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Exhaust Air Dust Monitoring is Superior to Soiled Bedding Sentinels for the Detection of *Pasteurella pneumotropica* in Individually Ventilated Cage Systems

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Reliable detection of unwanted organisms is essential for meaningful health monitoring in experimental animal facilities. Currently, most rodents are housed in IVC systems, which prevent the aerogenic transmission of pathogens between cages. Typically soiled-bedding sentinels (SBS) exposed to soiled bedding collected from a population of animals within an IVC rack are tested as representatives, but infectious agents often go undetected due to inefficient transmission. *Pasteurellaceae* are among the most prevalent bacterial pathogens isolated from experimental mice, and the failure of SBS to detect these bacteria is well established. In this study, we investigated whether analysis of exhaust air dust (EAD) samples by using a sensitive and specific real-time PCR assay is superior to conventional SBS monitoring for the detection of *Pasteurella pneumotropica* (*Pp*) infections. In a rack with a known prevalence of *Pp*-positive mice, weekly EAD sampling was compared with the classic SBS method over 3 mo. In 6 rounds of testing, with a prevalence of 5 infected mice in each of 7 cages in a rack of 63 cages, EAD PCR detected *Pp* at every weekly time point; SBS failed to detect *Pp* in all cases. The minimal prevalence of *Pp*-infected mice required to obtain a reliable positive result by EAD PCR testing was determined to be 1 in 63 cages. Reliable detection of *Pp* was achieved after only 1 wk of exposure. Analysis of EAD samples by real-time PCR assay provides a sensitive, simple, and reliable approach for *Pp* identification in laboratory mice.

Abbreviations: EAD, exhaust-air dust; Pp, Pasteurella pneumotropica; SBS, soiled-bedding sentinel

In recent decades, the use of IVC rack systems in laboratory rodent facilities has increased. Within these microisolation units, each cage is ventilated separately and therefore represents an individual microbiologic unit. If cages are handled appropriately, the spread of infectious agents between cages is prevented. For health monitoring of IVC-housed colonies, quarterly monitoring of sentinels exposed to soiled bedding (SBS) remains the most common method of indirect testing when various factors, such as housing conditions, immunodeficiency of resident animals, or low numbers of animals prevent direct animal sampling.[21] The detection of unwanted organisms by using sentinel mice relies on the sentinels becoming infected with the pathogen, regardless of the testing method used. The major disadvantage of this health monitoring strategy is that not all agents infect sentinels through soiled bedding transfer. Viruses (such as lymphocytic choriomeningitis virus and Sendai virus), bacteria (*Pasteurella pneumotropica* [*Pp*]), and murine fur mites often remain undetected.[1,7,9,15,19,29] The use of contact sentinels or colony animals to improve the detection of unwanted organisms is not always feasible, and health monitoring in IVC-housed rodent colonies remains challenging.

Pp is one of the most prevalent bacterial pathogens in experimental facilities worldwide.[14,27] Although the pathogenicity of most *Pasteurellaceae* species is low, *Pp* is associated with various clinical manifestations, including eye, genital tract, and respiratory infections.[2,22,24] Immunodeficient animals infected with *Pp* develop mild to severe or even lethal disease.[6,12,17,20] Even subclinical *Pp* infections in immunocompetent mice can represent an unwanted experimental variable that can critically influence research data.[26] In Europe, the Federation of European Laboratory Animal Science Associations recommends quarterly screening for this pathogen.[21] However, even when *Pp* was transferred to SBS, its subsequent detection and analysis can be problematic. *Pasteurellaceae* are usually detected through bacterial culture of swabs taken from the nasopharynx, genital tract, or large intestine, with subsequent analysis of suspect colonies by using biochemical test kits. However, the commonly used kits are optimized for human samples and frequently fail to identify rodent samples to the correct species or even family level.[4,13,21] The analysis of subcultured bacterial colonies by matrix-assisted laser desorption–ionization–time-of-flight mass spectrometry has good specificity,[11,32] but the equipment is very expensive, and reference databases lack sufficient murine datasets. Serological tests are inappropriate for the detection of *Pp* infections, because the seroconversion of infected sentinel mice is unreliable.[29] PCR technology is the most reliable and sensitive method currently, and genus- and species-specific assays are available.[3,5,10,18,30] Recently, we demonstrated that the combination of a real-time PCR assay that amplified a variable region in the 16S rRNA sequence with a high-resolution melting curve analysis is sufficient for the identification and differentiation of murine *Pasteurellaceae* species.[23]

