PCR Prevalence of Murine Opportunistic Microbes and their Mitigation by Using Vaporized Hydrogen Peroxide

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Exposing immunodeficient mice to opportunistic microbes introduces risks of data variability, morbidity, mortality, and the invalidation of studies involving unique human reagents, including the loss of primary human hematopoietic cells, patient-derived xenografts, and experimental therapeutics. The prevalence of 15 opportunistic microbes in a murine research facility was determined by yearlong PCR-based murine and IVC equipment surveillance comprising 1738 specimens. Of the 8 microbes detected, 3 organisms—*Staphylococcus xylosus*, *Proteus mirabilis*, and *Pasteurella pneumotropica* biotype Heyl—were most prevalent in both murine and IVC exhaust plenum specimens. Overall, the 8 detectable microbes were more readily PCR-detectable in IVC exhaust airways than in murine specimens, supporting the utility of PCR testing of IVC exhaust airways as a component of immunodeficient murine health surveillance. Vaporized hydrogen peroxide (VHP) exposure of IVC equipment left unassembled (that is, in a ‘static-open’ configuration) did not eliminate PCR detectable evidence of microbes. In contrast, VHP exposure of IVC equipment assembled ‘active-closed’ eliminated PCR-detectable evidence of all microbes. Ensuring data integrity and maintaining a topographically complex immunodeficient murine research environment is facilitated by knowing the prevalent opportunistic microbes to be monitored and by implementing a PCR-validated method of facility decontamination that mitigates opportunistic microbes and the risk of invalidation of studies involving immunodeficient mice.

Abbreviations: AHU, air handling unit; BI, biological indicator; CI, chemical indicator; VHP, vaporized hydrogen peroxide

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The creation of increasingly immunodeficient mice by progressively disabling major components of the immune system has contributed to major advances in the understanding of human hematopoietic stem cell engraftment and multilineage differentiation.^{65,67} Hematopoietic ‘humanized’ mice⁶⁶ that are recipients of patient-derived tumor xenografts, which in early passage retain features of the primary tumor, serve as optimal platforms for cancer immunotherapeutic development.³⁶ Beginning with the discovery in 1962 of athymic T-cell-deficient nude mice²⁹ due to a spontaneous mutation in the forkhead box N1 (*Foxn1*) transcription factor gene¹⁴ and the subsequent descriptions in 1983 of C.B-17-Prkdc^{scid} (*scid*) mice defective in T- and B-cell receptor rearrangements,⁴ in 1995 of NOD/LtSz-*scid* mice with reduced macrophage function, complement-dependent hemolytic activity, and NK cell activity;^{21,69} and in 2005 of NOD/LtSz-*scid*/IL2R γ ^{null} (NSG) mice lacking mature lymphocytes, NK cells, and the interleukin 2 receptor γ -chain,^{68,69} increasingly immunodeficient mice have supported ever higher levels of human hematopoietic cell engraftment and differentiation and have permitted a wider range of tumors to grow, progress, and undergo therapeutic trial in vivo.^{36,65-67}

As murine immunity has been progressively disabled, whether intentionally to improve engraftment of human hematopoietic cells or patient-derived tumor xenografts or

unexpectedly during the creation of genetically engineered mutants,^{16,32,52} the murine primary enclosure has evolved from a rudimentary wire-topped wooden box into a complex, gasket-sealed, filter top-enclosed, IVC with HEPA supply and exhaust.^{25,33,35,61} IVC contribute to pathogen exclusion, allergen containment, colony productivity, microenvironment quality, and the protection of complex microbiota.^{38,54,55,64,71,72} Regardless, some IVC exhaust plenums may be a potential nidus where microbes aerosolized with dander from the primary enclosures can accumulate. As such, sentinels or filters exposed to IVC exhaust air can be monitored and tests of these used as an indication of the microbial status of mice housed on the IVC rack.¹² IVC exhaust airway tests supplement indirect sentinel testing, which does not detect microbes that are inefficiently transmitted to sentinel animals.^{12,44} Consequently, PCR testing of the interiors of IVC exhaust airways has increasingly become a component of murine health monitoring.^{2,12,24,31,40,44,45,64}

