxes. To our knowledge, this report is the first description of contaminated materials in shipping boxes resulting in rotaviral infection of mice during export.

**Abbreviations:** EDIM, epizootic diarrhea of infant mice virus; MRV, mouse rotavirus

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Mouse rotavirus (MRV) is a group A rotavirus that replicates in the villus epithelial cells of the small intestine.<sup>2,6,9,20,24</sup> It has also been shown to undergo extraintestinal replication as well.<sup>8</sup> Epizootic diarrhea of infant mice virus (EDIM) is commonly used as a generic name for all mouse rotaviruses.<sup>2,5,20,22,24</sup> However, EDIM is just one of many rotaviral strains that can cause clinical signs in infected mice.<sup>15,22,24–26</sup> All ages of mice can become infected, but clinical signs typically occur only in infant mice younger than 2 wk and in pups from nonimmune dams.<sup>2,9,13,14,20,24</sup> Because maternal antibodies protect pups, clinical signs of EDIM tend to disappear once the virus becomes enzootic in a colony.<sup>9,20,24</sup> Common clinical signs of EDIM include diarrhea, abdominal bloating, fecal staining, and delayed growth. Morbidity may be high, but mortality typically is low because the infection is self-limiting.<sup>2,9,13,14,20,24</sup> MRV is spread orofecally and is highly contagious. The duration of viral shedding from subclinically infected adults varies widely and is between 2 and 7 d.<sup>2,9,13,14,18,19</sup> Some strains of immunodeficient mice may shed the virus for longer periods, and such animals may develop chronic infections.<sup>2,6</sup> In addition, shedding duration and volume are mouse-strain-dependent.<sup>2,15,24</sup> MRV is generally considered to be an excluded pathogen for most standard barrier facilities. Research complications associated with MRV infection include effects on the immune system, development of type I diabetes, and numerous effects on intestinal function, intestinal content, and gut permeability.<sup>2–4,20,24</sup>

In addition, animals infected with MRV may be unacceptable for transfer to other institutions and result in the loss of unique mouse strains.<sup>19</sup>

A 2009 study indicated an EDIM prevalence rate of 0.56% among North American and European samples submitted to a large commercial diagnostic lab over a 5-y period.<sup>16</sup> Transmission to dirty-bedding sentinels is ineffective for the detection of MRV-infected mice, but the same report demonstrated that contact sentinels were effective methods for transmission of MRV.<sup>7</sup> Transmission of MRV can occur through contact with infected fomites or infected animals. The introduction of MRV into a facility can occur through the import of infected animals, wild mice, contaminated fomites such as feed and bedding, or by personnel handling infected animals or materials.<sup>6</sup> Cell lines are considered an unlikely source for rotaviral contamination—rotavirus has not been detected among thousands of cell line samples screened at a large commercial laboratory by using PCR assays.<sup>6</sup>

Contaminated unsterilized feed and bedding have been linked to outbreaks of murine viruses. For example, unsterilized feed was linked to a large-scale multifacility mouse parvovirus outbreak in barrier-housed mice.<sup>23</sup> In addition, contaminated corncob bedding was implicated in a geographically diverse outbreak of MRV in 2013.<sup>1</sup>

As members of a large academic institution, our investigators frequently export barrier-maintained rodents to other institutions throughout the world. Here we report a case of MRV contamination of shipping box materials used to export rodents. This report is the first of which we are aware to demonstrate contaminated shipping box materials as a source of rotaviral infection in mice.

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## Case Report

Over 4 wk in April 2017, 7 different institutions informed us that mice exported from our SPF barrier facilities had tested positive for MRV either by serology or fecal PCR analysis.

Animals in our SPF barriers are housed in IVC with water treated by reverse osmosis and UV sterilization. Bedding is autoclaved, and feed is irradiated. Entry to the rooms is by keycard access, and personal protective equipment is required. Rodents from approved commercial vendors enter SPF barriers directly, and rodents originating from other sources undergo a 10- to 12-wk quarantine or are rederived. The health status of the SPF colony is monitored by using soiled-bedding sentinels. Testing for the following excluded agents is performed quarterly: mouse hepatitis virus, pneumonia virus of mice, mouse parvovirus, minute virus of mice, MRV, Theiler murine encephalomyelitis virus, ectromelia, ectoparasites, and endoparasites.

