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PCR Testing of Media Placed in Soiled Bedding as a Method for Mouse Colony Health Surveillance

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Rodent colony health surveillance has traditionally been accomplished by testing sentinel animals that have been exposed to soiled bedding from colony animals. Collecting samples from exhaust plenums on ventilated caging systems, followed by PCR analysis, has emerged as another promising method for health surveillance. However, environmental testing at the rack level is not effective for all ventilated rack designs. In this study, we tested whether media placed in soiled bedding is effective in detecting 3 adventitious agents: mouse norovirus (MNV), *Helicobacter* spp., and fur mites. Soiled bedding was collected from pathogen-positive colony mice and distributed to traditional sentinel mouse cages and mouse-free experimental cages every 1 to 2 wk for static and ventilated cages, respectively. Experimental cages contained 10 flocked swabs ('passive swabs') and 1 piece of filter media. After 90 d, fresh feces, pelage swabs, and blood were collected from the sentinel cages, and the passive swabs and filter media were collected from the experimental cages. Concurrently, 10 additional flocked swabs ('active swabs') were stirred through the cumulated soiled bedding of each experimental cage. Sentinel mice were positive for MNV and *Helicobacter* spp., but negative for fur mites by pelage swab PCR. All samples from experimental cages were positive for *Helicobacter* spp. and fur mites in both caging types. For MNV, passive swabs were most effective at detection (100%), followed by active swabs (80% to 100%) and filter media (60% to 80%). These findings suggest that testing media in pooled soiled bedding samples is more effective than traditional sentinel methods for colony health surveillance and is a viable option when sampling at the rack level is ineffective.

Abbreviations: MNV, murine norovirus

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Research animal care programs typically take steps to exclude certain infectious agents from mouse colonies. Such colonies are often referred to as specific pathogen-free (SPF). Colony health surveillance programs are designed to monitor for the presence of excluded pathogens and are the basis for determining whether the desired SPF status remains intact. Surveillance is also important for maintaining the integrity of the science for which the animals are being used.

Testing all colony animals as part of a health surveillance program would be time- and cost-prohibitive. Therefore, strategies for assessing fewer, select animals that reflect the health status of the entire colony were devised decades ago.[11,15,21] At their core were the practices of using of 'sentinel' animals and 'soiled bedding transfer.' These methods involve collecting bedding that contains urine, feces, and dander from colony animals, usually as part of routine husbandry activities. This bedding is then added to the cage of a sentinel, which is an animal originating from outside the colony under survey and that is known to be free of excluded pathogens. The approach assumes that the sentinel animal, through direct contact with any infectious agents in the transferred material, will become infected and yield a positive result upon testing for said agents. With some periodicity, typically every 3 to 6 mo, biologic samples such as blood for serology, fecal pellets, pelage swabs, and oral swabs for PCR, pelage tapes for microscopy, and tissues for histopathology may be collected from the sentinel mice and assayed for pathogens of interest. The results from this testing are then considered to represent the health status of the colony to which the sentinel animal was exposed.

These traditional surveillance methods, referred to as 'sentinel programs,' have been valuable and reasonably effective given the relevant time and cost constraints; however, sentinel testing has several limitations. Most important is the inconsistent transferability of pathogens via soiled bedding,[5,40] intermittent shedding of some agents from colony mice,[39] and the varying susceptibility to infection or seroconversion rates of sentinel mouse strains.[3,10] For example, murine norovirus (MNV),[26,34] mouse hepatitis virus (MHV),[5,6] and *Helicobacter* spp.[5,20,41] are reliably transferred via soiled bedding, as is mouse parvovirus (MPV), under optimal conditions.[39] Conversely, Sendai virus,[1,5,6,8] lymphocytic choriomeningitis virus (LCMV),[13] lactate dehydrogenase elevating virus (LDV, LDHV),[5] *Spironucleus muris*,[33] *Rodentibacter pneumotropicus* and *R. heylii* (previously *Pasteurella pneumotropica*),[38] and fur mites[8,19,28,40] do not transfer readily and subsequently are difficult to detect. In addition, transfer of soiled bedding is labor-intensive for animal husbandry technicians, and sentinel animals are susceptible to unexpected illness and death.