As immunodeficient and genetically engineered murine inventories continue to grow and as the complexities of co-evolving host-microbiota homeostasis are further defined,^{9,10,26} additional challenges to murine biosecurity, data integrity, and study replication likely will emerge.⁶³ In immunodeficient murine settings, members of the microbiota may function as opportunistic pathogens,^{15,74} and commensal symbionts may function together as pathobionts.²⁶ In immunodeficient mice, dysbiosis (that is, altered abundance of multiple commensals) may result in high morbidity,⁴⁶ encouraging a variant of Koch’s postulates—traditionally concerned with disease attributable to a single infectious agent—that instead addresses polymicrobial

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disease attributable to the microbiome.^{48,63,70} As a result, the list of microorganisms to monitor in murine facilities has expanded well beyond traditionally excluded pathogens and adventitious microbes (that is, 'nonnative agents arriving accidentally from outside animals') to include opportunistic microbes and members of the microbiota (that is, 'native agents within animals'). For example, the symbionts *Proteus mirabilis* and *Klebsiella pneumoniae* act in concert to induce colitis in *Tbet*^{-/-} × *Rag2*^{-/-} mice,^{18,62} the microbiota members *Enterococcus* spp. and *Klebsiella oxytoca* can cause nephritis,¹⁵ and the opportunist *Pasteurella pneumotropica* may cause pneumonia in aged NSG mice.⁵⁹ In addition, the murine skin commensal *Staphylococcus xylosus* can cause dermatitis, abscess formation, or cystitis in immunodeficient strains,^{1,5,20,56,58} and the opportunist *Corynebacterium bovis* causes hyperkeratotic acanthotic dermatitis in nude mice¹¹ and skin disease in haired *Pkrdc*^{scid} mice⁶⁰ and hairless immunocompetent SKH1-*Hr*^{hr} mice¹⁹ and becomes disseminated facilitywide.^{6,43} Moreover, a number of microbes either harbored by humans or common to the environment are opportunistic pathogens in immunodeficient mice, including *Pseudomonas aeruginosa*, β -hemolytic *Streptococcus* spp., *Staphylococcus aureus*, and *Pneumocystis carinii*.⁶⁴

Stringent husbandry practices for immunodeficient mice tend to focus on the primary enclosure, including the use of a sterile primary enclosure opened in a laminar flow hood, weekly cage changes to prevent murine exposure to a minimal inoculating dose of opportunistic microbes, and frequently decontaminated forceps, gloves, and sleeves.^{17,47} Biosecurity in academic research facilities is made challenging though by exposing mice to bidirectional trafficking to imaging, microscopy, behavioral, and other procedural cores and by maintaining topographically complex housing equipment with IVC exhaust plenums, hoses, and air handling unit (AHU) prefilter chambers that are likely—but have not been documented—to accumulate a broad spectrum of opportunistic agents.

Furthermore, some murine commensals that serve as opportunistic pathogens in immunodeficient settings, including *Staphylococcus xylosus*, are not monitored by even approved commercial vendors of mice.¹³ In addition, when still other opportunistic agents that are monitored become detected in vendor production barriers, including *Klebsiella pneumoniae*, *Pneumocystis murina*, *Proteus mirabilis*, *Pseudomonas* spp., *Staphylococcus aureus*, *Streptococcus pneumoniae*, and β -hemolytic *Streptococcus* spp., shipping to research facilities is not stopped.¹³ In addition, new pathogens have emerged (and will likely continue to emerge), including a *Corynebacterium* species known as HAC2 that causes hyperkeratotic dermatitis and thus invalidates studies involving NOD and NSG mice that are engrafted with patient-derived xenografts and that are culture-negative for *Corynebacterium bovis*.²⁸

Prevalent microbes affecting murine research have been identified in surveys of health monitoring practices and microbe outbreaks at research programs^{7,8,30,41} or, alternatively, based on diagnostic laboratory results that detect microbes in specimens submitted by research programs.^{33,51} Still others have identified microbes prevalent in tissues of NSG mice.⁷³ Among the bacterial outbreaks experienced by programs, *Helicobacter* spp., *Corynebacterium bovis*, and *Pasteurella pneumotropica* were reported most frequently.⁴¹ Similarly, *Helicobacter* spp. was the bacterial agent most frequently detected by diagnostic laboratories in murine specimens submitted by research programs,^{33,51} followed by either *Proteus mirabilis*³³ or *Pasteurella pneumotropica*.⁵¹ Neither of 2 reports^{33,51} included *Corynebacterium bovis* in its summaries.

