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Surveillance of a Ventilated Rack System for *Corynebacterium bovis* by Sampling Exhaust-Air Manifolds

Christopher A Manuel,[1,2,*] Umarani Pugazhenthi,[3] and Jori K Leszczynski[1,2]

Corynebacterium bovis **causes an opportunistic infection of nude (*Foxn1*, nu/nu) mice, leading to nude mouse hyperkeratotic dermatitis (scaly skin disease). Enzootic in many nude mouse colonies, *C. bovis* spreads rapidly to naive nude mice, despite modern husbandry practices, and is very difficult to eradicate. To facilitate rapid detection in support of eradication efforts, we investigated a surveillance method based on quantitative real-time PCR (qPCR) evaluation of swabs collected from the horizontal exhaust manifold (HEM) of an IVC rack system. We first evaluated the efficacy of rack sanitation methods for removing *C. bovis* DNA from the HEM of racks housing endemic colonies of infected nude mice. Pressurized water used to flush the racks' air exhaust system followed by a standard rack-washer cycle was ineffective in eliminating *C. bovis* DNA. Only after autoclaving did all sanitized racks test negative for *C. b*ovis DNA. We then measured the effects of stage of infection (early or established), cage density, and cage location on the rack on time-to-detection at the HEM. Stage of infection significantly affected time-to-detection, independent of cage location. Early infections required 7.3 ± 1.2 d whereas established infections required 1 ± 0 d for detection of *C. bovis* at the HEM. Cage density influenced the quantity of *C. bovis* DNA detected but not time-to-detection. The location of the cage on the rack affected the time-to-detection only during early *C. bovis* infections. We suggest that qPCR swabs of HEM are useful during the routine surveillance of nude mouse colonies for *C. bovis* infection.**

Corynebacterium bovis is an opportunistic pathogen of immunodeficient mice and is primarily recognized as the causative agent of hyperkeratotic dermatitis (scaly skin disease) in athymic nude (*Foxn1*, nu/nu) mice. With a worldwide distribution,[2,10,14] *C. bovis* causes a clinical illness of short duration followed by what is believed to be lifelong subclinical skin colonization.[2,4] Despite the limited duration of clinical signs, *C. bovis* is thought to have significant effects on xenograft tumor development, leading to delayed, slowed, or failed xenograft and allograft tumor growth.[7,8]

Eradication of *C. bovis* from infected nude mouse colonies has proven to be challenging. Variable success has been demonstrated even with an ideal remediation plan of depopulation, decontamination, and repopulation.[14,16] Additional challenges face institutions that attempt phased decontamination, including efficient horizontal spread of infection despite modern husbandry practices and the ineffectiveness of antibiotics to cure clinically and subclinically infected nude mice.[2,3] In addition, *C. bovis* is known to produce diffuse environmental contamination throughout facilities by airborne deposition of bacterially populated skin flakes.[1,3] Airborne transmission has even been documented within biosafety cabinets, which should be considered one of the primary methods of cage-to-cage transmission.[3]

Early detection is crucial to maintain nude mice colonies free of *C. bovis*, given the potential for rapid spread through experimental manipulations, general animal care practices, and extensive equipment and environmental contamination.[3,16] After rapid detection, prompt restriction of animal manipulations and movement would allow time for the identification and removal of infected cage(s), followed by localized decontamination of housing and research equipment. However, colony-based *C. bovis* detection is neither rapid nor efficient, with ubiquitous soiled-bedding sentinel programs that are based on a paradigm of serologic response, in which soiled bedding typically is gathered every 1 to 2 wk at the time of a cage change and is followed by a traditional 3-mo monitoring interval. Although the monitoring interval might be shortened to enhance surveillance, only a fraction of the cages on an IVC rack can contribute bedding to the sentinel cage at any specific time point, due to volume limitations.[5,9] Therefore, multiple cage-change cycles must occur for all cages on a rack to equally contribute to the sentinel cage. Furthermore, little is known about the duration that immunocompetent mice will carry *C. bovis* on the haircoat to facilitate detection. To more accurately represent the mice under surveillance, nude mice have been used as soiled-bedding sentinels, with the successful detection of *C. bovis* within nude mouse colonies.[3] However, according to the cited report,[3] the inherent limitations of a soiled-bedding sentinel program were not overcome, given that only about half of the cages on a rack contributing soiled bedding during weekly cage changes, with a maximal surveillance interval of 1 mo. Moreover, concerns remain that nude sentinel mice may aid in propagating disease, because the potential for environmental contamination from a *C. bovis*-infected sentinel nude mouse would mirror that of naturally infected nude mice.[6] Finally, an additional obstacle to the use of soiled-bedding sentinels is data that suggests that soiled-bedding accumulation points in biologic safety cabinets during cage changing may aid in the horizontal spread of infection.[3]

PCR-based diagnostic surveillance of IVC rack air exhaust for mouse pathogens has yielded some success, through the

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[1]Office of Laboratory Animal Resources, [2]Department of Pathology, and [3]Department of Medicine, Division of Endocrinology, Metabolism, and Diabetes, Anschutz Medical Campus, University of Colorado, Aurora, CO
**Corresponding author. Email: Christopher.Manuel@ucdenver.edu*

