# *Corynebacterium bovis* Surveillance and Rapid Detection by Sampling IVC Rack Exhaust Air Manifolds

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## Background and Significance

*Corynebacterium bovis* is an opportunistic infection of nude (*Foxn1*, nu/nu) mouse populations' worldwide. Identified as the causative agent of nude mouse hyperkeratotic dermatitis or "scaly skin disease", *C. bovis* causes clinical illness of short duration followed by what is believed to be life-long subclinical skin colonization. Despite the limited duration of clinical signs, the impact on xenograft tumor development can be significant leading to delayed, slowed, or failed tumor growth. As highlighted in the 2010 National AALAS Meeting Panel Discussion, "Control and Eradication of Corynebacterium-associated Hyperkeratosis (CAH) in Athymic Nude Mice," *C. bovis* plagues both academic and industry research facilities as a bacterial contaminant that is extremely difficult to eliminate.

Modern rodent sentinel monitoring programs that rely on soiled bedding sentinels are not designed for early detection of a rapidly-spread, environmentally stable, and airborne-transmitted bacteria like *C. bovis.* There is a need to establish a method that can reliably be used to more efficiently monitor nude mouse colonies for *C. bovis* beyond soiled bedding sentinels. To address this problem, we proposed monitoring the individually ventilated cage (IVC) rack exhaust air system by PCR for the presence of *C. bovis*. Previous attempts to monitor IVC rack air exhaust for mouse pathogens such as *Helicobacter* spp., murine viruses, and endo- and ectoparasites by PCR have been described with varying success.

The rationale for IVC rack air exhaust system sampling for *C. bovis* detection is based on findings reported by Burr *et al.* 2012 indicating that *C. bovis* is efficiently spread by airborne transmission within air currents of biosafety cabinets. It is believed that *C. bovis* populated skin flakes are distributed by air currents, resulting in airborne transmission. We hypothesized that forced air movement through ventilated cages will effectively carry *C. bovis* contaminated particulate into the IVC exhaust air system for easy diagnostic sampling and rapid detection.

## IVC Rack Air Supply and Exhaust System

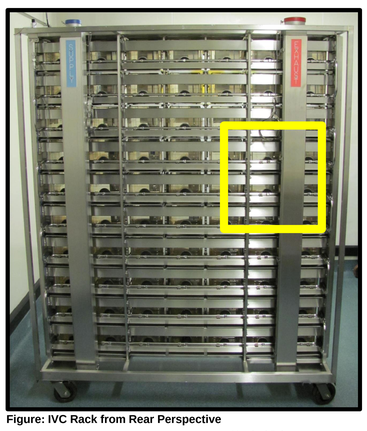


Figure: IVC Rack from Rear Perspective

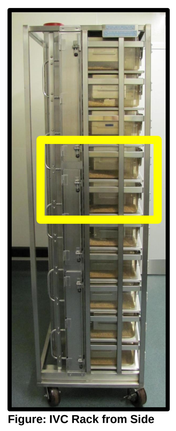


Figure: IVC Rack from Side

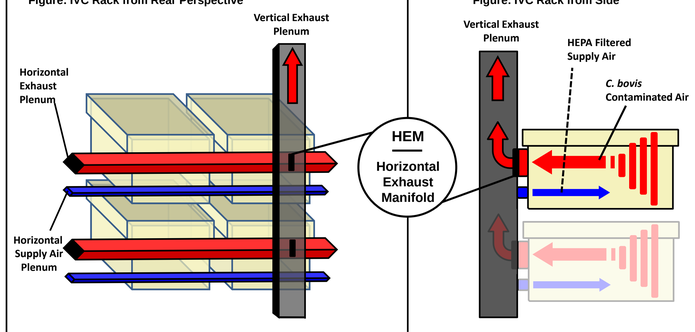



Diagram of the HEM at the junction of the horizontal and vertical exhaust plenums.