We recently described a method of sterilizing IVC racks and AHU that used vaporized hydrogen peroxide (VHP) and was validated by *Staphylococcus xylosus* PCR monitoring,⁵³ a method we then applied to the first successful facility-wide eradication of *Corynebacterium bovis*.⁴² VHP is used in human clinical settings to mitigate the nosocomial agents methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus* species, norovirus, *Acinetobacter* spp., and *Clostridium difficile*.^{43,50} VHP involves the conversion of a liquid concentrate that is 35% H₂O₂ to a gaseous phase by flash vaporization,³ which is dispersed in a dry air stream onto all exposed surfaces sealed within a room. In its gaseous phase, VHP is rapidly and broadly antimicrobial and sporicidal through oxidation, with a 6-log reduction of microbes occurring in minutes at 150 to 400 ppm, after which, H₂O₂ degrades to water and oxygen.^{3,22} Compared with other surface sterilants, such as chlorine dioxide and formaldehyde, VHP has a much more favorable safety profile, a higher Permissible Exposure Limit, and a higher Immediately Dangerous to Life or Health level and is safe on many surfaces, resulting in no substantive change to their physical or chemical properties.^{3,22,27,34,49,50}

Even with best practices involving the murine primary enclosure, immunodeficient murine biosecurity is facilitated by monitoring prevalent opportunistic microbes in mice and the environment and by secondary housing equipment sterilization routines that reduce the risk of disseminating microbes that can modulate data and invalidate results. Herein we describe a yearlong effort involving PCR testing of murine and IVC exhaust plenum specimens to determine prevalent opportunistic microbes in an SPF, viral antibody-free murine research facility with a comprehensive pathogen exclusion list, including the exclusion of *Helicobacter* spp., murine norovirus, and *Corynebacterium bovis*. In addition, we provide a PCR-validated method for eliminating the evidence of opportunistic microbes from IVC exhaust plenums by using active-closed exposure to VHP.

Materials and Methods

Facility. The 29,931 ft² murine vivarium includes 7117 ft² of murine housing space and 4510 ft² of procedural, surgical, imaging, irradiation, and genetic engineering space. At capacity, this facility accommodates 62,496 mice in 15,624 IVC in 18 housing rooms, including 8064 immunodeficient mice in 2016 IVC in 4 isolation housing rooms separate from immunocompetent strain housing. The program and facilities for animal care and use are fully AAALAC-accredited. All animals are housed and used in accordance with IACUC-approved protocols. It is an SPF murine facility that is also viral antibody-free, with a separate 6-room, 1593 ft² quarantine facility located outside of the facility itself.

Infectious agent exclusion. Standard procedures, including monthly PCR tests of all IVC exhaust plenum interiors, exclude *Helicobacter* spp., murine norovirus, *Corynebacterium bovis*, *Corynebacterium* HAC2, *Syphacia* spp., *Aspiculuris tetraptera*, parainfluenza virus type 1 (Sendai), coronavirus (mouse hepatitis virus), *Mycoplasma pulmonis*, paramyxovirus (pneumonia virus of mice), parvovirus (minute virus of mice and mouse parvovirus), poliovirus (Theiler murine encephalomyelitis virus strain GDVII), reovirus type 3, lymphocytic choriomeningitis virus, mouse adenovirus types 1 and 2, poxvirus (ectromelia virus), rotavirus (epizootic diarrhea of infant mice virus), papovavirus (polyoma virus), Hantaan virus, *CAR bacillus*, *Clostridium piliforme* (Tyzzer disease), and *Encephalitozoon cuniculi*.

Procedural space. Murine procedural space includes 5 common procedural rooms; the Mouse Models Core, a 303 ft²

laboratory that provides services to generate transgenic, gene-targeted mutant, or CRISPR/Cas9 founders; and the Small Animal Imaging Laboratory comprising 2035 ft² providing 3D, high-resolution anatomic and molecular imaging in mice. A separate necropsy room is equipped with a dual-draft necropsy workstation and hematology and serum chemistry analyzers.

Housing. IVC units (Blueline, Tecniplast, Buguggiate, Italy) are used for murine housing and are changed in animal transfer stations or in class II type A2 biologic safety cabinets. A double-sided rack configuration holding 126 microisolation units (that is, 63 cages per rack side) is used, and each pair of racks is ventilated and exhausted by a separate AHU. AHU exhaust prefilter changes occur every 2 wk and use a bag-out procedure while spraying with OxivirTb (Diversey Sealed Air, Charlotte, NC). Mice were fed a sterilized rodent diet (Teklad Global 18% protein, Envigo, Somerset, NJ), housed on sterilized pelleted paper bedding (Teklad 7084, Envigo), and provided with sterilized enrichment items and nesting material.