Many drawbacks are associated with SBS, but an alternative health monitoring strategy has yet to be developed. Conse-

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quently PCR testing of environmental samples, such as exhaust air dust (EAD) samples from IVC racks, has been proposed.[7] In a previous study, mouse hepatitis virus, Sendai virus, mouse parvovirus,[7] and mouse norovirus[33] were detected in the exhaust-air prefilter, and *Radfordia affinis* and *Myobia musculi* were detected in swabs of the horizontal exhaust plenum.[16] Although *Pp* could not be detected in environmental samples by using conventional PCR,[25] we investigated whether EAD samples are suitable for the detection of *Pp* infections in IVC-housed mice by using a specific and highly sensitive real-time PCR assay.

Here we compared real-time PCR analysis of dust samples taken from the exhaust-air prefilter of an IVC rack containing a known number of *Pp*-infected mice with the conventional SBS health monitoring strategy. We also investigated the minimal prevalence of infected animals necessary for reliable detection of *Pp*.

Materials and Methods

Mice. All mice used were excess mice obtained from breeding and research colonies at our facility. The *Pp*-positive colony consisted of immunocompetent female AVM:ICR mice housed in groups of 5 animals per cage; they were approximately 3 mo old at the beginning of the study. The *Pp*-negative colony consisted of immunocompetent mice of both sexes and various strains, ages, and genetic backgrounds, to simulate a typical research mouse colony. Mice were housed in sex-matched groups of 2 to 5 animals per cage; in exceptional cases, single animals were housed separately for a short time. Female AVM:ICR mice (age, 3 mo) housed in groups of 3 animals per cage were used as *Pp*-negative bedding sentinels. The *Pp*-positive colony and the sentinel mice were obtained from a breeding colony in which no FELASA-listed agents[21] were detected during routine health monitoring surveillances. The *Pp*-negative colony was obtained from a research colony with a known history for *Helicobacter* spp. and *Pseudomonas aeruginosa.* All experiments and animal housing were performed in strict accordance with the directive 2010/63/EU.

Animal housing. All animals were housed in an IVC rack (SealSafe Plus, Tecniplast, Buggugiate, Italy) under SPF conditions in IVC cages (GM 500, Tecniplast), with a maximal cage density of 5 adult mice per cage (socially housed in stable groups of compatible animals; 100 cm^2 floor area per mouse) and environmental conditions (12:12-h light:dark cycle, 20 to 24 °C, 45% to 65% humidity) according to directive 2010/63/EU. Routine health monitoring surveillance was performed for all agents listed in the FELASA recommendations[21] with the recommended quarterly and annual tests via SBS.[21] Air-handling units were run with 60 air changes per hour in positive pressure mode (15 to 22 Pa). Autoclaved wood fiber (Lignocel 3/4 S, J Rettenmaier and Söhne, Rosenberg, Germany) and nesting material (Crinkle Nature, J Rettenmaier and Söhne) were used as bedding and environmental enrichment. Sterile-filtered tap water and an irradiated standard diet for rodents (Altromin 1314, Altromin Spezialfutter, Lage, Germany) were available without restriction. Access to the animal room was gained through an air shower, and all personnel entering the room performed a complete change of clothes and shoes, and wore gloves, surgical masks, and bonnets the entire time. All cage changes and sample collections were performed in a HEPA-filtered cage-changing station. All cages and racks were cleaned and autoclaved prior to use.

Sample types and collection. Several sample collection methods were used to monitor the *Pp*-positive and -negative colonies, the sentinels, and the EAD. For detection of *Pp* by using oral swabs, the mice were scruffed at the neck, and a sterile flocked swab (FLOQSwabs Mini Tip 80 mm, Mast Diagnostica, Reinfeld, Germany) was gently inserted approximately 1 cm into the oral cavity, rotated, and quickly removed. The flocked tip of the swab was inserted into a 2-mL microcentrifuge tube for further analysis. For detection of *Pp* in feces, pooled samples of 10 fecal pellets per cage or single fecal pellets from individual sentinel mice were collected by using disposable forceps and processed as described later. Once each week, approximately 10 mL soiled bedding was collected from every cage in use (49 cages for the *Pp*-negative colony; 7 cages for the *Pp*-positive colony) during routine cage changing. The sentinel cage was filled with the soiled bedding mixed with an equal amount of fresh bedding. Two sample types were used for analysis of EAD: (1) autoclaved gauze pieces (2 × 2 cm; ES Kompressen, Paul Hartmann, Heidenheim, Germany) attached to the 'dirty side' of the exhaust air prefilter directly above the exhaust-air hose opening and (2) 2 × 2-cm samples cut from the prefilter by using clean and disinfected scissors and forceps (Figure 1).

