PCR Testing of Filter Material from IVC Lids for Microbial Monitoring of Mouse Colonies

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Testing sentinel animals exposed to soiled bedding from colony animals is the most common method used for health monitoring in rodent facilities. Although environmental sampling is being explored—and, in many cases, has been implemented—as an alternative, exhaust plenum sampling is not effective for all ventilated rack designs. This study evaluated PCR testing of filter paper from sentinel cages on ventilated racks. We hypothesized that testing filter paper from cages containing soiled bedding would be as effective as testing sentinel mice and that periodic shaking of cages would generate sufficient particulate movement to substitute for the presence of live animals. Three cages containing soiled bedding were maintained in each of 8 rooms; one cage contained 2 Cr:NIH(S) mice, one had no mice and was shaken twice weekly, and the remaining one had no mice and was left undisturbed. For 3 consecutive months, a piece of filter paper from the undersurface of the cage lid was tested monthly for adventitious agents and then replaced. A second piece remained on the cage undersurface for 3 mo. Fecal pellets and oral and fur swabs were collected from sentinel mice at months 1 and 3 and tested for the same agents. At month 3, serology was performed on the sentinel mice; feces and oral and fur swabs from colony animals were tested concurrently for comparison. Filter paper from cages without mice and shaken were at least as effective than all other methods in detecting the presence of endemic agents, including mouse norovirus, *Helicobacter* spp., *Pasteurella pneumotropica*, *Entamoeba muris*, and *Spironucleus muris*. For IVC systems where exhaust plenum testing is ineffective, PCR testing of IVC filter tops should be considered as an alternative to soiled bedding sentinels. Environmental sampling may provide increased reliability and reduce the number of rodents used for routine health surveillance.

Abbreviation: MNV, mouse norovirus

DOI: 10.30802/AALAS-JAALAS-18-000008

Routine rodent colony health surveillance is an important measure to ensure the health of animals involved in research and to avoid inadvertent effects of pathogens on research study outcomes. Traditional methods for microbial monitoring include the use of soiled bedding transfer and sentinel mice,^{7,16,21,23} particularly where direct sampling of resident animals is not a reasonable option. However, not all pathogens transfer well by using this indirect sentinel animal method, especially when prevalence is low. For example, *Pasteurella pneumotropica* is not readily transmitted through the dirty bedding method and often goes undetected in laboratory rodent populations.¹⁹ Comparison of detection methods for mouse norovirus (MNV) found the exhaust air of IVC to be superior to serology from sentinels housed on soiled bedding.²⁴ Other pathogens that have shown poor transmission by soiled bedding include lymphocytic choriomeningitis, Sendai virus, MNV, cilia-associated respiratory bacillus, and murine fur mites.^{1,5,6,10,13,15,20,22,23,24} In addition, for some adventitious agents such as parasites and bacterial pathogens for which serologic testing is infeasible and other detection methods must be used (for example direct detection, culture, or PCR testing), the organism must be present at the time of testing to illicit a positive result. PCR testing for pathogen detection has greatly expanded and improved our repertoire of options, but when used for testing samples collected directly from sentinel

animals, it likewise is capturing only organisms present at the time of testing.^{4,9,21}

Alternatively, rodent health surveillance can be conducted through environmental sampling, for example, exhaust plenum sampling from IVC racks, a method that continues to evolve as different rack designs and methods of sampling are evaluated.^{2,5,12,19} In this method, samples taken from collection devices, filters, or plenums are used in PCR assays to determine whether pathogenic nucleic acid is present. In one study, this method was shown to have a 94.1% probability of detecting murine fur mites.¹² Several significant advantages of testing rack exhaust dust for infectious agents include decreased use of animals for routine health surveillance (if environmental monitoring replaces use of sentinel animals), detection of agents not easily transmitted through soiled bedding, and elimination of soiled bedding collection during cage change procedures (which could itself lead to inadvertent exposure of colony animals). However, rack design is of critical importance when considering switching to exhaust air sampling. Some IVC rack systems are designed such that cage exhaust air is filtered prior to entering the plenums (Figure 2); thus dust and organic matter are not deposited to any sufficient degree. In this context, exhaust plenum testing at our institution was ineffective, with every sample showing negative results despite positive sentinel results for agents endemic in our colonies.

Given the limitations of both traditional sentinel animal testing and exhaust manifold testing of IVC where air flow is filtered prior to reaching the exhaust manifolds, we hypothesized that testing cage top filters from soiled bedding cages by using PCR

Received: 12 Jan 2018. Revision requested: 12 Feb 2018. Accepted: 20 Mar 2018.