use of 2 sample collection methods primarily.[5,9,11] Small pieces of filter fabric placed in front of exhaust-air rack filters successfully led to the detection of *Helicobacter muridarum*, Sendai virus, mouse hepatitis virus, and mouse parvovirus but failed to detect *Helicobacter hepaticus* and mouse rotavirus in experimentally infected mice.[5] More recently, direct sampling of a rack system's horizontal exhaust manifold (HEM, Figure 1) with a sterile swab for PCR has been used successfully to detect the fur mites *Myobia musculi* and *Radfordia affinis* from naturally infected mice.[9] However, in another study using HEM sampling, the mouse pinworm *Aspiculuris tetraptera* was not detected from naturally infected mice.[11] Despite the mixed results, we were interested in using quantitative PCR (qPCR) techniques to evaluate the HEM for *C. bovis*, given the known distribution of *C. bovis*-contaminated skin flakes by air currents.[3] In addition, this method would preclude entering individual cages for sample collection, subsequently decreasing the potential for cross-contamination between cages. Furthermore, surveillance intervals would not be limited by cage-change frequency, and all cages on an IVC rack could be monitored simultaneously. We also wanted to determine the effects of cage location, mouse cage density, and stage of infection on how quickly *C. bovis* could be detected by PCR analysis of HEM swabs. To further aid in the practical implementation of this surveillance technique, we also evaluated whether our standard rack-sanitation procedure eliminated *C. bovis* DNA from the HEM of racks that housed infected nude mice.

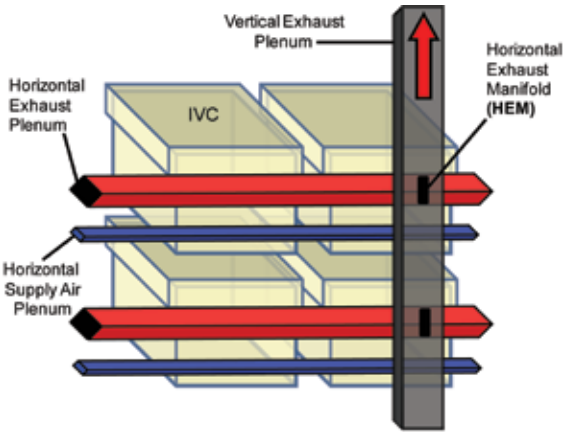

Figure 1. Cut-view illustration of the air supply and exhaust plenums of an IVC rack system (Allentown) viewed from the rear. HEPA-filtered supply air is forced into each cage from the horizontal supply air plenum. Negative pressure draws air from each IVC cage into the horizontal exhaust plenum of the rack. Exhaust air from all cages on the row passes through the row's HEM to enter the common vertical exhaust plenum flowing in the direction of the red arrow.

Materials and Methods

IVC rack sanitation and *C. bovis* detection. We used qPCR to test the ability of the rack sanitation process to eliminate *C. bovis* DNA from the HEM of *C. bovis*-exposed racks. We randomly selected 5 racks known to have housed *C. bovis*-positive nude mice for an undetermined period while in service for 124 ± 49 d. Serial samples were collected by opening the door to the vertical exhaust plenum and using a dry sterile swab (BBL Culture Swab EZ, Becton Dickinson, Franklin Lakes, NJ) to wipe the inside of all HEM of the rack according to the technique described in detail in the section *Technique for sampling HEM*. For each rack, a swab was collected prior to rack sanitation, after pressurized rinse and sanitation by rack-wash only, and after rinsing, sanitation, and autoclaving. For rack sanitation, which occurred in a dedicated cage-wash area, all doors to supply and exhaust air plenums were opened and prerinsed with pressurized municipal water by using a handheld wand (Strahman, Bethlehem, PA), and the automatic watering system pipe manifolds were opened and allowed to drain. Racks were washed in a cage-washer (Basil 9500, Steris, Mentor, OH) by using an alkaline detergent (7.4 mL per 3.78 L of water; Clout, Pharmacal, Naugatuck, CT), a wash temperature that reached 49 °C (120 °F), and a final rinse water temperature that remained at 88 °C (180 °F) for at least 1 min. After the rack was washed, its automatic watering system was connected to an automatic chlorine-injection station (Edstrom Industries, Waterford, WI), which performed an initial flush, a soak with a 1:25 dilution of a 5.25% hyperchlorite solution (2000 ppm free chlorine), and a final flush with 20 ppm hyperchlorinated water; the system then was allowed to drain. Racks were then steam sterilized in a bulk autoclave (AMSCO series, Steris) using a prevacuum cycle at 132 °C (270 °F) for 10 min and a dry time of 5 min. Racks were allowed to cool at ambient temperature prior to HEM sampling for the detection of *C. bovis* by qPCR analysis.

Mice, housing, and husbandry. Female, athymic nude mice (age, 6 to 7 wk; Hsd:Athymic Nude-*Foxn1*^{nu}; Harlan Laboratories, Indianapolis, IN) were obtained and documented to be free of endoparasites and ectoparasites by microscopy and of lactate dehydrogenase elevating virus, *Helicobacter* spp., *Corynebacterium bovis, Pneumocystis murina*, *Streptobacillus moniliformis* by PCR, and *Bordetella bronchiseptica*, *Citrobacter rodentium*, *Corynebacterium kutscheri, Klebsiella oxytoca, K. pneumonia*, *Pasteurella multocida*, *P. pneumotropica*, *Proteus mirabilis*, *Pseudomonas aeruginosa, Salmonella* spp., *Staphylococcus aureus*, *Streptococcus* spp. group B β, and *Streptococcus pneumonia* by culture. Serology from immunocompetent sentinel mice of the athymic nude stock confirmed the absence of lymphocytic choriomeningitis virus, minute virus of mice, mouse parvovirus, mouse hepatitis virus, mouse adenovirus type 1 and 2, mouse cytomegalovirus, mouse polyoma virus, mouse rotavirus, mouse thymic virus, murine norovirus, pneumonia virus of mice, respiratory enteric virus III, Sendai virus, Theiler murine encephalomyelitis virus, cilia-associated respiratory bacillus, *Clostridium piliforme*, *Mycoplasma pulmonis*, and *Encephalitozoon cuniculi*. All mice were confirmed by qPCR analysis to be *C. bovis*-negative on arrival and at the start of the experiment. For all studies, mice were housed in JAG 75 (Allentown, Allentown, NJ) cages on 70-cage, single-sided, individually ventilated racks (MicroVent, Allentown) providing 40 air changes hourly. All racks used in the following studies were sanitized and autoclaved as described earlier and confirmed to be *C. bovis*-negative by HEM sampling prior to use. Cages for autoclaving contained aspen chip bedding, a compressed cotton square, and a box feeder. Irradiated rodent diet (2920X, Teklad Extruded Diet, Harlan Laboratories), a sterilize Mouse Igloo (BioServ, Frenchtown, NJ), and mice were placed into sterile cages in an area determined to be *C. bovis*-free by *C. bovis* surveillance with qPCR analysis of swab samples. Mice were provided free access to reverse-osmosis–purified, hyperchlorinated water from an automated watering system (Edstrom Industries) by using a water valve attached to the rack. Mice were housed at 1 or 5 mice per cage, depending on the experimental design. The macroenvironment of the animal housing room was maintained at 22.2 ± 1 °C (72 °F) and 30% to 40% humidity with at least 12 fresh-air changes hourly and a controlled 14:10-h light:dark cycle. All mouse manipulations were performed in an animal transfer station (ATS2, Allentown), and all work surfaces and nitrile-gloved hands were kept moist with a general disinfectant (1:18:1; Clidox S, Pharmacal, Naugatuck,