**Horizontal Exhaust Manifold (HEM) Sampling:** A sterile, dry, non-sticky, non-flocked swab is introduced into the HEM. All 4 sides of the manifold, from front to rear are sampled. To standardize sampling each HEM is sampled for 5 seconds.

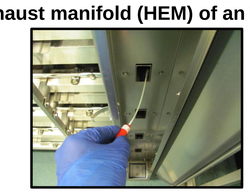
**Why the HEM?** The HEM is a defined location at the union of the vertical and horizontal exhaust plenums. Each row of an IVC rack has a single HEM. Thus, each HEM could be swabbed individually to determine the *C. bovis* status of each row of a rack. Similarly a common swab could be used to sample all HEMs to determine the *C. bovis* status of the entire rack.

## Hypotheses

- **PCR testing of a ventilated caging system's horizontal exhaust manifold (HEM) will rapidly detect a single cage of *C. bovis* infected mice.**
- **The number of *C. bovis* infected mice per cage will not affect the rate of detection of *C. bovis* DNA.**
- **The cage position in relation to the HEM will not affect the rate of detection of *C. bovis* DNA.**

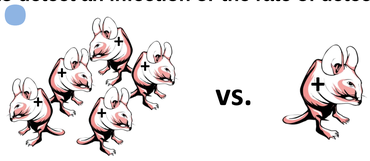
## Experimental Goals

**1. Can *C. bovis*, from infected nude mice, be detected in the horizontal exhaust manifold (HEM) of an IVC rack?**

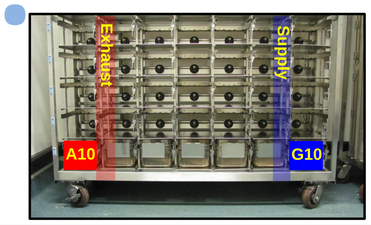


**2. What level of rack sanitation is required to eliminate *C. bovis* DNA from the rack exhaust system?**


**3. Will the number of mice in an infected cage affect the ability to detect an infection or the rate of detection?**




**4. Will cage position on the rack (proximity to the HEM) affect the rate of *C. bovis* detection?**




## Materials and Methods

**Racks, Caging, and Rack Sanitation:** Allentown Inc., seventy cage, single-sided, individually ventilated MicroVent racks and JAG 75 cages were used in all experiments. The rack sanitation process was evaluated for the elimination of *C. bovis* DNA from the HEM of the rack air exhaust system. IVC racks (n = 5) were sampled at the HEM while in-service for 124 ± 49 days using a single, sterile, dry foam swab (Becton Dickinson, BBL™ CultureSwab™ EZ , Ref# 220144). Racks housed cages of nude mice in enzootically infected housing rooms with clinical history of scaly skin disease. All HEMs from each rack were sampled prior to removing the rack for sanitation and then following sanitation by a rack washer and bulk autoclave.

**Animals:** Five, group housed, 6 week old, male, Hsd:Athymic Nude mice (*Foxn1*$^{nu}$) were experimentally exposed to 15 mL of soiled bedding from a nude mouse cage infected with *C. bovis*. Weekly, post inoculation (PI), mice were orally and dermally sampled by a foam swab and serially confirmed to be *C. bovis* positive by qPCR. On day 90 PI, 2 mice were separated from the cage of 5 mice and housed individually. Weekly, oral and dermal sampling continued for singly housed mice.

**Single Cage *C. bovis* Detection at the HEM**: To test the rate of detection of a single cage containing 5 *C. bovis* positive mice, a sanitized, autoclaved, rack was connected to the facility IVC supply and exhaust air system. Prior to *C. bovis* positive cage placement, the HEMs were sampled using a single foam swab and confirmed *C. bovis* negative. The single *C. bovis* positive cage was placed on row 10 of the rack, either in the bottom left cage position A10 (n = 2, closest to the HEM) or in the bottom right cage position G10 (n = 2, furthest from the HEM). Daily for either 4 to 8 days, the HEM from row 10 was swabbed. As an environmental control, the HEM of row 9 of the same rack was swabbed at the end of the sampling period. At the end of the sampling period, swabs were submitted for qPCR detection of *C. bovis* and the rack was re-sanitized, autoclaved and returned for each replicate. At the conclusion of the 5 mice per cage replicates (n = 4), the experiment was repeated with 1 mouse per cage at cage position A10 (n = 2) or G10 (n = 2).