Surface decontamination. Housing and procedural area surface decontamination before and after each use are accomplished by using OxivirTb (Diversey). All surfaces—including AHU, animal transfer stations, biosafety cabinets, procedural countertops, gloved hands, core imaging equipment, and the external surfaces of the occupied IVC—are decontaminated before and after each use by using OxivirTb (Diversey).

Equipment sanitization. Cage wash is arranged soiled to clean to sterile. IVC are sanitized at 180 °F final rinse in redundant tunnel washers with dryers (Basil 6000, Steris, Mentor, OH). IVC racks are sanitized at 180 °F final rinse in a rack washer with dryer (Basil 9500, Steris). Washed and dried caging equipment exit these machines and enter a clean IVC preparation room. Clean IVC are bedded, reassembled, and clamped shut. In separate loads, prepared IVC and municipal water-filled bottles are then sterilized by passage through 2 roll-in prevacuum bulk steam sterilizers with bio-seals (Century SLH Scientific, Steris); this process is ensured by using Verify Integrator strips and Verify biologic steam test pack indicators (Steris). Sterile IVC and sterile filled water bottles are labeled with the date sterilized, covered by an autoclavable cover (Ancare, Bellmore, NY), and stored in the sterile cage ready room.

IVC racks, plenums, and connecting hoses are washed in a mechanized rack or tunnel washer with 180 °F final rinse temperature. The exterior surfaces and interior prefilter chambers and hose connecting ports of AHUs are hand-sanitized (OxivirTb, Diversey) during AHU change-outs. All washed IVC racks, connecting hoses, and hand-sanitized AHU are then VHP-decontaminated in a 'flex room,' as described later.

VHP sterilization. During the yearlong environmental and murine surveillance and assessment of VHP methods, washed equipment was VHP-exposed (Bioquell, Horsham, PA) by using a process validated by biologic indicators, chemical indicators, and PCR monitoring for microbes, as previously described in detail.³⁴ Biologic indicators detected VHP levels capable of a 6-log kill of *Geobacillus stearothermophilus* spores sealed inside pouches (Bioquell). Chemical indicators provided a semiquantitative, visual, ink-on-card indication of 2-, 4-, or 6-log microbial kill VHP levels (Bioquell). Prior to sealing of the flex room and initiating each VHP cycle, biologic and chemical indicators were placed in the flex room with equipment to be decontaminated, as previously described.³⁴

Two methods of VHP exposure of equipment were assessed, that is, with equipment in either a 'static-open' or 'active-closed' configuration.³⁴ During months 2 through 6, static-open VHP exposure of equipment was evaluated, with the AHU turned off,

hoses disconnected, and IVC rack plenums unassembled and unsealed. During months 7 through 10, active-closed VHP exposures were evaluated, with IVC rack plenums fully assembled and sealed, hoses connecting IVC racks to an AHU, and with the AHU set on to the VHP cycle. VHP decontamination was implemented by using a model Z2 generator and R30 aeration catalytic accelerator (Bioquell, Horsham, PA). VHP exposure of portable IVC and AHU equipment was conducted in a flex room (that is, an empty housing room) as a decontaminating chamber.

PCR surveillance for microbes. Murine fecal pellets were collected from primary enclosures of immunodeficient mice for PCR testing. Immunodeficient strains from which fecal pellets were collected included nude, SCID beige, NOD/LtSz-scid, NOD/LtSz-scid/IL2R γ null (NSG), NOD-scid IL2R γ null-3/GM/SF (NSGS), RAG2-KO matrix metalloproteinase 2 (MMP2)-KO, and RAG2-KO/MMP13-KO mice.

Sterile FLOQ swabs (Copan Flock Technologies, Brescia, Italy) were used to collect specimens from the interiors of IVC exhaust plenums when soiled, after mechanized washing, and after either static-open or active-closed VHP exposure. Specimens were collected by tracing a circular pattern with the swab for 3 circumferences as the tip was rolled; swabs were then PCR analyzed.