CT). Personal protective equipment required to enter the facility included a hair bonnet, disposable gown, and shoe covers over personal clothing and shoes. All animal studies were approved by the University of Colorado Denver IACUC.

Study design. We tested the effects of 3 variables on how quickly *C. bovis* could be detected by qPCR analysis at the HEM of a single-sided IVC rack after the placement of an infected cage. These variables included the: 1) stage of *C. bovis* infection (either early infection [immediately after acute exposure] or an established infection); 2) cage density (1 or 5 nude mice per cage); and 3) proximity of the cage containing nude mice to the HEM of the IVC rack. For the infection detection studies, autoclaved IVC racks were placed into a recently sanitized holding room where only nude mice in the study were housed.

Early and established C. bovis infections. Similar to a previously described methodology, nude mice were acutely exposed to *C. bovis* by placing them for 1 min in a 7-d-old soiled cage containing a single, subclinically infected nude mouse.[2] Exposed mice then were placed in clean, sterile cages and housed singly or with 4 unexposed nude mice. We relied on intracage transmission to spread infection among the 5 cohoused mice. Mice were considered to have an early infection immediately after exposure and were used in the early-infection study. However, to quantify the day of infection as determined by qPCR detection, the exposed mouse from each cage was sampled on days 1, 3, 5, 7, and 10 after exposure by using a single swab to wipe the mouth and skin on each day. All mice from the early-infection study were confirmed to be *C. bovis*-infected by qPCR analysis and were reused at 49 to 63 d after exposure to represent mice with infections of known duration for the established-infection study.

Stage of infection, cage density, and cage position. One cage, containing either 1 or 5 nude mice with either early or established *C. bovis* infections, was placed in the lower left (position A10; column A, row 10) or lower right (position G10; column G, row 10) corner of a single-sided IVC rack. The remaining 6 cage positions on row 10 were filled with empty, sterile cages. Rack position A10 is immediately adjacent to the HEM, and rack position G10 is furthest from the HEM. The experimental variables of cage density and cage position were each tested in triplicate with early and established infections. Prior to use, all IVC racks were sanitized by rack-wash, autoclaved, and confirmed to be negative for *C. bovis* by qPCR analysis.

Technique for sampling the HEM. Similar to methods previously described, to access the HEM of each rack for sampling, the connections for the air exhaust followed by the air supply were disconnected from the rack to prevent air currents during sample collection. The door to the vertical exhaust plenum then was opened to gain access to all HEM of the rack. For approximately 5 s, a dry sterile swab (BBL Culture Swab EZ) was used to wipe all inside surfaces (60 cm^2) of the HEM of row 10. After sample collection, the door to the vertical exhaust plenum was closed, and the connections for the supply air were reconnected to the rack, followed by those for the exhaust air.[9,11] After cage placement, the HEM were swabbed daily for 11 d during the early-infection study and for 3 consecutive days for the established-infection study. For both the early- and established-infection studies, the HEM of row 9 for each experimental rack and row 10 of a control rack with no cages were swabbed by using the same method described, before and after each trial as negative controls. Swabs were evaluated by qPCR analysis for *C. bovis* DNA.

Single-cage crossover study with rack positions A10 and G10. This experiment was performed to confirm that the difference in the quantity of *C. bovis* DNA detected at the HEM was due to the proximity of the infected cage to the HEM and not to variability in *C. bovis* generated by mice of different cages or to fluctuations in shedding within a single cage of mice. Infection was established in a cage of 5 nude mice by adding to their home cage 15 mL of soiled bedding from *C. bovis*-infected mice. Beginning 3 wk after exposure, 3 of 5 mice in the cage were sampled weekly for 10 wk by pooled oral and skin swabs to represent the maximal bacterial burden of the mice. As in both the early- and established-infection studies described earlier, mice were transferred to a clean, autoclaved cage and placed onto the autoclaved rack. For 4 consecutive days after cage placement, swabs were collected from the HEM followed by control swabs collected from HEM of row 9. After the 4 d of sample collection, the cage was removed and the rack sanitized, autoclaved, and returned. By using the same rack and cage of mice, the procedure was repeated 4 times, cage placement was alternated between rack positions A10 and G10. Swabs were evaluated by qPCR analysis for *C. bovis* DNA.