**Air Supply and Exhaust:** Rack ventilation was assessed at the beginning of each replicate with a Allentown rack flow meter and cage flow detector. Rack ventilation remained consistent throughout the study with a supply airflow range of 18.6 – 22 ft$^3$/min and an exhaust airflow range of 31.7 - 33.4 ft$^3$/min, corresponding to approximately 35-40 air changes per hour at the cage level. Air flow rate did not differ between position A10 and G10.

**Quantitative PCR (qPCR):** *C. bovis* primers and probe were designed with Prism 7900 sequence detection software (Primer Express, PE ABI). Primer and probe sequences used: *C. bovis* F 5'-AACGCGAAGAACCTTACCTGG-3'; *C. bovis* R 5'-ACCACCTGTGAACAAGCCCA-3' and the probe 6FAM-GGCAGGACCGGCGTGGAGA-TAMRA. All samples were run on a ABI Prism 7900 sequence detector (Applied Biosystems). Reactions were performed in MicroAmp optical plates in a 20 ul mix containing 1X TaqMan Buffer A, 300 uM each of dATP, dGTP, dCTP and 600uM dUTP, 5.5 mM $MgCl_2$, 900 nM forward primer, 900 nM reverse primer, 200 nM probe, 1.25 U AmpliTaq Gold DNA Polymerase and 10 uL template DNA. Thermal cycling conditions: 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 sec and 60°C for 1 min.

## Experimental Results

| Rack # | Days In Use | *C. bovis* Status In Room (Copy Number) | *C. bovis* Status After Rack Wash (Copy Number) | *C. bovis* Status After Autoclave |
|---|---|---|---|---|
| 1 | 53 | + (56,944) | + (45) | Negative |
| 2 | 172 | + (659,400) | Negative | Negative |
| 3 | 188 | + (482) | + (34) | Negative |
| 4 | 97 | + (279,774) | + (844) | Negative |
| 5 | 127 | + (316,227) | Negative | Negative |
| Summary | 124 ± 49 | 100% Positive | 60% Positive | 0% Positive |

**Rack Sanitation and *C. bovis* Detection Table:** *C. bovis* qPCR results from individual racks (#1-5) that housed *C. bovis* infected nude mouse cages prior to testing. The HEMs of each rack was sampled with a common swab while in the housing room, then again following sanitation by rack washer and after the rack was autoclaved. These results demonstrate that *C. bovis* can be detected at the HEM when infected cages are placed on the rack. In addition, sanitation by rack washer only will not be adequate to completely eliminate *C. bovis* DNA. Autoclaving is required to prevent false positive results from a rack with potential historical exposure to *C. bovis* infected cages.

**5 Mice/Cage - Rapid Detection of *C. bovis***
Figure 1: Mean ± SD *C. bovis* copy number from a pooled sample collected directly from mice's skin was 669,467 ± 462,199 (n = 10, pool 3 of 5 mice). The mean copy number for samples collected from the HEM was 13,787 ± 9,784 (n = 16). With the exception of the first trial (24 hrs not sampled), *C. bovis* was detected 24 hrs after the infected cage was placed on the rack.
Figure 2: With 5 mice/cage, cage placement closest to the HEM in slot A10 demonstrated a significant increase in copy number as compared to cage placement furthest from the HEM in slot G10.