FLOQ swabs of IVC exhaust plenum interiors and fecal pellets of immunodeficient mice were submitted to the IDEXX BioResearch testing laboratory (Columbia, MO), where they were real-time PCR-tested for evidence of 15 opportunistic microbes, 14 bacteria, and a fungus, including *Staphylococcus xylosum*; *Klebsiella oxytoca*; *Klebsiella pneumoniae*; *Corynebacterium kutscheri*; *Proteus mirabilis*; *Pseudomonas aeruginosa*; *Staphylococcus aureus*; β -hemolytic *Streptococcus* groups A, B, C, and G; *Pasteurella pneumotropica* biotypes Heyl and Jawetz; *Pneumocystis murina*; and *Citrobacter rodentium*. For each microbe, the PCR assay used a FAM/TAMRA-labeled hydrolysis probe that targeted a region of the 16S rRNA gene conserved among the agent of interest's genomic sequences deposited in GenBank. Hydrolysis probe-based real-time PCR assays targeting the bacterial 16S rRNA gene were used to ensure DNA recovery and the absence of PCR inhibitors in extracted nucleic acids. Real-time PCR analysis was performed by using a standard primer, standard probe concentrations, and a master mix (LC480 ProbesMaster, Roche Applied Science, Indianapolis, IN) on a real-time PCR platform (LightCycler 480, Roche).

Statistics. Unconditional exact tests using R 3.4 statistical software (R Foundation for Statistical Computing, Vienna, Austria) were performed to compare the percentage of opportunistic microbe PCR-positive specimens among tested specimens overall and for each microbe detected for (1) murine specimens compared with soiled IVC exhaust plenum specimens, (2) washed exhaust plenum compared with soiled exhaust plenum specimens, and for (3) exhaust plenum specimens after static-open compared with active-closed VHP exposure. A *P* value less than 0.05 was considered to define a statistically significant difference.

Results

A yearlong, facility-wide PCR surveillance tested 1738 specimens for 15 opportunistic microbes, evaluating 197 murine and 1541 IVC exhaust plenum specimens (Table 1). Murine fecal pellet specimens were collected from the primary enclosures of nude, SCID beige, NOD/LtSz-scid, NOD/LtSz-scid/IL2R γ null (NSG), NOD-scid IL2R γ null-3/GM/SF (NSGS), RAG2-KO matrix metalloproteinase 2 (MMP2)-KO, and RAG2-KO/MMP13-KO mice. Equipment specimens were

Table 1. PCR Detection of microbes in mice or on IVC exhaust plenums

Microbe	Mice	IVC exhaust plenum			
		Soiled	Washed	VHP-treated static-open ^a	VHP-treated active-closed ^b
<i>Staphylococcus xylosum</i>	89/194 (46%)	892/1215 (73%) ^c	39/140 (28%) ^d	28/110 (26%)	6/76 (8%) ^e
<i>Klebsiella oxytoca</i>	5/197 (3%)	37/1215 (3%)	0/140 (0%)	1/110 (1%)	0/76 (0%)
<i>Klebsiella pneumoniae</i>	0/184 (0%)	9/1215 (1%)	0/140 (0%)	0/110 (0%)	0/76 (0%)
<i>Proteus mirabilis</i>	17/184 (9%)	219/1215 (18%) ^c	2/140 (1%) ^d	1/110 (1%)	0/76 (0%)
<i>Pseudomonas aeruginosa</i>	0/184 (0%)	37/1215 (3%) ^c	4/140 (3%)	0/110 (0%)	0/76 (0%)
<i>Staphylococcus aureus</i>	2/184 (1%)	94/1215 (8%) ^c	1/140 (1%) ^d	3/110 (3%)	0/76 (0%) ^e
<i>Pasteurella pneumotropica</i> biotype Heyl	7/184 (4%)	428/1215 (35%) ^c	3/140 (2%) ^d	0/110 (0%)	0/76 (0%)
<i>Pasteurella pneumotropica</i> biotype Jawetz	0/184 (0%)	100/1215 (8%) ^c	0/140 (0%) ^d	0/110 (0%)	0/76 (0%)

Data are given as no. of specimens that were PCR-positive/total no. of samples tested (mean proportion of PCR-positive samples as a percentage)

^aThat is, AHU off, with IVC plenums and hoses disconnected

^bThat is, AHU on VHP cycle, with IVC plenums and hoses connected to AHU

^c% PCR-positive samples differed significantly ($P < 0.01$) between soiled plenums compared with murine specimens

^d% PCR-positive samples differed significantly ($P < 0.01$) between washed compared with soiled plenums