qPCR. The tip of each sample swab was placed into a sterile -mL microcentrifuge tube (Safe-Lock, Eppendorf, Enfield, CT). With continuous pressure, the lid of the tube was closed and the shaft of the swab was bent repeatedly to break the swab shaft, leaving the tip in the tube. DNA was extracted from swab tips (QIAamp DNA Mini Kit, Qiagen, Germantown, MD) according to the manufacturer's recommendations. Quantitative real-time PCR reactions were performed by using a sequence detector (ABI Prism 7900, Life Technologies, Grand Island, NY) and analyzed by using the accompanying software. Primers and probe designed to target the 16S rRNA gene of *C. bovis* were designed by using the sequence detection software (Primer Express, Life Technologies). The following *C. bovis*-specific primer and probe sequences (Life Technologies) were used: forward primer, 5′ AAC GCG AAG AAC CTT ACC TGG 3′; reverse primer, 5′ ACC ACC TGT GAA CAA GCC CA 3′; and probe, 5′ GGC AGG ACC GGC GTG GAG A 3′. The probe labelled with 6-carboxyfluorescein at the 5′ end and with 6-caboxy-tetramethylrhodamine at the 3′ end. Amplification reactions were performed in MicroAmp optical plates (Life Technologies) in a 20-µL mix containing 1× TaqMan Buffer A (500 mM KCl, 100 mM Tris-HCl, 0.1 M EDTA, 600 nM passive reference dye ROX; pH 8.3 at room temperature), 300 µM each of dATP, dGTP, and dCTP, 600 µM dUTP, 5.5 mM $MgCl_2$, 900 nM forward primer, 900 nM reverse primer, 200 nM probe, 0.25 U of AmpErase uracil-N-glycosylase, 1.25 U AmpliTaq Gold DNA Polymerase, and 5 µL of template DNA (25% of the total reaction volume). All experimental samples were run concurrently with a no-template control and 1000 template copies of a positive-amplicon control.

Thermal cycling conditions were established to hold for 2 min at 50 °C followed by activation of TaqGold at 95 °C for 10 min. Subsequently 40 cycles of amplification were performed at 95 °C for 15 s and 60 °C for 1 min. The detection threshold was set above the mean baseline fluorescence determined from the first 15 cycles, which consistently exhibited a mean fluorescence less than 0.2. Amplification reactions in which the fluorescence intensity increased above the fluorescence threshold, and a cycle threshold less than 40 was defined as a positive reaction. A standard curve was generated by using the fluorescence data from the 10-fold serial dilutions of *C. bovis* DNA amplicon (range, 10^0 to 10^9 template copies; $R^2 = 0.99$). The standard curve then was used to calculate the absolute copy number of *C. bovis* in test samples.

Monitoring of IVC rack air supply and exhaust rates. Blowers located in the interstitial space provided HEPA-filtered supply

air from the conditioned air within the housing room, and exhaust air was evacuated directly into the building HVAC system. The rates of air supply and exhaust to each experimental and control rack were measured by using a rack flow detector (Allentown) positioned at the top of the rack and connected to the supply and exhaust hoses as though attached to the rack. Cage flow detectors (Allentown) were placed in rack positions A10 and G10 to determine the number of air changes hourly at the cage level.

Statistical analysis. All graphs and statistical analyses were performed by using SigmaPlot 11.2 (Systat Software, Point Richmond, CA). Kaplan–Meier curves were generated to visualize the detection of *C. bovis* over time either on mice or within the HEM of racks and were followed by a log-rank test to compare the infection distribution between the 2 experimental groups of each independent variable (cage density and cage position). A 2-tailed, unpaired Student *t* test was performed to compare the qPCR copy number between 2 samples at any given time point after the data were evaluated to confirm normal distribution and equal variance. The threshold for significance was a *P* value of less than 0.05 for all statistical analyses.

Results

Rack sanitation for elimination of *C. bovis* DNA. Prior to sanitation, the HEM of all 5 racks were positive for *C. bovis* DNA (Table 1), whereas the horizontal air-supply manifolds of all racks were negative (data not shown). Subsequently 40% (2 of 5) of the racks were negative for *C. bovis* DNA after being rinsed with pressurized water and processing through the rack-washer, whereas 100% of the racks were negative with the addition of autoclaving after rinsing and washing.

***C. bovis*-exposed mice.** The time required for acutely exposed mice to test positive for *C. bovis* by qPCR was 4.0 ± 1.3 d (n = 12). All exposed mice were positive by day 5. After exposure, the time required for mice to test positive for *C. bovis* did not differ depending on cage density (individual or group-housing; Figure 2 A) or on rack position (A10 or G10; Figure 2 B).

Detection of *C. bovis* at the HEM of racks housing mice with early infections. The number of mice in the infected cage did not significantly affect the amount of time required to detect *C. bovis* at the HEM of the rack. *C. bovis* was detected at 7.3 ± 1.5 d (n = 6) for singly housed mice and 7.3 ± 0.8 d (n = 6) for group-housed mice (Figure 2 A). In contrast, cage position in a row of the rack significantly ($P \leq 0.05$) affected the amount of time before *C. bovis* was detected at the HEM: 6.7 ± 0.8 d (n = 6) for position A10 and 8.0 ± 1.1 d (n = 6) for position G10 (Figure 2 B). Once *C. bovis* DNA was detected by qPCR, all racks remained positive until the end of the monitoring period (day 11).

The position of the cage on the rack influenced the amount of *C. bovis* DNA detected at the HEM for cages containing 1 or 5 nude mice. The amount of DNA on the swab was greater when infected cages were placed closer to the HEM (Figure 3). However, this difference was significant only on days 10 and 11 when cages contained 1 mouse each (Figure 3 A). We compared the quantity of *C. bovis* DNA detected at the HEM of racks depending on the housing density and cage position. Cages with 5 mice generated significantly ($P \leq 0.05$) more *C. bovis* DNA at the HEM on day 11 for rack position G10 only (data not shown), even though a clear trend was present at all time points and cage positions between days 7 through 11. Furthermore, swabs collected from the HEM of row 9 and the control rack were negative at all time points during this experiment.