SBS were tested by using real-time PCR analysis of fecal samples and oral swabs, as well as by bacteriological culture. For bacteriologic examination, a Columbia blood agar plate containing 5% sheep blood (VWR International, Darmstadt, Germany) was inoculated with an oral swab immediately after sampling. Plates were incubated for 24 h at 37 °C. *Pasteurella*-like colonies were picked and analyzed by using a multiplex PCR assay based on the 16S-23S rRNA internal transcribed spacer region that can be used to differentiate between the *Pp* biotypes Jawetz and Heyl as well as *Actinobacillus muris* and other *Pasteurellaceae*.[3] For serologic examination, serum samples of *Pp*-positive mice and sentinels were analyzed by an external diagnostic laboratory. Two sentinel animals were examined for all FELASA-listed organisms[21] at the end of the exposure time.

DNA extraction. DNA isolation from oral swabs, gauze pieces, and prefilter material was performed by using the phenol–chloroform extraction method[28] with minor modifications: sample material was incubated with lysis buffer (10 mM EDTA, 10 mM Tris-HCl pH 7.6, 0.5% SDS, 10 mM NaCl, and 0.3 mg/mL Proteinase K) for 30 min at 55 °C and shaken at 600 rpm by using a thermomixer. For oral swabs, gauze pieces, and prefilter materials, 400 µL, 500 µL, and 650 µL, respectively, of lysis buffer was used. After incubation, 300 µL of lysis mixture was transferred into a 1.5-mL microcentrifuge tube. An equal volume of phenol–chloroform–isoamyl alcohol solution was added, and the mixture was vortexed. After being centrifuged for 6 min at 15,300 × *g*, 200 µL of the upper aqueous phase was transferred to a new tube containing 500 µL of NaCl-saturated 100% ethanol and centrifuged for 10 min at 15,300 × *g* and 4 °C for precipitation. The DNA pellet was washed twice with 70% ethanol and subsequently dissolved in 35 µL of ultrapure water. DNA isolation of fecal samples was performed by using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) with the following amendments: after pipetting 1 mL of InhibitEX buffer onto the fecal pellets, a 7-mm stainless steel bead (Qiagen) was added. The contents were ground for 5 min at 50 Hz by using a TissueLyser LT (Qiagen). After heating the suspension for 5 min at 70 °C and centrifuging it for 1 min at 15,300 × *g*, 200 µL of supernatant was collected and processed according to the manufacturer's instructions.

Real-time PCR. A *Pp* Jawetz-specific real-time PCR assay[10] for amplification of a sequence within the 16S rRNA gene (forward primer, 5' CGG GTT GTA AAG TTC TTT CGG T 3'; reverse primer, 5' GGA GTT AGC CGG TGC TTC TTC 3') was performed by using a 6-FAM-BHQ-1 dual-labeled fluorogenic

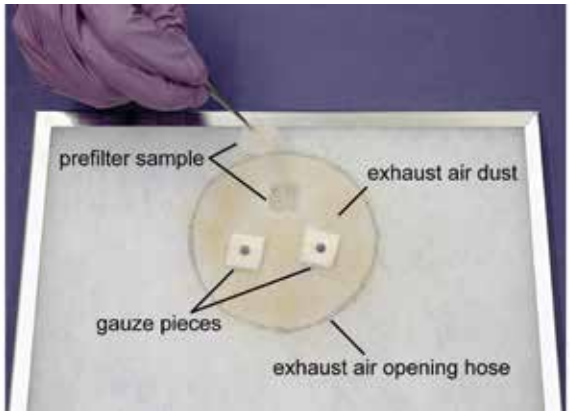

Figure 1. The 2 methods for exhaust air dust (EAD) sampling of the IVC exhaust-air prefilter. The black circle indicates the position of the exhaust-air hose relative to the prefilter.