Figure 1

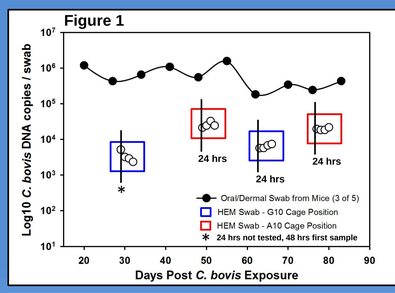
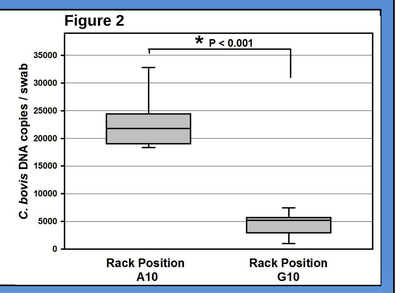



Figure 2




**1 Mouse/Cage - Rapid Detection of *C. bovis***
Figure 3: Mean ± SD *C. bovis* copy number from samples collected directly from the skin of 2 individual mice was 64,385 ± 64,686 (n = 10). The mean copy number for samples collected from the HEM was 502 ± 369 (n = 16). *C. bovis* was detected at the HEM 24 hrs after placement of the cage.
Figure 4: With 1 mouse/cage, cage placement on the rack, either closest (A10) or furthest away (G10) from the HEM did not have a significant impact on the copy number.

Figure 3

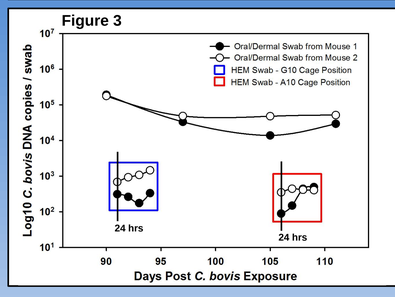



Figure 4

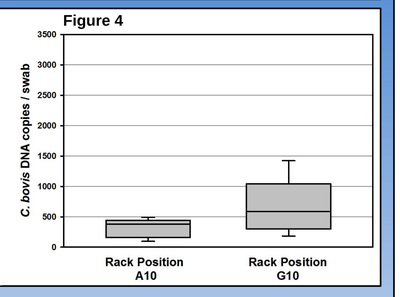
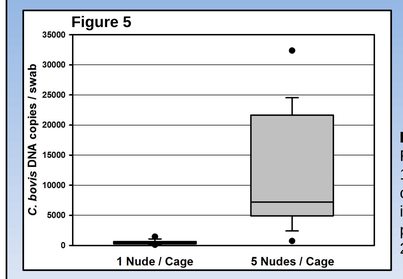



Figure 5




**Impact of the Number of Infected Mice/Cage on Copy Number Detected at the HEM**
Figure 5: The mean ± SD *C. bovis* copy number from HEM samples with 1 or 5 mice/cage was 502 ± 369 and 13,787 ± 9,784 respectively. The number of infected mice per cage demonstrates a marked impact on the quantity of *C. bovis* DNA shed from the cage into the IVC rack air exhaust system. A 5 fold increase in the number of mice in the cage resulted in a 27 fold increase in the *C. bovis* DNA that reached the HEM at identical time points after placement of an infected cage on the rack. Independent of cage density, *C. bovis* DNA was to be reliably detected 24 hrs after placement.

## Conclusions

- **HEM sampling reliably detected *C. bovis* at the HEM from all racks housing naturally and experimentally infected mice.**
- **At 24 hrs, all racks containing a single *C. bovis* positive cage housing either 1 (n = 4) or 5 nude mice (n = 3) were positive by qPCR.**
- **After 24 hrs, all subsequent samples remained positive during the 4-8 day sampling period.**
- **Neither the number of mice per cage nor cage proximity to the HEM affected the rate of detection of *C. bovis*.**
- **The cage position closest to the HEM resulted in a significantly higher copy number of *C. bovis* DNA for trials containing 5 mice/cage only (P < 0.05).**
- ***C. bovis* DNA shedding into the rack air exhaust system is 27x higher in cages containing 5 infected nude mice as compared to cages containing 1 infected nude mouse.**