The 7 shipments were exported as early as 17 February 2017 and as late as 22 March 2017, and each shipment originated from a different barrier housing room representing different principal investigators. Furthermore, the housing rooms were located within 4 different buildings on 2 different campuses. As part of our standard soiled-bedding sentinel monitoring program, our facility screens quarterly for MRV antibodies. MRV had never been identified in any of our barrier housing facilities or in any incoming shipments during the past 10 y.

On notification of these MRV-positive shipments, we immediately quarantined the 7 rooms from which the animals originated and placed additional soiled-bedding sentinels for testing. Sentinels were placed on each side of the rack and received dirty bedding from all cages on that side at cage change. After 4 wk of exposure to dirty bedding, fecal samples and blood were collected from the sentinels, which then were euthanized. Fecal samples submitted to a commercial laboratory for MRV PCR analysis, and serum was provided for serology. We performed this sentinel testing for MRV monthly for 2 mo in each of the 7 quarantined rooms.

The only commonality identified among the 7 shipments was the type of shipping box that was used to ship the mice. These boxes were acquired from a single vendor; are prepackaged with feed, paper chip bedding, and gel packs; and arrive closed and wrapped in several layers of plastic (Figures 1 and 2). Shipping boxes are received 24 to 48 h after ordered from the vendor. The vendor does not autoclave or irradiate the feed or bedding prior to shipment, and we do not autoclave the shipping boxes at our institution prior to their use. We hypothesized that the feed or bedding that the vendor placed into the shipping boxes may have been the source of MRV. We contacted the vendor, but the sources of the feed and bedding were not provided. The vendor stored the feed and bedding in an enclosed warehouse near an open field that was known to harbor wild rodents. Once we began to suspect contamination of shipping boxes, we immediately suspended all exports from using this particular shipping box and notified all institutions that had received mice from our institution during the 6 mo previous to the MRV findings.

To test our hypothesis, naïve mice were placed onto feed and bedding from unused, unopened shipping boxes in clean sterile caging. We identified 22 unused, unopened boxes at our facility that were part of a larger order and that had been associated with positive MRV detection in recipient mice. In addition, we had 4 boxes in storage that had remained unopened and unused; 3 of these boxes had been stored in building P and the remaining one in building R. We also ordered 3 additional unopened boxes from the vendor; we did not store these boxes but immediately



Figure 1. Open shipping box stocked with feed and bedding from the vendor.



Figure 2. Unopened shipping boxes received from the vendor.

opened for use in this study. All boxes were shipped directly by the vendor to the buildings housing the mice shipment and did not spend time in transit at other buildings.

## Materials and Methods

**Animals.** Female SPF Hsd:ICR mice (age, 4 to 5 wk; Envigo, Livermore, CA) were housed on corncob bedding in individually ventilated sterile and disposable cages (Innovive, San Diego, CA). The vendor reported that the colonies from which these mice originated were seronegative for mouse hepatitis virus, pneumonia virus of mice, mouse parvovirus, minute virus of mice, epizootic diarrhea of infant mice, Theiler murine encephalomyelitis virus, and ectromelia and were free of ectoparasites and endoparasites.

**Animal testing.** The mice were allowed 24 h to acclimate, after which blood was obtained from the submandibular vein, and fresh fecal samples were collected. Fecal samples were analyzed for MRV by PCR testing, and serum samples were tested for MRV antibodies. After the submission of baseline samples, mice were housed singly in cages and exposed to feed and bedding from 1 of the 29 unused boxes. Each cage received feed and bedding from the shipping box only; no other feed or bedding was provided in these cages.