probe (5′ AAT AAG GGT ATT AAC CTT ATC ACC TTC CTC ATC 3′) in a RotorGene Q instrument (Qiagen). DNA template (2 µL) was added to a reaction mixture consisting of 5 µL of 5× HOT FIREPol Probe qPCR Mix Plus (Solis BioDyne, Tartu, Estonia), 300 nM of each primer, 200 nM probe, and ultrapure water in a 25-µL total reaction volume. The DNA extracted was not quantified prior to application. The thermocycling parameters were: initial denaturation at 95 °C for 15 min, and 40 cycles with denaturation at 95 °C for 15 s and annealing–extension at 58 °C for 60 s. For quantitative analysis, a plasmid was constructed by cloning the 93-bp real-time PCR amplicon into the pCRII-TOPO vector (Invitrogen Life Technologies, Waltham, MA) according to the manufacturer's instructions. A triplicate plasmid DNA dilution series, ranging from 1×10^7 to 1×10^2 copies/µL, was used as a standard in each run. To identify contaminants in the reaction mixture, no-template, positive, and negative controls were included. Samples that yielded at least 10 copies/µL were judged to be *Pp*-positive; samples with fewer than 10 copies/µL were considered equivocal and retested; these samples were considered positive when the copy number was greater than 0 after retesting. Other samples were considered negative. Data were analyzed by using RotorGene Q Software (version 2.1, Qiagen).

Samples of the 4 different matrix types (fecal pellet, prefilter material, gauze piece, and oral swab) were spiked with *Pp* DNA isolated directly from a *Pp* Jawetz bacterial colony to confirm that the matrix did not inhibit the analysis (Figure 2). Different DNA extraction protocols were used for each matrix as previously described. The initial amount of *Pp* Jawetz DNA for the spike was individually calculated for the different sample types to obtain a final DNA concentration of 1 ng/µL after DNA extraction. PCR analysis was performed by including a positive control (2 µL of 1 ng/µl DNA isolated from the *Pp* Jawetz bacterial colony), a no-template control, and the 4 spiked sample types. PCR products were analyzed by using the QIAxcel Advanced System (Qiagen), which is based on capillary electrophoresis, and the QIAxcel DNA Fast Analysis Kit (Qiagen). *Pp* DNA was PCR-amplified from all sample types after spiking negative samples (fecal pellet, prefilter material, gauze piece, and oral swab) with DNA isolated from a *Pp* Jawetz bacterial colony. Positive (DNA isolated from the *Pp* colony) and negative controls were included.

Study designs. To determine the time needed for *Pp* to spread from an infected to a noninfected mouse within the same cage (that is, contact infection), we set up 4 cages each containing one *Pp*-positive mouse and 2 *Pp*-negative mice. Mice were tested

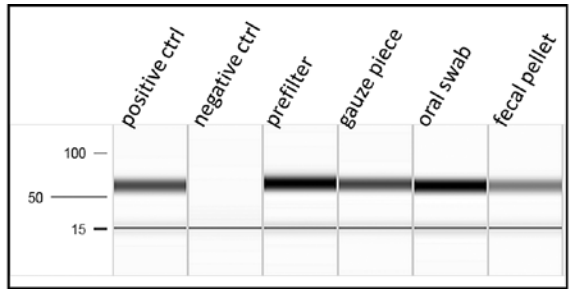

Figure 2. Capillary electrophoresis of PCR products from different types of sampling matrix spiked with *Pasteurella pneumotropica* Jawetz DNA prior to DNA extraction.

by using oral swabs immediately before grouping and every 3 d for a total of 21 d.

To compare EAD analysis and SBS monitoring, a cleaned and autoclaved IVC rack connected to a clean and disinfected (Purpsept-FD, Merz Hygiene, Frankfurt am Main, Germany) air-handling unit was populated with the *Pp*-negative colony. The *Pp*-negative status of the colony was confirmed by testing one mouse per cage during the baseline testing week at the beginning of each experimental round. Single mice were considered sufficient, given that our contact infection study revealed that a *Pp* infection spreads rapidly within a single cage. All mice were tested in rotation in subsequent rounds to verify the status of each individual mouse at least once during the experiment. Old or sick mice of the *Pp*-negative colony underwent veterinary examination and were replaced with new *Pp*-negative mice as necessary. All sick mice were confirmed by oral swab testing to be *Pp*-negative to exclude that clinical signs are caused by *Pp* infection. One horizontal row of the IVC rack was equipped with empty cages to provide space for sentinel mice. Three sentinel mice (2 for health monitoring surveillance and one backup) were determined to be free of *Pp* by testing of oral swabs and single fecal samples during the baseline week. After the IVC rack system was populated with the *Pp*-negative colony, a gauze piece was attached directly above the opening of the exhaust-air hose on a new exhaust air prefilter in the air-handling unit (Figure 1). This gauze piece and the prefilter were removed and analyzed after the baseline testing week, to exclude any remaining DNA contamination within the IVC rack system and the air-handling unit and to confirm the negative infectious status of the negative colony. Afterward, a new prefilter containing 2 new gauze pieces was applied. At the beginning of the test period, a *Pp*-positive colony consisting of 7 cages, each containing 5 positive mice, and the sentinel mice were placed in the rack. To confirm infection of the positive mice, the entire positive colony was tested every second week by using individual oral swabs and pooled fecal samples from each cage. Six experimental rounds were determined to be necessary to produce statistically relevant results. An optimal hygienic monitoring period lasts 10 to 12 wk to provide sufficient time for infection and seroconversion to various agents, such as *Mycoplasma pulmonis* and *Pp* [21]. Therefore, sentinel mice were retested by using individual oral swabs and individual fecal samples after 6 and 12 wk of exposure to soiled bedding; after 12 wk of exposure, serologic, bacteriologic, and complete health monitoring surveillance according to FELASA recommendations was performed.