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assays would be as least as effective than other health surveillance methods. Animal activity within the cage likely would increase the amount of dust and pathogen nucleic acid deposited on the cage top filter, which could then be detected by using PCR analysis. We further hypothesized that periodic shaking of cages would generate sufficient air and particulate movement within them to substitute for the presence of live animals. If effective, this method has the potential to reduce or replace sentinel mice used in rodent health monitoring programs.

Materials and Methods

Animals. Female Cr:NIH(S) (NIH Swiss) mice (age, 4 to 5 wk) were purchased (Charles River Laboratory, Frederick, MD) and used as sentinels. Sentinel mice were housed 2 per cage and were free of pathogens according to vendor testing for epizootic diarrhea of infant mice, *E. muris*, *Helicobacter* spp., mouse hepatitis virus, MNV, mouse parvovirus, minute virus of mice, *Pasteurella pneumotropica*, *Spiroplasma muris*, and Theiler encephalomyelitis virus and endo- and ectoparasites. Mice were housed in a combination of barrier and nonbarrier conditions (4 rooms each) on ventilated racks (SuperMouse 750, Lab Products, Seaford, DE). In barrier rooms, all cage supplies were autoclaved. Research colony mice were housed on corn cob bedding (1/8 in., Bed-o'-Cobs, The Andersons Lab Bedding Products, Maumee, OH), were fed irradiated chow (diet 5053 [barrier mice] or diet 5001 [conventionally housed mice], LabDiet, St Louis, MO), and received filtered chlorinated water (Hydropacs, Lab Products, Seaford, DE) and nesting material for enrichment (EnviroDri, Shepherd Specialty Papers, Cleveland OH). All cage changes were performed in a HEPA-filtered animal transfer station or biosafety cabinet. Room conditions were $22 \pm 2^\circ\text{C}$, relative humidity of 30% to 70%, and a 12:12-h light:dark cycle (lights on, 0600). All animal use was approved by the Georgetown University IACUC. The animal care and use program is AAALAC-accredited. Animals are housed and maintained in accordance with the *Guide for the Care and Use of Laboratory Animals*, 8th edition.¹¹

Soiled bedding collection and cage handling procedures. Three experimental cages were maintained concurrently in each of the 8 rooms included in this study. The cages were placed next to each other on a lower row of a single IVC rack in the room. One of the 3 cages contained 2 sentinel mice (cage A); one cage had no mice and was shaken sufficiently to mix bedding and distribute particulates around all inner surfaces for 15 seconds twice weekly (cage B), and the third cage had no mice and was left alone (no shaking; cage C). A pooled sentinel bedding container (empty mouse cage) received an equal amount (one heaping tablespoon holding approximately 22 g [30 mL]) of soiled bedding from all other cages on that side of the rack (maximum, 80 cages) once every other week. This pooled bedding was mixed and then distributed equally to the 3 test cages to create a layer of bedding approximately 1/3 in. deep. At the biweekly changes, the old bedding in the test cages was discarded and replaced with new pooled soiled bedding. Three pieces (3 × 2 in. each) of filter material (Reemay 2024, Maryland Plastics, Federalsburg, MD) were inserted on the undersurface of the cage filter lid, between the full sheet of filter material and the inner metal grid (Figure 1); these smaller pieces are of the same material used within the cage filter tops for air filtration. One piece was removed for testing and replaced once monthly for 3 consecutive months. The second piece of filter paper (no. 3) remained on the cage undersurface for the entire 3 mo before being tested. The remaining piece of filter (no. 2) originally was intended to be used as a 2-mo cumulative time point but, in light

of the 1-mo results, ultimately was left in place as a backup to the no. 3 piece. The IVC racks (SuperMouse 750, Lab Products) have HEPA-filtered air supply and exhaust units and are designed so that cages receive positive pressure, while the rack is under negative pressure with regard to room air. Cage air is pulled through the filter paper in the cage lid prior to entering the exhaust plenums of the rack, and thus most particulate matter is trapped at the level of the filter lid (Figure 2).