After 7 d of exposure to feed and bedding from the shipping boxes, fresh fecal samples collected from each animal were submitted for PCR testing. After 14 d of exposure, blood and

**Table 1.** Fecal PCR and serology results for sentinels housed with feed and bedding from 29 shipping boxes

Shipping box location	Date of arrival	No. of shipping boxes	No. of sentinels	No. MRV-positive by fecal PCR assay on day 7	No. MRV-seropositive on day 14	No. MRV-positive by fecal PCR assay on day 14
Building R	4 Jan 17	1	1	0	0	0
Building G	6 Feb 17	22	22	12 (55%)	20 (91%)	4 (18%)
Building P	10 Feb 17	1	1	0	0	0
Building P	23 Feb 17	2	2	1 (50%)	2 (100%)	0
Delivered by vendor	5 Apr 17	3	3	1 (33%)	2 (66%)	0
Total		29	29	14 (48%)	24 (82%)	4 (14%)

fecal samples were collected from all mice, which then were euthanized by CO<sub>2</sub> overdose and cervical dislocation. In addition, from the mice representing the 22 remaining boxes from a single shipment, we submitted mesenteric lymph nodes after 14 d of exposure as well as the cage filter and tested these samples for MRV by PCR assay.

We did not perform all 29 exposures simultaneously; instead, testing was completed in 2 distinct groups. The first group represented the 7 mice exposed to feed and bedding from the 3 boxes obtained specifically for testing and the 4 unused boxes stored in buildings P and R. During the 14 d of exposure, cages were not changed or opened except on days 7 and 14 for testing. A class II type A/B3 safety cabinet (NuAire, Plymouth, MN) was used for all cage handling and animal sample collections. The safety cabinet was wiped down with 0.025% sodium hypochlorite between cages. Gloves were changed between cages or animals and before handling clean supplies. The remaining 22 exposures began approximately 1 wk after the first 7 exposures began. These 22 cages were housed on a separate rack in the same room. Mice in group 1 were never tested or handled on the same day as group 2 animals.

**Testing of feed, bedding, and shipping boxes.** Swabs of feed, bedding, and the interior of each shipping box were collected from the 3 unused boxes stored in building P and were submitted for PCR testing.

**Serology and PCR analysis.** All diagnostics were performed by Charles River Laboratories (Wilmington, MA). Serum samples were tested for antibodies specific to MRV by using a multiplexed fluorometric immunoassay.<sup>27</sup> Indeterminate, equivocal, or unexpected samples were retested by using a complementary immunofluorescent assay.<sup>12</sup> Details of sample processing, total nucleic acid isolation, and reverse transcription prior to PCR analysis have been described previously.<sup>27</sup> Briefly, fecal pellets were homogenized in PBS (approximately 4 times the sample volume) and partially clarified through centrifugation to eliminate large particles; 100 µL of the resulting supernatant was added to 275 µL of vendor-provided lysis solution (Thermo Fisher Scientific, Waltham, MA). Swabs and filters were vortexed in 500 µL of vendor-provided lysis buffer and briefly centrifuged to bring fluid to the bottom of the processing tube. Total nucleic acid was isolated from each lysed sample by using magnetic bead isolation, and a portion was reverse-transcribed to cDNA. The resulting cDNA reaction was PCR-amplified by using a group A MRV real-time PCR assay. Positive PCR reactions were confirmed by repeating the testing process, beginning with nucleic acid isolation.

**Facilities and housing.** All animal care and experimental procedures were in accordance with federal policies and guidelines governing the use of animals and were approved by the University of California San Francisco's IACUC. This institution has an AAALAC-accredited animal care and use program and

operates in accordance with the recommendations provided in the *Guide*.<sup>11</sup> Animals had continuous access to food and filter-purified, acidified water. The housing room was maintained at 66 to 74 °F (18.9 to 23.3 °C), with a humidity range of 30% to 70% and a 12:12-h light:dark cycle. All racks were maintained in a conventional facility, historically free of MRV.

## Results

At baseline, all 29 sentinel mice tested negative for MRV by serology and fecal PCR testing on arrival from the vendor. All animals remained healthy throughout the 14-d study period and showed no clinical signs of illness.

**Testing of feed, bedding, and shipping boxes.** All PCR swabs collected from the feed, bedding, and interior of the 3 shipping boxes from building P were negative for MRV by PCR analysis.

**Results after 7 d of exposure.** After 7 d of exposure to feed and bedding from the shipping boxes, 14 of the 29 sentinels tested positive for MRV by fecal PCR analysis (Table 1).

**Results after 14 d of exposure.** After 14 d of exposure, 4 of the 29 mice tested positive for MRV both by fecal PCR assay and serology. These 4 animals also were MRV-positive by fecal PCR analysis at the 7-d time point. A total of 24 of the 29 animals tested positive for MRV by serology (Table 1).