For EAD analysis, a gauze piece was collected each week and a prefilter sample was collected every 2 wk. EAD testing was considered positive and the experimental round was stopped when dust samples yielded positive results 2 wk in a row. This experiment was repeated 5 times by using cleaned and autoclaved IVC racks (Figure 3). Each experimental round

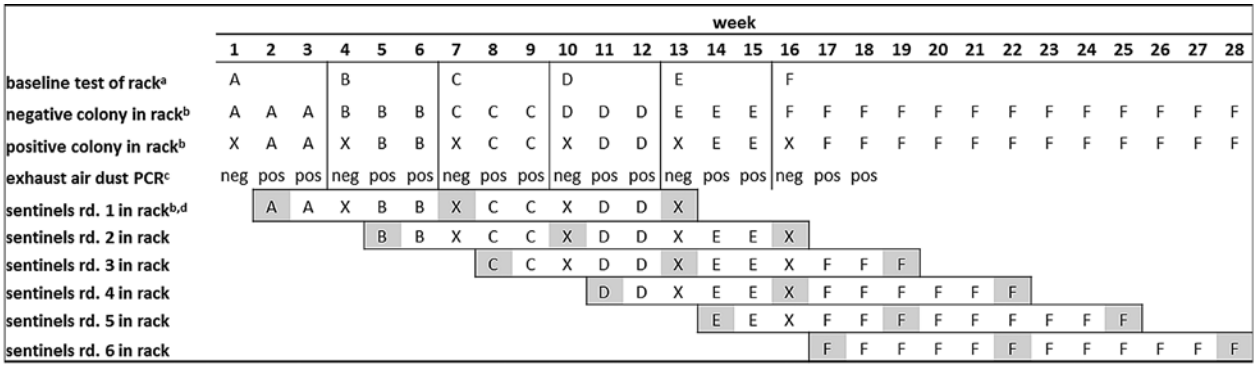

Figure 3. Experimental design for comparing EAD analysis with sentinel monitoring. Letters A through F indicate different IVC racks. Each new round of experimentation began with a clean and autoclaved IVC rack, after detection of 2 positive EAD samples in the previous round; X indicates an IVC rack where *Pp*-positive and sentinel mice were held during the baseline test week. [a], Samples for baseline testing were collected after the rack (containing the *Pp*-negative colony) operated for 1 wk; the Pp-positive colony and the sentinel mice were then placed in experimental IVC rack. [b], Localization of *Pp*-positive and - negative as well as sentinel mice. [c], Real-time PCR results of EAD sample. [d], Sentinel mice in all 6 rounds were exposed to soiled bedding over a period of 12 wk. During baseline testing weeks, the sentinel mice were transferred to and held in rack X and further exposed to soiled bedding from the *Pp*-positive and -negative colonies; highlighted letters indicate sentinel testing (immediately before placement in the experimental rack, as well as after 6 and 12 wk of exposure time). *Pp*, *Pasteurella pneumotropica*; EAD, exhaust air dust; rd, round; pos, positive EAD real-time PCR result; neg, negative EAD real-time PCR result; bar, rack change.

consisted of an initial baseline testing period, to confirm that the negative colony and the IVC were negative for *Pp*, and a testing period, during which the rack was repopulated with the positive colony. All sentinels were housed for 12 wk on soiled bedding derived from all cages, including the positive colony, even when the positive colony was located in another rack during the baseline test weeks. At the end of the 6 rounds, blood samples for serologic examination were collected from 12 mice from the *Pp*-positive colony, which had tested positive by repeated oral swab and fecal analysis over a 6-mo period.