Testing procedures. In addition to the filter paper samples collected, we tested fresh feces and oral swabs, and fur swabs from the mice in cage A for the same agents at months 1 and 3; blood samples were collected from the mice at month 3 for serology. Lastly, at month 3, feces and oral and fur swabs were collected randomly from colony mice in 10 different cages on the rack and tested for the same panel of agents. All samples were sent to IDEXX BioResearch (Columbia, MO). Feces, oral and fur swabs, and filter paper were tested by PCR methods for epizootic diarrhea of infant mice virus, *Entamoeba muris*, *Helicobacter bilis*, *H. ganmani*, *H. hepaticus*, *H. mastomyrinus*, *H. rodentium*, *H. typhlonius*, mouse hepatitis virus, MNV, mouse parvovirus, minute virus of mice, *P. pneumotropica*, *S. muris*, Theiler mouse encephalomyelitis virus, and murine endo- and ectoparasites. Blood samples were tested for MNV by using a serologic assay.

Diagnostic assays. Total nucleic acids were extracted according to standard protocols from fecal pellets, swabs of the fur or oral cavity (FLOQSwabs, Copan, Murrieta, CA), and filter papers by using a commercially available platform (Nucleo-Mag VET, Macherey-Nagel, Bethlehem, PA). PCR testing for infectious agents used proprietary service platform (IDEXX Laboratories), with all assays having an analytical sensitivity of 1 to 10 copies per PCR reaction. Briefly, all microbes were detected with real-time PCR assays using hydrolysis probes. A hydrolysis-probe-based real-time PCR that targeted a housekeeping gene (16S rRNA) was used to confirm nucleic acid integrity and ensure the absence of PCR inhibitors in the test sample. Real-time PCR using standard primer and probe concentrations and a commercially available master mix (LC480 ProbesMaster, Roche Applied Science, Indianapolis, IN) was performed on a commercially available PCR platform (LightCycler 480, Roche). Serologic testing was performed by IDEXX BioResearch by using validated standard operating procedures.

Results

This study examined endemic pathogens that were expected to be observed in our existing mouse colonies. These pathogens included *Helicobacter* spp., MNV, *P. pneumotropica*, *E. muris*, and *S. muris*. No other microbial agents were detected (Tables 1 and 2). After 2 wk, all animal technicians responsible for the experimental cages were fully trained and performing this task well. However, due to inconsistencies in shaking of cages and amount of soiled bedding added to the cages during the initial 2 wk, filter paper results from the first month were not included in data analysis. Results for the filter sample that was replaced monthly (no. 1) were collected at month 2 of the study. Results for filter sample no. 3 provided cumulative data for the entire 3-mo period; filter sample no. 2 was not tested. Oral and body swabs from sentinel mice were tested for agents other than those known to be present in our mouse colonies and were negative for all pathogens tested at all time points. At the 3-mo cumulative time point, the most effective testing methods were PCR assays of random colony samples (feces, oral and fur swabs) and filter paper taken from cages with no mice present and shaken twice weekly. Filter paper taken from cages housing

Figure 1. Layout of filter paper sections on the inside of a filter top lid. (A) A completely assembled lid, with the extra filter pieces enclosed within the lid of the cage. (B) Layout of the 3 filter pieces within the lid, which is open. The sample labeled 1 was replaced every month. The filter pieces labeled 2 and 3 stayed on the lid for the entire 3-mo testing period; ultimately filter 2 was not tested.

Figure 2. Schematic diagram of air flow into and out of the microisolation cages. Printed with permission of the manufacturer.

sentinel mice was moderately effective, detecting *Helicobacter* spp. at the same rate as cages B and MNV in 7 of the 8 cages but detecting *P. pneumotropica*, *E. muris*, and *S. muris* less reliably than for mouse-free but shaken cages. The unshaken cages were the least effective method, yielding just 1 positive test each for *Helicobacter* spp. and *P. pneumotropica*. Filter paper was as effective as (sentinel cages) or more effective than (mouse-free but shaken cages) feces collected from the sentinel mice, in regard to detecting all 5 microbial agents.

We then compared the results of testing filter papers after 1 mo with those after 3 mo (Table 2). Notably, after 1 mo, filter samples from mouse-free but shaken cages had higher rates of detection for all pathogens except *S. muris* than those from sentinel cages and sentinel feces. Filter paper samples from sentinel cages exceeded detection rates from sentinel feces for all tested pathogens but MNV. At the end of month 3, sentinel mice underwent serologic testing, and 7 of the 8 rooms tested positive for MNV. This finding differed from results of routine sentinel testing performed 1 mo earlier by using nonstudy sentinel mice in each of the 8 rooms. At that time, all 8 rooms tested positive, but depending on room and population size, multiple sentinel mouse cages were tested from each room.