Detection of *C. bovis* at the HEM of racks housing mice with established infections. Independent of housing density or cage position on row 10, sampling the HEM detected mice with established *C. bovis* infections by 1 d after cage placement on all racks (n = 12) and remained positive for the 3 d of serial rack sampling (Figure 4 A and B). When cages contained 1 infected mouse each, the quantity of *C. bovis* DNA detected at the HEM was 1.9- to 2.2-fold higher when the infected cage was placed at position A10 than at position G10 (Figure 4 A). Similarly, when cages contained 5 infected mice each, the *C. bovis* DNA copy number was 3.0- to 4.8-fold higher ($P \leq 0.05$) at the HEM when the cage was in position A10 than when placed at position G10 (Figure 4 B). Furthermore, swabs collected from the HEM of row 9 and the control rack were negative at all time points during this experiment.

Table 1. The effect of IVC rack sanitation on the elimination of *C. bovis* DNA from the HEM

Rack	No. of days in use	*C. bovis* status (copy no. by qPCR)		
		Before sanitizing	After rack-washing	After autoclaving
1	53	56,944	45	undetectable
2	172	659,400	undetectable	undetectable
3	188	482	34	undetectable
4	97	279,774	844	undetectable
5	127	316,227	undetectable	undetectable

Overall mean (± 1 SD) duration of rack in use was 124 ± 49 d

Throughout the 10-wk crossover study that occurred between weeks 4 and 11 after exposure, the *C. bovis* DNA copy number in infected mice fluctuated between 10^5 and 10^6 copies per swab. Overall, the 8 swabs collected from the HEM (1 swab each day) consistently yielded 10^4 copies per swab with the infected cage at position A10 and 10^3 copies per swab at position G10. The copy number detected at the HEM was significantly ($P \leq 0.05$) higher when the cage was placed in position A10 than when the cage was placed in position G10 (Figure 5). During the crossover study, swabs collected from the HEM of row 9 were negative for *C. bovis* at all time points.

Rack and cage ventilation. For the experiments evaluating the detection of early and established *C. bovis* infections, the rack air-supply rate, exhaust rate, and the airflow at the cage level did not differ between racks when the cage was placed in position A10 compared with G10 (Table 2).

Discussion

Recent reports provided by an international reference laboratory indicate that *C. bovis* continues to be a common contaminant of nude mouse populations.[6,13] Because this infection has the potential to negatively affect the growth of both tumor allografts and xenografts, academic and commercial institutions attempt to exclude it from their facilities. In the current study, we investigated the utility of sampling the HEM of an IVC rack system that housed nude mice with either early or established *C. bovis* infections. In addition, we evaluated 2 housing densities of mice and 2 cage locations on the rack to determine the influence of these variables on how quickly *C. bovis* was detected. Our findings suggest that swab samples collected from the HEM can be used to rapidly and reliably detect early and established *C. bovis*-infected nude mice housed on an IVC rack. In practice, interpretation of these results is aided by the removal of all *C. bovis* DNA within the rack air-exhaust system at the time of sanitation. Our data demonstrate that this goal can be achieved by full-rack autoclaving but is not consistently met by directing pressurized water into the exhaust-air plenums followed by processing through a typical rack washer cycle.

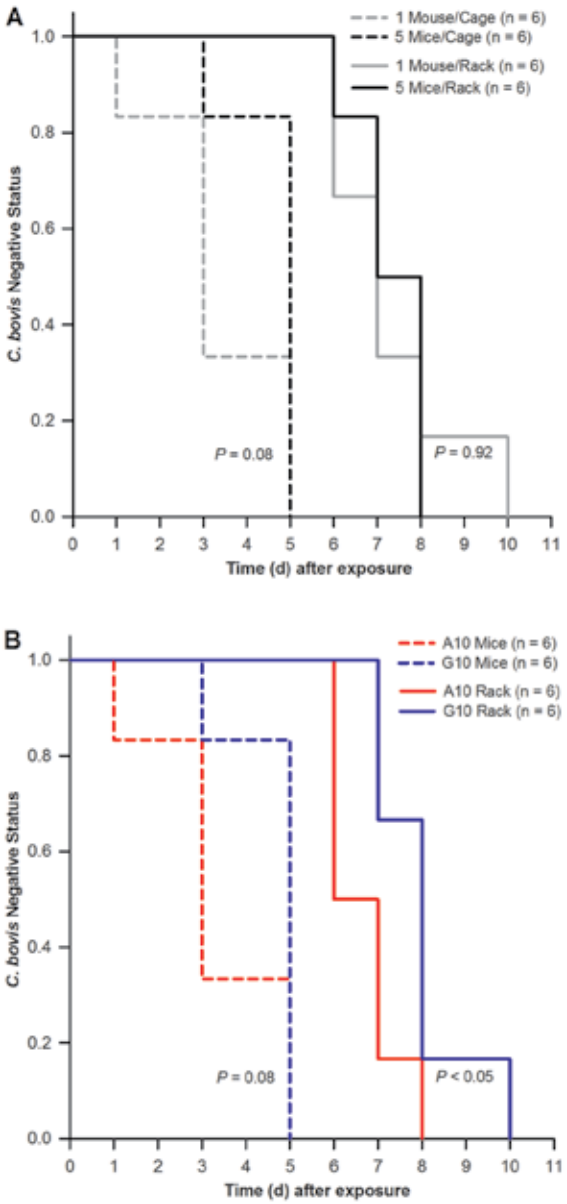

Figure 2. Kaplan–Meier curves depicting a decrease in *C. bovis*-negative status for nude mice (dashed lines) after exposure to *C. bovis* and for IVC racks (solid lines) after cages were placed on the racks with *C. bovis*-exposed mice. (A) The effect of the number of mice/cage on how quickly *C. bovis* is detected at the HEM of the rack. (B) The effect of infected cage location on how quickly *C. bovis* is detected at the HEM of the rack. Mice were tested by qPCR analysis of swabs of the skin and mouth, whereas racks were tested by qPCR analysis of swabs of the HEM. *P* values are shown near the pair of curves compared; $P \leq 0.05$ represents a statistically significant difference.