For these reasons, colony health surveillance programs based on environmental, rather than sentinel, sampling have gained popularity over the past decade. This method has been made possible by the development of reliable PCR assays for

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the adventitious agents that are most often monitored in SPF colonies and by the refinement of sampling methods. Several studies have shown that environmental sampling is a viable alternative to traditional sentinel programs.[16,17,23,25,27-32,35,42] The collection and assay of only environmental samples eliminates the need for sentinel animals, leading to a desirable overall reduction in the number of animals used, and the associated reduction in husbandry, labor, and supplies.[22]

Although environmental sampling can be done in many ways, the exhaust plenums of individually ventilated caging (IVC) systems have been shown to be a useful sampling location.[4,14,25,35] The so-called 'plenum testing' method has therefore been adopted by some animal care programs as a colony health surveillance technique. The logic behind this technique is that bedding, dust, and dander from cages carry pathogens as they circulate into the plenums, from which they can be collected, and that these samples can be considered representative of the entire rack. However, attention to detail is essential to the success of this approach. Racks and plenums should be sanitized and autoclaved, and ideally, confirmed sterile by PCR analysis before deployment at the beginning of a sampling cycle.[9] Placement of collection media into the exhaust plenums may be labor-intensive depending on the rack model. Media retrieval must also be done meticulously to eliminate the inclusion of contaminating DNA.

The IVCs themselves create additional challenges to program success, as they are designed to substantially reduce the transmission of agents that would otherwise spread throughout the population. Because the exhaust air of each cage is filtered prior to entering the plenum, debris may not be deposited in sufficient quantities within certain rack models to be reliably detected at the final exhaust filter.[8] In light of such concerns, studies to document that samples yield reliable data have been reported, with variable results depending on the location of the samples on the rack.[36] Common areas for sample collection on IVC racks include the horizontal exhaust manifold,[2,14] the cage filter top,[8,9] and the exhaust air prefilter.[2,4,17,24,29,42] Manufacturer variation in IVC rack design ultimately dictates where sampling should occur, as airflow pattern, location of dust accumulation, filter type and location, and plenum access must be considered.[2] All of these factors, perhaps including organizational resistance to change, can be hurdles to the widespread adoption of environmental testing for colony health surveillance.

Discontinuing the use of sentinel mice for colony health surveillance by transitioning to PCR-based environmental testing has been a long-standing objective in our program. Plenum testing would be a possibility, but is not sufficient for our program, as static microisolator caging is also in use. In addition, we exclusively use Lab Products AllerZone IVCs. Discussions with the manufacturer suggested that the substantial filtration at the individual cage level would result in minimal debris accumulation in the plenums, precluding this as a sample collection site. This challenge has been addressed in a study that describes an alternative sampling method used in caging systems similar to ours.[8] However, in a pilot study, we found that assembling and disassembling of the cage lid filter as described in the published study[8] was extremely labor-intensive and therefore, this method was considered untenable for our program.

The desire to find an alternative sampling strategy that would simplify sample collection was the major impetus behind the current study design. To obviate the need for lid manipulation, 2 different media—flocked swabs and filter media—were placed directly into pooled soiled bedding collected from colony cages using traditional transfer methods. Media were assayed after 90 d in both static and IVC caging. Flocked swabs were also used to collect dust and dander from cages on the final day of the experiment. The primary goal was to evaluate these media for their ability to detect 3 adventitious pathogens of laboratory mice: mouse norovirus (MNV), *Helicobacter* spp., and the fur mite species *Radfordia/Myobia* and *Myocoptes*. The results presented show that the media were effective, and either equivalent or superior, to our traditional sentinel program at detecting these agents in both static microisolator and IVC caging systems.

Materials and Methods

Animals. This study used female, heterozygous Crl:NU(NCr)-*Foxn1*^{nu} mice (Charles River Laboratories, Frederick, MD) of 2 ages, 5 mo and 6 wk, as colony (n = 20) and sentinel (n = 4) animals, respectively. These heterozygous mice are immunocompetent, fully hirsute, and routinely used at our institution for colony health surveillance. The colony mice in this study were retired sentinel mice that had tested positive by PCR for MNV, *Helicobacter* spp., and *Radfordia* fur mites. Sentinel mice were received from the vendor and entered directly into the study. These mice were free of the standard panel of pathogens according to vendor health surveillance. Sentinel mice from the vendor are routinely screened upon arrival at our institution and have historically tested negative for all pathogens.