Table 1. Results of diagnostic testing from all samples collected at the end of the 3-mo study period

	Sentinel mice	Colony animals ^a	Sentinel mice ^b	Sentinel mice	Shaken mouse-free cage	Undisturbed mouse-free cage
	Serology	Mixed PCR	Fecal PCR	Filter PCR	Filter PCR	Filter PCR
<i>Helicobacter</i> spp.	na	88%	71%	75%	75%	13%
MNV	88% ^c	100%	100%	88%	100%	0%
<i>P. pneumotropica</i>	na	50%	14%	38%	75%	13%
<i>E. muris</i>	na	25%	14%	13%	25%	0%
<i>S. muris</i>	na	13%	0%	0%	13%	0%

na, not applicable

A total of 8 rooms were tested; bolded values indicate the most sensitive testing method(s) for each agent.

^a10 random cages were sampled (feces, body and oral swabs) from the same rack side that held the sentinel cage.

^bOnly 7 of 8 cages were tested.

^cResults obtained from nonstudy sentinel mice in the same rooms 1 mo prior to study start revealed MNV in all 8 rooms.

Table 2. Comparison of 1-mo with 3-mo time periods for filter paper from 3 cage set-ups and sentinel mouse feces

	Sentinel cage		Shaken mouse-free cage		Undisturbed mouse-free cage		Sentinel cage	
	Filter PCR		Filter PCR		Filter PCR		Fecal PCR	
	1 mo	3 mo	1 mo	3 mo	1 mo	3 mo	1 mo	3 mo
<i>Helicobacter</i> spp.	88%	75% ^b	88% ^a	75% ^b	0%	13%	63%	71%
MNV	25%	88%	100% ^a	100% ^b	0%	0%	75%	100% ^b
<i>P. pneumotropica</i>	38%	38%	75% ^a	75% ^b	0%	13%	0%	14%
<i>E. muris</i>	25%	13%	38% ^a	25% ^b	0%	0%	13%	14%
<i>S. muris</i>	13% ^a	0%	0%	13% ^b	0%	0%	0%	0%

^aBolded values indicate the most sensitive testing method(s) for a 1-mo period

^bBolded values indicate the most sensitive testing method(s) after a 3-mo cumulative period.

Discussion

Rodent health surveillance is vital to maintaining the health of laboratory animal populations and ensuring that pathogen status is known and can be accounted for when interpreting research study results. Although the transfer of dirty bedding to sentinel animal cages is still the most commonly used method for rodent health surveillance programs,¹⁷ a recent systematic review of literature conducted to evaluate efficacy of soiled bedding transfer to detect microbial pathogens in rats and mice concluded that this method was effective for some infectious agents but ineffective or inconclusive for others.⁷ The results of the current study demonstrate an effective way to conduct rodent health surveillance that may reduce the number of sentinel mice used and might detect adventitious agents that would otherwise go unnoticed.^{10,20}

As expected, detection rates improved by using cumulative 3-mo samples compared with 1-mo samples for *Helicobacter* spp., MNV, and *P. pneumotropica* for all filter papers and feces taken from the sentinel cages. This improvement did not occur with filter papers taken from the shaken cages, where results were either similar or slightly decreased (1 fewer positive sample). However, detection rates were high for both time points from these cages. Results for *E. muris* and *S. muris* were less predictable, probably due to their overall low prevalence levels. Filter paper from both the sentinel cages and shaken cages had more positive results for these pathogens at 1 mo than at the 3-mo cumulative time point. We hypothesize that the low rate of prevalence in the colony along with test sensitivity limits might impair the accuracy of detection.

Rack design plays a pivotal role in the success of IVC exhaust manifold testing.¹⁴ Systems in which the exhaust air leaving individual cages is filtered prior to reaching exhaust plenums

are much less likely to be amenable to exhaust sampling methods. Indeed, this testing method proved to be ineffective in our own facility. The design-dependence of exhaust sampling is substantiated by the findings of a study comparing 2 rack designs—one with unfiltered air flow, and the other with filtered air flow.² A variety of rodent pathogens including viruses, bacteria, pinworms, fur mites, and protozoans all failed to be detected in the IVC system with filtered air flow.²