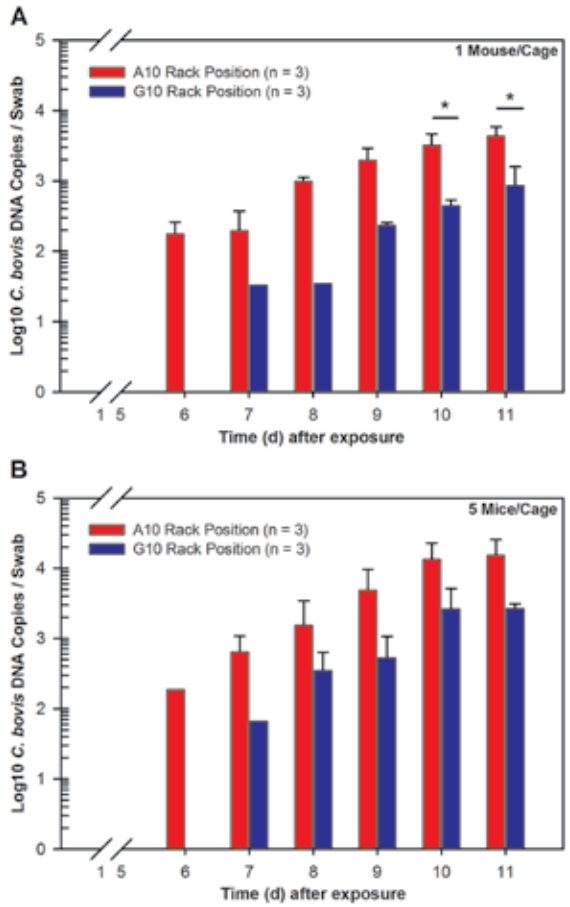

Figure 3. Early-infection study. *C. bovis* DNA copy number (mean ± 1 SD) detected by qPCR from HEM swabs that sampled racks holding *C. bovis*-exposed mice in cages containing (A) 1 mouse per cage and (B) 5 mice per cage placed at rack position A10 or G10. All samples collected on days 0 through 5 were negative for *C. bovis*. Bars without error bars indicate that only 1 of the 3 racks tested positive for *C. bovis* at that time point. *, significant ($P \leq 0.05$) difference between the numbers of DNA copies per swab detected at the same time point from cages in opposite positions on the rack.

For this project, we elected to expose naïve mice to *C. bovis* by direct contact with both an asymptomatic, infected mouse and soiled bedding in a single, brief exposure as compared with inoculation with cultured *C. bovis*. The rationale was based on the proposed mechanism of airborne transmission of *C. bovis* on shed keratin skin flakes and the hypothesis that clinical hyperkeratosis may aid in the distribution of bacteria in the rack air exhaust system. Previous reports of nude mice directly inoculated with cultured *C. bovis* have been unable to reliably generate clinical signs of hyperkeratosis. With direct transmission of infection from mouse to mouse during this study, mice in 67% of cages (8 of 12) developed a mild to severe clinical hyperkeratosis. Clinical signs were more common in cages containing 5 mice (100%; 6 of 6) as compared with cages with a single mouse (33%; 2 of 6). For cages of 5 mice, the number of mice with clinical signs in each cage was not recorded, but clinical signs varied from asymptomatic to severe hyperkeratosis within the same cage between 10 and 14 d after exposure. For exposed mice that were singly housed, mild clinical signs were first observed on day 14. Therefore, for singly or group-housed mice, our observation of clinical hyperkeratosis by 14 d after exposure is consistent with previous published and anecdotal reports ranging between 7 and 13 d.[2,4,6,14] Nevertheless, the detection of *C. bovis* infection occurred prior to and without the presentation of clinical signs of infection. Because our rack-monitoring period ended on day 11 after exposure, the effect of clinical hyperkeratosis on the quantity of *C. bovis* present in the HEM could not be evaluated.

The time required before an early *C. bovis* infection could be detected at the HEM ranged from 6 to 10 d, depending most notably on cage proximity to the HEM. Much of the time required for the detection of early infections is attributed to the time required for detectable bacterial skin burden after direct exposure. The relatively slow rate of growth on the skin of naïve nude mice is consistent with the modest rate of bacterial