^e% PCR-positive plenums differed significantly ($P < 0.05$) between active-closed compared with static-open VHP exposure

collected monthly for 12 mo from each of the soiled IVC exhaust plenums of mouse-occupied IVC racks, with 61 to 66 racks occupied. Exhaust plenums also were tested when soiled, washed, or VHP-exposed through either static-open or active-closed methods. Of the 15 microbes monitored by PCR testing, 7 were never detected in either murine or plenum specimens during the yearlong surveillance, including *Corynebacterium kutscheri*; β -hemolytic *Streptococcus* groups A, B, C, and G; *Pneumocystis murina*; and *Citrobacter rodentium*.

Only 3 (*Staphylococcus xylosum*, *Proteus mirabilis*, and *Pasteurella pneumotropica* biotype Heyl) of the 15 tested microbes were detected in murine specimens and resulted in PCR-positive monthly medians that were greater than 0% (Figure 1). A 4th microbe, *Klebsiella oxytoca*, was detected sporadically in murine specimens during a single month in a single room. After the affected inventory was depopulated, *Klebsiella oxytoca* was no longer PCR-detected in murine specimens.

In contrast, 8 of the 15 tested microbes were detected in soiled IVC exhaust plenums, of which 7—*Staphylococcus xylosum*, *Klebsiella oxytoca*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Pasteurella pneumotropica* biotype Heyl, and *Pasteurella pneumotropica* biotype Jawetz—yielded PCR-positive monthly medians greater than 0% (Figure 2). The 8th microbe, *Klebsiella pneumoniae*, was PCR-detected only sporadically from the soiled IVC exhaust plenums in a single housing room during 2 separate months.

Overall, the 8 PCR-detected microbes were more readily ($P < 0.0001$) detected in soiled IVC exhaust plenum PCR tests (1816 of 9720; 19%) compared with murine PCR tests (120 of 1495; 8%). Individually, 6 microbes were PCR-detected in a higher ($P < 0.05$) percentage of soiled IVC exhaust plenum specimens compared with murine specimens. These microbes were *Staphylococcus xylosum*, *Staphylococcus aureus*, *Pasteurella pneumotropica* biotype Heyl, *Pasteurella pneumotropica* biotype Jawetz ($P < 0.001$ for all comparisons), *Proteus mirabilis* ($P = 0.002$), and *Pseudomonas aeruginosa* ($P < 0.01$; Table 1).

Mechanized washing did not eliminate the PCR evidence of microbes in IVC exhaust plenums. After mechanized washing with a final rinse temperature greater than 180 °F, 5 microbes—*Staphylococcus xylosum*, *Proteus mirabilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Pasteurella pneumotropica* biotype Heyl—were still PCR-detected in exhaust plenums. Regardless, for the 8 microbes PCR detected in soiled plenums, mechanized

Figure 1. Boxplot depiction of the median percentage of PCR-positive murine specimens for each microbe that was PCR-detected during the 12-mo surveillance. The median percentage-positive value is represented by the thick horizontal line inside each box, and the lower and upper limits of each box portray the interquartile range (that is, the 2nd and 4th quantiles). Murine specimens were collected monthly for 12 mo. Blue dots indicate the percentage of tests that were PCR-positive during each month for each microbe.

washing substantially ($P < 0.01$) reduced the overall percentage from 19% PCR-positive tests of soiled plenums to 4% PCR-positive tests of washed plenums. Individually, compared with soiled plenums, mechanized washing reduced the percentage of PCR-positive tests for *Staphylococcus xylosum*, *Proteus mirabilis*, *Staphylococcus aureus*, *Pasteurella pneumotropica* biotype Heyl, and *Pasteurella pneumotropica* biotype Jawetz ($P < 0.01$ for all comparisons; Table 1).