Colony mice were housed only in IVC caging, whereas sentinel mice were housed in both IVC and static conditions, using Lab Products caging systems (RAIR HD Ventilated Rack and Micro-Isolator Cages, Lab Products, Seaford, DE). The IVC rack generates 34 air changes per hour. No colony mice were housed under static conditions, as we had no reason to believe that condition would impact study outcome. All cage materials were autoclaved, and husbandry methods standard for Emory University were used. All mice were housed on corncob bedding (1/8″ Bed-o'Cobs, The Andersons Lab Bedding Products, Maumee, OH), fed irradiated chow (PicoLab Rodent Diet 20 (5053), LabDiet, St Louis, MO), and provided autoclaved reverse osmosis water. Cotton squares (Ancare, Bellmore, NY), Bed-r'Nests (The Andersons Lab Bedding Products), InnoDomes, and InnoWheels (Bio-Serv, Flemington, NJ) were offered for enrichment. Husbandry staff accessed cages only under a HEPA-filtered, class II, type A2, biologic safety cabinet (NuAire, Plymouth, MN). Emory is accredited by AAALAC International, and all environmental conditions were maintained in accordance with *The Guide for the Care and Use of Laboratory Animals, 8th edition*.[12] This study was approved by the Emory University IACUC.

Experimental Design. Five cages, each containing 4 mice, comprised the experimental colony; these mice were arranged in a single IVC rack row. One cage containing 2 sentinel mice was placed on the same rack on a lower row, while another was placed on a static rack. Ten experimental cages that did not contain mice served as soiled bedding material receiving vessels; 5 cages were placed on both IVC and static systems. Otherwise, no other mice or cages were present on the IVC rack. Each experimental cage contained 10 flocked swabs (PurFlock Ultra, Puritan Medical Products Company, Guilford, ME) and 1 piece of 3 × 6″ filter media (Reemay 2024, acquired from Lab Products) and were placed in the cage at random with no particular positioning. In consultation with the diagnostic lab, 10 flocked swabs were recommended as the optimal sample size for best results.

During routine colony cage changes, 12 Tbsp (or approximately 180 mL) of soiled bedding were obtained from each colony cage, ensuring that approximately 25% of the nest material was sampled as well. This created a pooled reservoir of material for

transfer to the experimental and sentinel cages. Four and 10 Tbsp were respectively transferred to the mouse-free experimental cages and the sentinel mouse cages. Sentinel cages received a larger volume of soiled bedding to account for dilution caused by clean bedding that was provided during cage change, as per our standard operating procedure. The mouse-free experimental cages were shaken back and forth lightly after receiving soiled bedding to ensure mixed materials (bedding and media); cages were then returned to their racks. Static cages were handled identically, except that soiled bedding was collected during weeks in which no routine colony husbandry was occurring. This approach mimicked our standard weekly cage changing practices for mice housed in static cages. During the intervening weeks, 6 Tbsp of soiled bedding and cotton square enrichment material were obtained from each colony cage, pooled, and transferred only to the static experimental and sentinel cages. Soiled bedding transfer continued for 90 d, the standard duration of our current sentinel program. Bedding in experimental cages was 1 and 2″ deep for IVC and static cages, respectively, at the end of the experiment; these depths were a function of the variable bedding transfer schedule for each cage type. The lead researcher (WHH) performed all soiled bedding collection and distribution procedures, with the assistance of 2 veterinary technicians under direct supervision.