**Testing of exhaust filters and mesenteric lymph nodes.** At 14 d of exposure, we submitted mesenteric lymph nodes from the 22 sentinels in the 2nd group (see Methods) for PCR testing. In addition, the exhaust filters from the IVC housing these 22 sentinels were submitted for PCR testing. A total of 20 of the 22 animals tested positive for MRV after PCR analysis of mesenteric lymph nodes, and 12 of the 22 filters were MRV-positive by PCR testing.

**Testing of quarantine sentinels.** All housing rooms from which mice testing positive for MRV had been exported were placed under quarantine for a minimum of 2 mo. Dirty-bedding sentinels (2 per rack) were tested monthly for MRV by serology and fecal PCR analysis. All sentinels in all rooms were negative for MRV by both fecal PCR and serology. Room quarantines were released after the receipt of 2 mo of negative results.

## Discussion

On receiving the first few reports of positive MRV tests from exported mice, we considered several possible sources for the contamination. Although uncommon, infected vendor animals might have contributed to multiroom viral and parasite outbreaks in recent years.<sup>17</sup> All vendor imports into the housing rooms from which the infected exports originated were evaluated. No common vendor or mouse strain was identified among imported vendor animals. Approved vendors were contacted and were confirmed to have had no positive MRV tests in recent health screenings. We also assessed the possibility of mouse

transfers between the 7 housing rooms from which the infected animals were exported. At our institution, investigators are permitted to transfer animals to other investigators and other housing rooms provided they are at the same barrier status. All of our cage cards are barcoded, and cage movement through transfer and location can be tracked electronically. All transfer records for the 7 rooms were evaluated, and mice had been transferred between these housing rooms within the previous 6 mo. Other possible explanations for a sudden viral outbreak include contaminated feed, water, and cell lines and spread through the use of shared equipment. All of these possibilities were ruled out given, that the rooms from which the infected mice originated were distributed across multiple buildings and campuses, with different water and feed supplies and no shared resources or equipment. MRV contamination of cell lines has not been reported.<sup>6</sup>

The fact that all positive mice were exported animals implicated contamination of the shipping boxes themselves or of the mice during the shipping process. The 7 affected shipments were sent on different dates by using several different carriers. The only commonality among the 7 exports was the shipping boxes. The shipping boxes were not stored in a centralized location at our institution but were located at several different buildings and generally arrived 24 to 48 h prior to the animal shipment date. No evidence suggests that the shipping boxes became contaminated while at our facilities, and this conclusion is further supported by the results from mice exposed to feed and bedding from 3 boxes ordered from the vendor specifically for sentinel testing. These boxes were not stored, and feed and bedding were immediately removed and placed in 3 separate cages with 3 vendor-supplied sentinel mice. Two of the 3 mice housed with materials from 2 of these 3 boxes seroconverted to MRV.

The results of this study demonstrate that contaminated feed or bedding from the shipping boxes was the source of the MRV infection in the exported mice. Feed and bedding from 29 unused boxes were tested, and 24 naive animals housed in cages with those materials seroconverted to MRV. In addition, 14 of the 24 animals that seroconverted tested positive for MRV by fecal PCR analysis after the 14-d exposure period.

Not all of the 29 shipping box materials we used in this study appear to have been contaminated with MRV, or the amount of virus was below an infectious dose (Table 1). Specifically, only 24 of the 29 boxes showed evidence of MRV contamination according to sentinel seroconversion. The 2 shipping boxes that arrived at our facility on 4 January and 10 February did not show evidence of contamination with rotavirus, whereas both shipping boxes that arrived on 6 February 6 and 22 of the 24 that arrived on 23 February contained MRV-contaminated materials. Finally, 2 of the 3 shipping boxes delivered to us directly from the vendor on 5 April contained MRV-contaminated materials. One potential explanation for the variability among boxes to infect or not infect the mice is that the shipping boxes contained materials representing different lots of feed or bedding. The arrival dates of the contaminated shipping boxes show that this vendor had ongoing contamination with MRV-infected materials for roughly 2 mo. To our knowledge, our institution is the only location to have received shipping boxes that were prepacked with feed and bedding from this vendor, and the vendor confirmed that other institutions that use these shipping boxes receive them empty and provide feed and bedding at their own institutions.