Two methods of VHP exposure of equipment were evaluated for their ability to eliminate PCR evidence of microbes in exhaust plenums. During months 2 through 6, to determine whether more time-efficient VHP exposure could be achieved without equipment assembly, washed IVC racks and sanitized AHU were left in the static-open configuration. That is, after delivery of washed and sanitized equipment into the flex room, IVC racks were left unassembled, plenums left unsealed, hose connections left open, and the AHU turned to 'off.' VHP biologic and chemical indicators were placed in the flex room, the flex room was sealed, and the AHU VHP cycle was initiated. Overall, PCR evidence of microbes was not substantively

Figure 2. Boxplot depiction of the median percentage of PCR-positive exhaust plenum specimens for each microbe that was PCR-detected during the 12-mo surveillance. The median percentage-positive value is represented by the thick horizontal line inside each box, and the lower and upper limits of each box portray the interquartile range (that is, the 2nd and 4th quantiles). Exhaust plenum specimens were collected monthly from each mouse-occupied IVC rack for 12 mo. Blue dots indicate the percentage of tests that were PCR-positive during each month for each microbe.

diminished in exhaust plenums after static-open VHP exposure compared with mechanized washing (Table 1). PCR evidence of 4 microbes remained in static-open VHP-exposed plenums; these organisms were *Staphylococcus xylosus*, *Klebsiella oxytoca*, *Staphylococcus aureus*, and *Proteus mirabilis*.

In contrast, active-closed VHP exposure of equipment during months 7 through 10—that is, with hoses and plenums assembled and sealed and the AHU set to the VHP cycle—significantly reduced overall the PCR evidence of microbes in plenums compared with static-open VHP exposure. Overall, significantly ($P < 0.01$) fewer PCR tests of exhaust plenums after active-closed VHP exposure (6 of 608 tests; 1%) were positive for a microbe compared with after static-open VHP exposure (33 of 880 tests; 4%). Individually, PCR evidence of *S. xylosus* and *S. aureus* (the more prevalent microbes PCR-detected residually after static-open VHP exposure) were less prevalent ($P < 0.05$ for both comparisons) after active-closed VHP exposure (Table 1).

The 3 microbes most prevalent in both murine (Figure 1) and soiled exhaust plenum specimens (Figure 2) were *Staphylococcus xylosus*, *Proteus mirabilis*, and *Pasteurella pneumotropica* biotype Heyl. After effective development of the active-closed VHP exposure configuration during month 7, none of the opportunistic microbes, including these 3 most-prevalent microbes, were PCR-detected in exhaust plenums after active-closed VHP exposure. The murine skin commensal *Staphylococcus xylosus* was the most prevalent microbe detected during the yearlong PCR surveillance (Figure 3). The percentage of murine specimens collected each month that was *Staphylococcus xylosus* PCR-positive was highly variable, ranged monthly from 0% to 100%, with a 12-mo median of 54% (Figure 1) and a 12-mo mean of 46% (Table 1). In contrast, the percentage of exhaust plenum specimens collected each month that was PCR-positive for *Staphylococcus xylosus* ranged from 50% to 83% (Figure 3), with a comparable 12-mo median of 72% (Figure 1) and 12-mo mean of 73% (Table 1), suggesting that this skin commensal is readily aerosolized from murine primary enclosures. After effective development of the active-closed VHP exposure method during month 7, even this microbe became PCR undetectable in exhaust plenums after active-closed VHP exposure during months 8 through 10 (Figure 3).

Figure 3. Monthly mean percentage of PCR-positive tests for *Staphylococcus xylosus* in soiled, washed, and VHP-exposed exhaust plenums during the 12-mo surveillance. *Staphylococcus xylosus* was the most prevalent microbe detected but became PCR-undetectable in exhaust plenums after active-closed VHP exposure during months 8 through 10.

Discussion

Documenting the prevalent opportunistic microbes in a murine SPF–viral-antibody-free facility contributes to appropriate development of murine health surveillance routines and the accurate interpretation of results of detection of such agents. Three microbes—*Staphylococcus xylosus*, *Proteus mirabilis*, and *Pasteurella pneumotropica* biotype Heyl—were most prevalent in both the murine and IVC exhaust plenum specimens that we tested. All 8 opportunistic microbes recovered were overall and individually more readily PCR-detectable in IVC exhaust airways than in murine specimens. These findings extend prior reports regarding the utility of PCR testing of IVC exhaust airways as a component of murine health surveillance^{2,12,24,31,39,44,45} and are especially applicable to improved health monitoring of immunodeficient mice, in which such agents serve as opportunistic pathogens.^{1,5,15,20,56,58,59,74} Although sanitization by mechanized washing reduced the PCR evidence of these microbes, most species (that is, 5 of the 8 obtained) remained PCR-detectable. VHP exposure with equipment left unassembled (that is, in the static-open configuration) did not substantively further reduce the PCR evidence of microbes in exhaust plenums. In contrast, assembled IVC racks connected to AHU set on to the VHP cycle (that is, during active-closed VHP exposure) ensured that VHP was effectively drawn through the IVC manifolds and plenums and AHU filters and chambers, thus eliminating PCR-detectable evidence of all microbes.