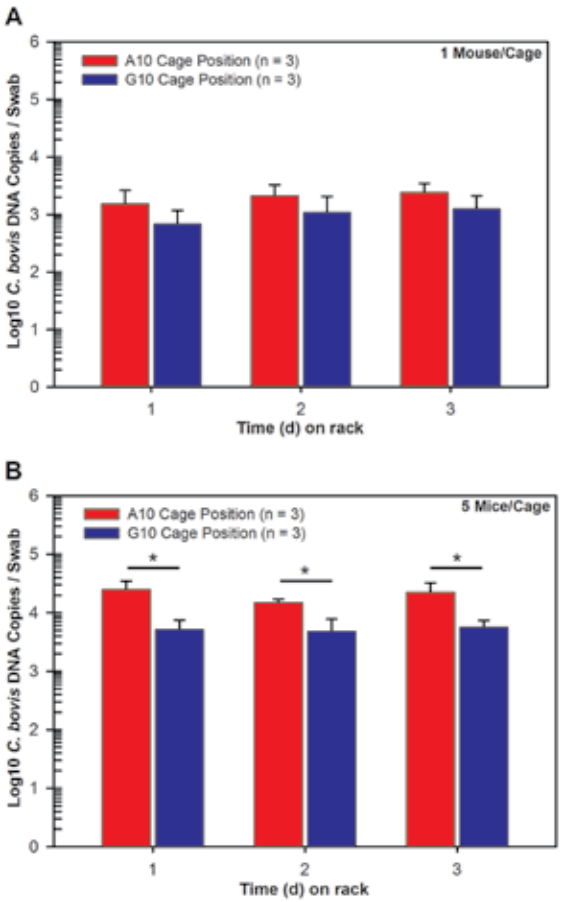

Figure 4. Established-infection study. *C. bovis* DNA copy number (mean ± 1 SD) detected by qPCR from HEM swabs that sampled racks holding *C. bovis*-infected mice in cages containing (A) 1 mouse per cage and (B) 5 mice per cage placed at rack position A10 or G10. *, Significant ($P \leq 0.05$) difference between the numbers of DNA copies per swab detected at the same time point from cages in opposite positions on the rack.

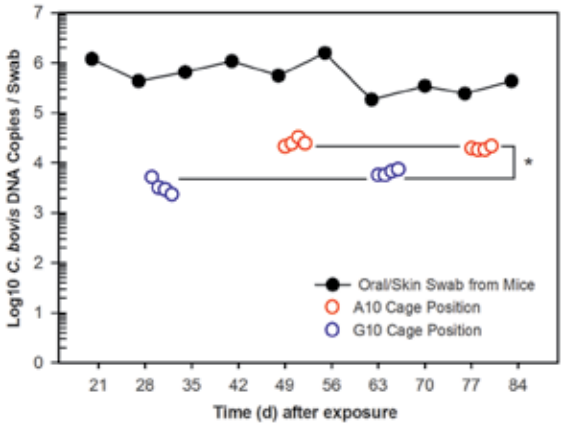

Figure 5. Time course of *C. bovis* skin and oral bacterial burden in mice according to a pooled (3 of 5 mice) sample and detected at the HEM by qPCR analysis as a single cage was alternated between rack positions A10 ($n = 8$) and G10 ($n = 8$). Serial circles represent HEM swabs collected daily during the 4 d prior to rack sanitation, autoclaving, and movement of the cage to the opposite location in the rack. *, Significant ($P \leq 0.05$) difference between the numbers of DNA copies per swab detected at the same time point from cages in opposite positions on the rack.

growth on 5% blood agar, which can take 2 d before colonies can be visually detected.[2,6] In addition, the slow skin colonization observed during early infections may explain why cage density did not influence how quickly *C. bovis* was detected at the HEM. We expected that housing 5 mice (compared with 1) in a cage would generate more *C. bovis* DNA to disseminate into the HEM, resulting in more rapid detection. Paradoxically, *C. bovis* was not detected more rapidly on racks housing a cage of 5 nude mice as compared with a single nude mouse. We attribute this finding to the method of exposure, in which only 1 mouse (index mouse) in a cage of 5 was exposed directly to *C. bovis*, followed by cohousing with 4 naïve mice. The lag time documented in the skin colonization of the index mice and bacterial detection in the HEM (Figure 2) suggests that naïve cage mates would not contribute to the quantity of bacteria emitted into the air-exhaust system until approximately 6 d after exposure to an infectious dose. Furthermore, 6 d represents the earliest time point at which *C. bovis* was detected at the HEM after index mouse exposure with 1 mouse per cage. Although qPCR samples were not directly collected from cage mates, this assumption is consistent with the finding that the quantity of DNA detected at the HEM of the early infection study did not differ between 1 and 5 mice per cage until day 11 after exposure. In contrast, during the established infection study, 8.7 times more *C. bovis* DNA was detected at the HEM with cages of 5 infected nude mice as compared with 1 mouse per cage.

Our study shares many similarities to a previous report that used HEM sampling for the detection of mouse fur-mite DNA in the same IVC rack system.[9] In that study, cage position G10 was selected for the infested cage because it was furthest from the HEM on the row. The authors speculated that the distance from the sampling site would affect the sampling method's sensitivity.[9] To further characterize HEM sampling for the rapid detection of *C. bovis*, we examined the effect of cage location relative to the HEM by placing infected cages immediately adjacent to (A10) and at the furthest point from (G10) the HEM of the row. In our study, cage placement on the row significantly affected the time required to detect *C. bovis* during early infections but not with established infections. We conclude that, during early infection, the physical distance that the fine-particulate matter, contaminated with *C. bovis*, must travel to reach the HEM is the cause of the delay in detection. However, mice with established infections are detected in 1 d, independent of cage location on the row. It is unclear whether the quantity of bacteria shed into the rack exhaust or the number of contaminated skin flakes shed by mice with established infections is responsible for overcoming this distance.

During both the early- and established-infection studies, the quantity of *C. bovis* DNA detected at the HEM was consistently highest when the cage was placed at position A10. However, the difference in quantity of DNA detected was statistically significant only for the duration of sample collection in the established-infection study with 5 mice in a cage (Figure 4 B). Because of the lack of consistent statistically significant results regarding the quantity of DNA detected between the distant rack positions, we suspected that a difference in bacterial skin burden between cages of mice, fluctuation in bacterial skin burden within the same cage of mice, or daily variation in the HEM sample collected would produce excessive variability and thus confound a direct comparison between these 2 rack positions. We addressed this concern by performing an experiment that used a single cage of 5 infected mice in crossover study design that alternated cage position between A10 and G10 on the same IVC rack. HEM swabs were collected for 4 consecutive days to further evaluate day-to-day variation in the DNA copy number per swab. During the study, weekly oral and skin

Table 2. IVC rack supply and exhaust air measurements with corresponding cage air flow at 2 cage positions (A10 and G10)