## Implementation

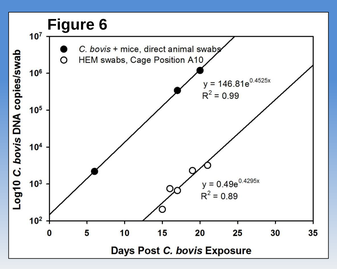
- **At UC Denver, HEM sampling is performed weekly on each rack that houses nude mice in established *C. bovis*–free colonies.**
- **Weekly monitoring for *C. bovis* provides confirmation of health status while concurrently monitoring for a break in *C. bovis*–free barrier rooms.**
- **Routine surveillance for *C. bovis* by HEM sampling must be part of a larger *C. bovis* remediation or management plan.**
- **A *C. bovis* management plan contains a variety of additional facets including:**
  - *C. bovis* positive and negative staffing teams
  - traffic patterns,
  - PPE or facility clothing
  - procedure room management
  - shared equipment management
  - equipment decontamination
  - and many more….

## Future Direction

**Detection of Established vs. Acute Infections:** The data presented above demonstrates the rapid detection of an established infection. Future work will determine the rate of detection of recently acquired infections.

**5 Mice/Cage – "Early Infection"**
Figure 6: Detection of *C. bovis* at the HEM from experimentally exposed mice first performed 15 days post *C. bovis* exposure. A parallel trend from exponential regression lines suggest a correlation between log bacterial growth on the skin of mice and bacteria presence at the HEM. Results suggest that early infection may be less rapidly detected until bacteria skin colonization becomes more abundant.

Figure 6

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**nude mouse artwork by Jane Wang, 2006**

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**ABSTRACT – P14**

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*Corynebacterium bovis* is an opportunistic infection of nude (*Foxn1*, nu/nu) mice that affects many academic and industry research facilities. Once present, it is highly contagious and extremely difficult to eliminate. Moreover, soiled bedding sentinel programs are not designed for the rapid detection required to monitor eradication efforts. To achieve more rapid *C. bovis* detection, swabs were collected from the horizontal exhaust manifold (HEM) of an individually ventilated caging (IVC) system and evaluated by quantitative PCR. First, rack sanitation methods were assessed for their capability to eliminate *C. bovis* DNA. Forty percent of *C. bovis* exposed racks which had housed enzootically infected colonies, tested positive at the HEM following sanitation by rack-wash only (n = 5). After autoclaving, all sanitized racks tested negative for *C. bovis* by qPCR (n = 5). HEM sampling for surveillance based testing requires IVC rack air ventilation, thus rates of supply and exhaust air movement were recorded. Rack ventilation remained consistent throughout the study with a supply airflow range of 18.6 – 22 $ft^3$/min and an exhaust airflow range of 31.7 - 33.4 $ft^3$/min, corresponding to approximately 35-40 air changes per hour at the cage level. To determine the rate of *C. bovis* detection and the infected mouse detection threshold of the HEM sampling technique, a cage containing either 1 or 5 experimentally infected, male, nude mice was placed at the first or last cage position on the bottom row of a 70 cage IVC rack. Sterile, dry swabs were used to sample the corresponding HEM beginning 24 hrs after cage placement. At 24 hrs, all racks containing a single *C. bovis* positive cage housing either 1 (n = 4) or 5 nude mice (n = 3) were positive. All subsequent samples remained positive during the 4-8 day sampling period. Neither the number of mice per cage nor cage proximity to the HEM affected the rate of detection. The cage position closest to the HEM resulted in a significantly higher copy number of *C. bovis* DNA for trials containing 5 mice per cage only ($P < 0.05$). Our findings suggest that HEM sampling is a promising method for routine surveillance and rapid detection of *C. bovis* in nude mouse colonies.

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