As immunodeficient and genetically engineered mice comprise an ever larger portion of murine inventories, microbes to be monitored in murine facilities will likely expand to include opportunistic microbes, members of the microbiome, and new, previously unrecognized microbial pathogens.^{1,5,6,11,15,18-20,39,40,43,46,56,58-60,62,63,74} We recently demonstrated that active-closed VHP sterilization of secondary housing equipment was an important component to facility-wide eradication of *Corynebacterium bovis*,⁴³ perhaps one of the more problematic opportunistic outbreaks in an immunodeficient murine colony. Our prior report described that despite the absence of marked, widespread morbidity attributable to *Corynebacterium bovis*, this organism can be harbored by a number of immunodeficient strains, is PCR-identifiable among unaffected immunocompetent strains, and is broadly disseminated to all facility areas during an outbreak.⁴³

Regardless, not every opportunistic microbe is amenable to eradication. The murine skin commensal *Staphylococcus xylosum*, which is not routinely monitored by even approved vendors,¹³ was the most prevalent microbe that we detected in both murine and plenum specimens. As documented in our prior report,⁵³ this organism's prevalence makes it an ideal indicator species for ensuring the sterilization efficacy of active-closed VHP exposure. To ensure effective mitigation of opportunistic agents that can invalidate studies involving immunodeficient mice, we now complete semiannual active-closed VHP sterilization of all secondary housing equipment during each murine housing room change-out.

We do not claim that implementing such VHP sterilization efforts eradicates commensals, but instead it limits their accumulation in murine secondary enclosures. As documented herein, IVC exhaust airways left unattended accumulate opportunistic microbes. A number of such microbes are either harbored by humans or are common to the environment, including *Pseudomonas aeruginosa*, β -hemolytic *Streptococcus* spp., and *Staphylococcus aureus*.⁶⁴ Many microbes, such as *Pasteurella pneumotropica* biotypes Heyl and Jawetz, are capable of forming biofilms, matrix-enclosed microbial growth, layered onto inanimate substrates when such microbes are outside the murine mucosa.⁵⁷ Biofilms contribute to microbial survival, confer resistance to antimicrobials,^{23,57} and form and grow exponentially in the distribution manifolds of automated murine drinking water systems that are left unattended.⁴² Completing semiannual active-closed VHP sterilization of all secondary housing equipment during each murine housing room change-out reduces the risk that microbes may accumulate and develop biofilms on IVC exhaust airway interior surfaces, including manifolds, plenums, connecting hoses, and AHU filter chambers. Validating the efficacy of VHP sterilization of secondary housing equipment by PCR testing of airway interiors for *Staphylococcus xylosum*, the most prevalent opportunistic microbe,⁵³ contributes to immunodeficient murine biosecurity.

Biosecurity of immunodeficient murine research involves more than the exclusion of adventitious agents and is augmented by monitoring prevalent opportunistic microbes. The prevalent murine opportunistic microbes previously identified in surveys of programs^{7,8,30,41} and diagnostic laboratories^{37,51} include *Helicobacter* spp., *Corynebacterium bovis*, *Proteus mirabilis*, and *Pasteurella pneumotropica* as frequent isolates. Herein, we made direct assessments in an SPF-viral-antibody-free murine facility that excludes *Helicobacter* spp. and *Corynebacterium bovis* and completed a yearlong PCR surveillance monitoring 15 microbes and comprising 1738 murine and exhaust plenum specimens. Our findings confirm the prevalence of *Proteus mirabilis* and *Pasteurella pneumotropica* biotype Heyl in a murine research facility and extend these prior observations to include the prevalent skin commensal *Staphylococcus xylosum*.

Prior to our current report, information existed regarding whether and which opportunistic microbes might accumulate in IVC exhaust airways and pose a risk to immunodeficient murine research was sparse. As described herein and previously reported,^{43,53} ensuring data integrity and maintaining topographically complex murine research housing and core procedural areas that require an array of shared supporting, imaging, anesthetic, surgical, and diagnostic electronic equipment requires an understanding of the prevalent opportunistic microbes to be monitored and the implementation of a PCR-validated method for facility-wide decontamination to mitigate the risk of invalidating studies involving immunodeficient mice.

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