Sample Collection and Testing. Fecal pellets, pelage swabs, and blood were collected from colony mice every 30 d to verify the continued presence of MNV, *Helicobacter* spp., and fur mites. The presence of fur mites was also visually verified by microscopic observation of pelage tapes at the end of the study. At the 90 d time point, the 10 flocked swabs (henceforth referred to as 'passive swabs') and the filter media were retrieved from each experimental cage. To avoid contamination, the flocked swabs were picked up by the handle only, and gloves were changed between pooled samples. The filter media was collected using a separate autoclaved forcep for each pooled sample. Ten additional flocked swabs (henceforth referred to as 'active swabs') were stirred through the soiled bedding of each experimental cage. All samples were pooled at the cage level. Ten fecal pellets, 10 pelage swabs, and blood samples were also collected and pooled for each sentinel cage. All samples were shipped to IDEXX BioAnalytics (Columbia, MO) for assay. The passive swabs (10 per cage), filter media (1 per cage), and active swabs (10 pooled per cage) were tested via PCR for MNV, *Helicobacter* spp., and fur mites (*Radfordia/Myobia* and *Myocoptes*). The fecal pellets (10 per cage) were tested via PCR for MNV and *Helicobacter* spp., while the pelage swabs (10 per cage) were tested via PCR for fur mites (*Radfordia/Myobia* and *Myocoptes*). Blood was tested by MFI[2] serology using the Opti-Spot card for MNV. All sampling was performed by the lead researcher (WHH) with the assistance of 2 veterinary technicians under direct supervision.

Results

Colony Cages. All colony mice were confirmed to be positive for MNV, *Helicobacter* spp., and fur mites by PCR and remained positive for the duration of the study (data not shown). In addition, the presence of fur mites was verified visually by microscopic observation at the 90 d time point. No attempt was made to speciate the mites, either visually or by PCR, but *Radfordia affinis* were identified in the pilot study and presumed to be the species present.

Sentinel Mouse Cages. All samples collected from sentinel mice housed in both cage types yielded positive results for MNV and *Helicobacter* spp. All samples were negative for fur mites (Tables 1 and 2) .

Experimental Cages. Detection rates of the sampling methods for both IVC (Table 1) and static cages (Table 2) were comparable. Passive swabs performed the best, with 100% of samples (5/5) from both caging types yielding positive results for the 3 agents studied. Active swabs were equally effective in static caging with 100% detection rate (5/5) for all 3 agents. Active swabs in IVC housing showed a 100% detection rate (5/5) for *Helicobacter* spp. and fur mites, but only 80% (4/5) for MNV. Filter media was the least effective in both cage types, but only for MNV. *Helicobacter* spp. and fur mites were successfully detected 100% of the time (5/5) by filter media, but only 80% (4/5) and 60% (3/5) of samples were positive for MNV in IVC and static caging, respectively.

Discussion

The primary aim of this study was to determine whether media placed in mouse-free cages receiving soiled bedding would detect 3 adventitious pathogens endemic in portions of our murine census: MNV, *Helicobacter* spp., and fur mites (ostensibly *Radfordia*). This novel approach to environmental sampling for colony health surveillance was necessitated by the design of caging systems in use at our institution. Results show that the methods described were highly effective in detecting the pathogens of interest. In the case of fur mites, this method was superior to the samples traditionally obtained from sentinel mice. Among the media types, both the passive and active swabs were superior to the filter media, and housing type had no significant impact on effectiveness.

Of the 3 agents investigated, MNV was the only pathogen with less than 100% detection, as both active swabs and filter media yielded some negative results. Whether these results are a function of the agent, the media, the sampling method, or the PCR assay itself should be considered. MNV is reported to transfer well via soiled bedding and is stable in fecal pellets, even after storage at room temperature for 14 d.[26] Furthermore, MNV was reliably detectable in diluted samples—1 MNV-positive fecal pellet to 19 MNV-negative fecal pellets—suggesting that the PCR assay was sensitive and that false negative results were unlikely.[26] In addition, the laboratory performing the PCR reports that the assay reliably detects 10 or fewer copies of target DNA or RNA.[2] These factors suggest that failure to detect MNV was unlikely to be due to poor transmission by soiled bedding, instability of the agent in fecal pellets, or low PCR sensitivity.

The properties of the sampling media and how it is deployed are also factors in MNV detection. In our study, passive swabs performed better than active swabs and filter media. The flocked swabs are composed of polyester in the form of 'webbing' that provides a greater surface area for specimen collection and subsequent elution. Passive swabs were used throughout the experiment, whereas active swabs were only used immediately before the end of the study. Passive swabs were therefore in contact with soiled bedding for a significantly longer time than were the active swabs, which is a possible reason for the differing detection rates. As with the passive swabs, filter media was used for the entire experiment. It is composed of spun-bonded polyester made into a stiffer, tighter, sheet structure with less density and surface area. It is designed for filtration and therefore exhibits excellent arrestance, but is perhaps inferior for collecting debris by direct contact. However, all media and methods were equally effective in detecting both *Helicobacter* and fur mites. Because of this, we can only speculate that the

Table 1. Testing results of samples collected in the IVC cage system at the end of the 90-d study period. Sentinel samples were pooled from both mice per cage (n = 1) and assayed using PCR and serology. Experimental cage samples were pooled per cage (n = 5) and assayed using PCR.