One limitation of this study was the lack of negative controls. Efforts to reduce the likelihood of cross-contamination between cages—including glove changes between animals, cages and

new equipment; opening of cages only at the time of testing and in the biologic safety cabinet; and cleaning with dilute sodium hypochlorite between cages—were in place. However, without the use of negative controls, we cannot completely rule out the possibility that some of the animals with feed and bedding exposure were infected by cross-contamination from other infected cages.

An assumption made through the course of this discussion is that the contaminating virus is murine in origin, given that the PCR assay and serology could have shown positive results with a heterologous rotaviral strain. Rotaviruses are typically species-specific, but studies have shown that mice have limited susceptibility to heterologous rotaviral strains.<sup>3-5,20,21,22,26</sup> The specific rotavirus strain in the current study was not sequenced, but given the history of wild mice in the field near the vendor's feed and bedding storage area, the likelihood that the virus is heterologous is low.

In the current study, rotaviral infection was identified most consistently by serology and PCR analysis of mesenteric lymph nodes at 14 d after exposure. Specifically, 20 of the 22 mice tested by mesenteric lymph node PCR assay were MRV positive, and these same 20 animals were seropositive for MRV. We also tested the filter from each of these 22 cages by PCR assay. Of the 20 cages with seropositive sentinels, only 12 had filters that tested PCR-positive for MRV. The short shedding period of MRV likely produces a reduced viral load in the cage that may compromise detection by using the cage filters. In addition, PCR testing of swabs of feed and bedding were not a useful diagnostic method for MRV contamination.

In comparison, among PCR samples, mesenteric lymph nodes appear to provide the largest window of detection, although verification at earlier time points was not investigated. In addition, PCR analysis of mesenteric lymph node may be useful for confirming seropositive mice. Due to the short shedding period of MRV, fecal pellet testing may be useful during quarantine testing only when fecal pellets are collected soon after arrival. Serology provided the best detection in the current study scheme; however, previous reports suggest that MRV does not transfer successfully to soiled-bedding sentinels.<sup>7</sup> Direct testing of mice in quarantine by PCR assay and serology may be important to demonstrate that mice are not shedding virus on arrival and that no previous exposure has occurred.

Unlike many other murine viruses, MRV can be eradicated at a facility without replacement of the entire colony with uninfected or rederived animals. Through cessation of breeding for several weeks and the removal of immunodeficient animals, MRV has been successfully eliminated from positive facilities.<sup>9</sup> Without susceptible neonates or immunodeficient animals, the virus is unable to spread. Immunocompetent adult mice shed the virus for a limited period of time only and then become immune.<sup>9</sup> One institution that received a large number of infected animals from our institution successfully eliminated the further spread of rotavirus by using this strategy.

An advantage for facilities in this case study that used a PCR-based quarantine is the rapid identification of rotavirus and elimination of positive mice to mitigate the spread of the virus within the quarantine holding space. A soiled-bedding sentinel system would retain the MRV-positive mice for the entire quarantine period, thus increasing the chance of spread to other cages. Although MRV is unlikely to transfer to bedding sentinels, some of the recipients of positive mice from our facility did observe transmission to soiled-bedding sentinels during quarantine.<sup>7</sup>

Rotaviruses are nonenveloped viruses that are stable in the environment and at low pH. Despite their stability, rotaviruses are readily inactivated by common disinfectants, including phenols, chlorine, and ethanol, or through autoclaving or irradiation of materials.<sup>6,24</sup> As a result of this incident, our institution has reevaluated our approach to sterilizing and disinfecting shipping boxes. We now order empty shipping boxes and autoclave all shipping boxes (regardless of vendor) at 115 °C for 5 min, followed by thorough cleaning with a standard disinfectant prior to use.

In summary, our current case report is the first of which we are aware to implicate contaminated shipping box materials as a source of rotaviral infection in mice. Although we were unable to differentiate whether feed or bedding was the source of the contamination, this event emphasizes the importance of assessing import–export procedures and practices to prevent exposure of animals to excluded pathogens. One notable and commendable aspect of this event was the outstanding communication from other laboratory animal veterinarians, who immediately notified of us of the MRV-positive results. Without this rapid notification from other institutions, several additional shipments of MRV-positive mice might have been exported.

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