Given the limitations of environmental sampling with IVC designed to filter air at the cage level, the ability to trap and subsequently test particulate matter in filter paper placed within a cage receiving soiled bedding from colony mice offers an attractive alternative to testing sentinel mice directly. Over time, because of dust generated from mice moving around a cage during their normal daily activities, sufficient microbial nucleic acid can be extracted from filter top media to reliably use PCR analysis for pathogen detection. A recent study demonstrated superior detection of murine pinworms and fur mites from filter paper extracts compared with PCR analysis of animal feces, fur swabs, and exhaust manifold filters.⁸ In the current study, our primary goal was to determine whether PCR analysis of particulate debris trapped in filter paper affixed to the lids of sentinel cages receiving soiled bedding was as effective as samples obtained directly from sentinel animals. A secondary goal was to evaluate whether periodic shaking of empty cages with soiled bedding was an effective proxy for live animals as a way to distribute organic material containing infectious-agent nucleic acid onto the cage lid filter paper. We determined that PCR testing of filter paper from cages shaken twice weekly was not only more effective than PCR testing of feces and fur swabs from sentinel mice but also was as effective as direct sampling and testing of colony animals. We compared PCR testing of

filter tops with serology for MNV because serology remains a component of our traditional health surveillance program. MNV was the only viral pathogen present in our colonies, so the ability of filter top sampling to detect other viral pathogens remains to be demonstrated.

The most effective method of pathogen detection in our study was testing filter samples from cages containing no mice but shaken twice weekly for 15 s each. Indeed, the cage shaking proved to be critical, because unshaken cages rarely tested positive for any of the 5 endemic agents. The choices of frequency and duration of shaking were arbitrary and was based partly on what seemed to be logistically feasible without being too labor-intensive. In retrospect, the choices proved to be effective, because the 1-mo time point showed the highest detection rates of any of the methods tested, with 88% and 94% positive detection rates for *Helicobacter* spp. and MNV, respectively. Future studies will focus on optimizing these parameters to maintain the sensitivity but minimize the frequency and duration of cage shaking to the extent possible.

Distinct advantages of testing filter material from cages exposed to soiled bedding that lack sentinel mice are that it reduces the use of sentinel mice, it can be used with any containment caging system, and it requires no structural modifications to the rack. Despite these advantages and the promising results obtained by using filter samples from cages containing no animals, a potential downside of this methodology is the heavy reliance on personnel to shake the cages routinely. Until another technical method is developed to stir up the particulate matter from soiled bedding, cage shaking is absolutely essential to our method's success. That being said, filter samples from cages with sentinel mice was as effective as PCR testing of sentinel fecal samples for all infectious agents tested, except MNV; after 3 mo, MNV was detected on 7 of the 8 sentinel cage filter samples compared with all 8 samples of sentinel feces. Likewise serology of the same mice at 3 mo was positive in only 7 of the 8 rooms. It should be noted that all 8 rooms tested positive for MNV by serology during routine health surveillance conducted 1 mo earlier on other sentinel mice. Larger sample sizes with more rooms may balance these results. The number of mice in the sentinel cage likely plays an important role in the method's success as well. Presumably, the presence of more mice increases the amount of organic debris aerosolized and deposited on the filter media. Although we used only 2 mice per cage in this study, other similar studies used either 3 mice² or 4 mice⁸ in their study designs.

As with all programs relying on soiled bedding for transfer and detection of infectious agents, the amount of soiled bedding transferred, the dilution rate (that is, how many cages soiled bedding is collected from per sentinel cage), and the frequency of transfer all may have significant effects on the sensitivity of the assay, regardless of whether samples are obtained from sentinel mice or from filter material from the sentinel cage. Although the testing of filter material from cage lids remains susceptible to some of the weaknesses of soiled bedding transfer for detection of endemic infectious agents, the revised method may decrease the rate of false negatives, which appears to have been the case in our study. For example, the presence of live agents is unnecessary, as long as nucleic acid is transferred with the soiled bedding and subsequently deposited onto the filter media. For bacterial pathogens such as *P. pneumotropica*, which does not survive well on some types of bedding,²² this advantage can be particularly important. In addition, other animal-specific parameters associated with the use of sentinel animals, including strain, age, immune status, and other physiologic traits are removed as variable factors.³

In conclusion, PCR analysis of filter material from cage lids exposed to soiled bedding may be an effective alternative to other common screening methods for routine rodent pathogen surveillance. Moreover, the presence of live sentinel animals may be unnecessary for accumulating microbial debris (that is, nucleic acid) and retaining a high level of test sensitivity. Testing this methodology for its ability to detect the presence of other important rodent pathogens—particularly fur mites, pinworms, and other viral agents—is warranted.

Acknowledgments

We thank the animal care technicians of the Division of Comparative Medicine who participated in this study by caring for the sentinel cages used in this study. We also thank IDEXX Biomedical Research for their generous technical and financial support of diagnostic testing.

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