	Sentinel mouse cage		Experimental cages					
	Blood	Feces/Pelage swabs	Passive swabs		Active swabs		Filter media	
MNV	+	+	5/5	100%	4/5	80%	4/5	80%
Helicobacter spp.	not applicable	+	5/5	100%	5/5	100%	5/5	100%
Fur mites	not applicable	-	5/5	100%	5/5	100%	5/5	100%

Table 2. Testing results of samples collected in the static cage system at the end of the 90-d study period. Sentinel samples were pooled from both mice per cage (n = 1) and assayed using PCR and serology. Experimental cage samples were pooled per cage (n = 5) and assayed using PCR.

	Sentinel mouse cage		Experimental cages					
	Blood	Feces/Pelage swabs	Passive swabs		Active swabs		Filter media	
MNV	+	+	5/5	100%	5/5	100%	3/5	60%
Helicobacter spp.	not applicable	+	5/5	100%	5/5	100%	5/5	100%
Fur mites	not applicable	-	5/5	100%	5/5	100%	5/5	100%

properties of MNV, in combination with those of the media and deployment method, led to the discrepant detection rates.

Pelage swabs collected from sentinel mice were negative for fur mites by PCR, even though the colony animals had been verified positive. Traditional soiled bedding transfer is known to provide inconsistent detection of fur mites in sentinel animals,[8,17-19,37] and as such our finding is not entirely novel. However, all media samples collected from the soiled bedding of mouse-free experimental cages were positive for fur mites by PCR. This suggests that fur mite nucleic acid is indeed reliably transferred with the collection of soiled bedding. Therefore, failure of detection in traditional soiled bedding sentinel programs is likely due to the lack of live fur mites on live sentinel animals, rendering pelage swabs ineffective. Another possible explanation for detection failure is the dilution of soiled bedding in sentinel cages, as the mice receive clean bedding at every cage change. Nonetheless, our result further supports the decision to transition from sentinel mice to environmental sampling in our program.

The role of physical agitation of the experimental cages in successful detection of agents was not tested in this study. Shaking mouse-free boxes of dirty bedding twice weekly was necessary to consistently detect pathogens on the filter media of IVC filter tops in a previous study.[8] Shaking was posited to function as an effective substitute for a resident mouse that would normally 'kick up' particulate matter in the course of daily activity and thereby deposit material on filter top media as air circulated in the cages. The design of the current study obviated the need for this step, as all media directly contacted soiled bedding. Instead, experimental cages were agitated only in conjunction with soiled bedding transfer to ensure mixing of contents. An additional study to determine whether agitation is necessary could be considered, as eliminating this step would save on time and labor and be more ergonomically sound. In the absence of such data, however, the authors are of the opinion that the act of bedding transfer provides sufficient mixing and that agitation is probably not vital.

Statistical analysis was not pursued for this study, given the limited sample size. Another aspect of the study design worth acknowledging is that soiled bedding was collected only from the 5 agent-positive colony cages. Therefore, pathogen-positive soiled bedding was not diluted with pathogen-negative soiled bedding, as would be expected in a real surveillance situation. While our experimental design is not representative of a real-life scenario, this investigation was considered a proof-of-technique study with results encouraging pursuit of a larger, in-facility study where realistic soiled bedding dilutions would occur.

This study showed that filter media and flocked swabs, used both passively and actively in soiled bedding, allow the successful detection of representative adventitious infectious agents of mice in both IVC and static caging systems. Although we studied only 3 pathogens, our results, taken together with other studies of similar design, suggest that these methods would be effective for many agents commonly assessed as part of a colony health surveillance program and could be successfully implemented for environmental sampling. Although the need for soiled bedding transfer remains, the methods described offer several advantages over our traditional surveillance program, all of them a function of eliminating sentinel mice from the program: reduced veterinary technician time in managing clinical cases, reduced husbandry labor, and the elimination of the emotional fatigue experienced at the time of sentinel euthanasia.[22]

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