	No. of mice per cage		Rack airflow (ft^3/min)		Cage airflow (no. of air changes hourly)	
			A10	G10	A10	G10
Early *C. bovis* infection						
	1	Supply	11.9 ± 0.9	12.2 ± 0.9	40.2 ± 1.6	40.7 ± 2.7
		Exhaust	29.2 ± 0.9	29.2 ± 1.5		
	5	Supply	12.5 ± 1.2	12.9 ± 1.1	41.6 ± 1.2	41.6 ± 2.0
		Exhaust	27.6 ± 1.6	27.4 ± 2.1		
Established *C. bovis* infection						
	1	Supply	11.1 ± 0.9	11.8 ± 0.6	41.0 ± 1.4	40.0 ± 1.1
		Exhaust	25.9 ± 1.4	26.6 ± 2.4		
	5	Supply	11.3 ± 0.7	11.4 ± 0.7	41.6 ± 1.2	41.6 ± 2.0
		Exhaust	28.4 ± 2.3	28.9 ± 2.6		

Data are shown as mean ± 1 SD. For the early infection trials, measurements were collected at the beginning, middle, and end of the 11 d of sample collection (n = 9 measurements per value). For the established infection trails, measurements were taken at the beginning and end of the 3-d sampling period (n = 6 measurements per value). The rack system does not have a mechanism for measuring the rate of air exhausted from the cage.

swabs directly monitored the bacterial skin burden of the mice. Over the 10-wk study, *C. bovis* DNA copy number fluctuated between 10^5 and 10^6 from the mice within the cage (Figure 5). Despite a 1-log fluctuation in copy number on the mice, plenum swabs consistently yielded 10^4 copies when the cage was placed at rack positions A10 and 10^3 copies when in the G10 position. According to the serial HEM swabs, the quantity of DNA detected stayed consistent from day to day despite 4 consecutive daily sample collections. Although we did not repeat this study with a single infected mouse per cage at different stages of infection, we conclude that inherent variation in the swab collection technique and fluctuations in shedding from the mice has a larger effect on the number of DNA copies detected when the number of mice in the cage is low and mice were recently infected. Therefore, with few mice and early infections, a direct comparison of copies between the distant cage positions will fail to achieve a statistically significant difference unless the numbers of observations are increased above what we performed in this study.

At the conclusion of the 11- and 3-d sampling periods in the early- and established-infection studies, respectively, we used 2 different negative controls to rule out the detection of environmental contamination, which is common in facilities with *C. bovis* infections, as compared with *C. bovis* generated within cages containing experimentally infected mice. The first control was an IVC rack that was positioned within the same housing room but that had no cages. The exhaust system of this control rack was drawing room air and remained negative for the duration of the study. The second control was a single swab from the HEM of row 9, directly above the HEM of row 10, which carried the cage of *C. bovis*-infected mice. The HEM of row 9 served as the most stringent control, given its proximity to the HEM of row 10 and because exhaust air ascends in the vertical exhaust plenum to be evacuated from the rack. For the duration of the early-infection study, cross-contamination between the HEM of rows 9 and 10 did not occur within the 5 d between the initial detection of *C. bovis* at HEM 10 (day 6) until the study's end (day 11). Similarly, during the chronic-infection study, during which more than 10^4 copies per swab was detected for 3 consecutive days at HEM 10, HEM 9 remained negative. Sampling of individual HEM has been proposed as an aid in localizing infections to a particular row of a rack, but no data have been presented to address the potential for cross-contamination between HEM.[9] Our results suggest that sampling individual HEM can differentiate between rows, given that cross-contamination between immediately adjacent HEM did not occur for the short duration of the current study. However, we did not evaluate the potential for cross-contamination between the other 8 HEM on the same rack. In addition, we suspect that cross-contamination between HEM will occur eventually, given that fine particulate material in the plenum is disrupted by opening and closing the access panel door and by physically disturbing the material through sample collection. Nevertheless, for the short duration of this study, the use of HEM sampling was effective in differentiating between 2 adjacent rows, a feature that might aid in localizing the source of infection on the rack.

IVC racks and caging from Allentown Caging were used in our study and in 2 recent studies using HEM sampling to detect mouse parasites.[9,11] The utility of this surveillance method may vary depending on the design of these equipment, which varies by manufacturer. We performed an informal assessment of several types of IVC racks, and in all cases and for all manufacturers, we identified an easily accessible exhaust-air location that could be swabbed to represent the entire rack and that did not compromise the supply-air plenums during sampling. However, a common concern among the rack designs evaluated was the inability to easily access a site that would distinguish the exhaust air from individual rows (or columns) of the rack. To identify the infected cage(s) on a rack, testing individual rows to trace an infection back to a series of cages would be considerably faster and less expensive than would individually sampling all cages. For future IVC rack designs, we encourage rack manufacturers to consider contemporary methods of collecting samples for health surveillance and to provide easily accessible locations for differential sampling of exhaust plenums that do not compromise the delivery of air to the rack.

This study provides the first data demonstrating the effectiveness of HEM sampling for the rapid detection of *C. bovis*. This validated method can be used as a routine surveillance technique for the detection of infection in nude mouse colonies and provides a solid foundation for a comprehensive *C. bovis*

remediation plan, which is needed to eradicate this agent from mouse colonies. From these data and in light of our own experience in using this technique, we suggest performing rack sampling every 7 d for populations of mice that are at risk of infection. Prolonging sampling intervals increases the sensitivity of swab detection, but reports of rapid spread of infection within colonies[4,14] reveal an increased risk of horizontal spread when an infection occurs between distant sampling events. *C. bovis* is not an opportunistic pathogen exclusive to nude mice. Additional studies are needed to determine whether HEM sampling can be used to detect *C. bovis* from other immunodeficient strains that are susceptible to infection.[12,15]

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