To investigate the minimal prevalence of infected animals that is necessary for reliable detection of *Pp* in the EAD, we used decreasing numbers of *Pp*-positive mice within a rack of 63 cages otherwise completely filled with *Pp*-negative cages. Three rounds of dust testing with 4, 2, and 1 cage, each occupied by 5 *Pp*-positive mice, were performed as described previously but without sentinel mice. As previously, the experimental round was terminated when we obtained 2 consecutive positive dust samples. At the end of the experiment, the entire negative colony was retested by using oral swabs to exclude *Pp* transmission within the rack during the experiment.

Results

Rapidity and duration of *P. pneumotropica* transmission by direct contact. To better understand the speed of transmission from infected to uninfected mice, the duration and intensity of the infection, and the duration and intensity of bacterial colonization of the nasopharynx, we cohoused *Pp*-infected mouse with 2 *Pp*-negative mice in a single cage, in a total of 4 cages (Table 1). At the beginning of the experiment, the infection status of all mice was confirmed by PCR analysis of oral swabs. Additional oral swabs were PCR-analyzed every 3 d for a total of 21 d. One animal with no detectable *Pp* infection had to be euthanized after 3 d due to emaciation and poor general condition. All initially negative mice tested *Pp*-positive between days 3 and 12; additional testing showed that *Pp* was continuously detectable in all mice until day 21 (Table 1).

Comparison of PCR analysis of EAD with SBS for detecting *Pp* infections in IVC-housed mouse colonies. Sentinels were exposed to soiled bedding from a colony with known *Pp* prevalence for a total of 12 wk. To minimize the time required, each experimental round was terminated and a new round started when 2 consecutive positive EAD results were obtained. However, the sentinels from each experimental round were retained until they had been exposed to soiled bedding for a total of 12 wk (Figure 3). After baseline EAD testing of rack A and the negative colony during week 1, the positive colony (35 mice in 7 cages) and the SBS (3 mice in one cage) for round 1 were transferred from the holding rack (rack X) to rack A at the beginning of week 2 (start of round 1). After positive EAD results were obtained at the end of weeks 2 and 3, the negative colony (49 cages containing the *Pp*-negative mice) was transferred to a fresh rack (rack B). Baseline testing for round 2 was performed in the new rack at the end of week 4. The positive colony and the sentinels were held in rack X and returned to rack B during week 5, together with the sentinels for round 2, after baseline testing. Therefore, during weeks 5 to 6, sentinels from rounds 1 and round 2 were placed in rack B. After 2 positive EAD results were

Table 1. *Pasteurella pneumotropica* infection status of initially negative mice during 21 d of contact with infected cage mates

Cage	Mouse	Infection status[a]	Real-time PCR results on day						
			3	6	9	12	15	18	21
1	1	–	–	nt	nt	nt	nt	nt	nt
	2	–	–	–	–	+	+	+	+
	3	+	+	+	+	+	+	+	+
2	1	–	–	+	+	+	+	+	+
	2	–	–	–	–	+	+	+	+
	3	+	+	+	+	+	+	+	+
3	1	–	+	+	+	+	+	+	+
	2	–	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+	+
4	1	–	–	–	+	+	+	+	+
	2	–	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+	+

–, negative real-time PCR result; +, positive real-time PCR result; nt, not tested (mouse euthanized)
[a]Infection status, as determined immediately before grouping

obtained during weeks 5 and 6, EAD testing of round 2 was terminated and round 3 was prepared, and so on. This pattern was completed a total of 6 times. EAD testing for round 6 was terminated in rack F during week 18. The SBS for round 1 were taken from rack D during week 13 for terminal testing, and the SBS from experimental round 6 remained in rack F until week 28. No cross-contamination occurred between the infected and noninfected colonies. The negative mice were confirmed to be free of *Pp* infection by the results of real-time PCR of all oral swabs tested throughout the experimental period.

The positive mice showed continuous colonization of the oral cavity, as determined by biweekly oral swab analysis of 33 of the 35 infected mice over 1 y. Only 2 infected mice tested negative, at one time point for 1 or 3 biweekly tests. These spurious results might reflect technical problems during DNA extraction (for example, loss of the DNA pellet during DNA isolation procedure) or intermittent shedding of *Pasteurellaceae*. All pooled fecal samples from cages containing infected mice (10 pellets per cage) tested every 2 wk yielded positive PCR results at all time points. Prior to the beginning of each experimental round, the experimental racks (A through F) containing the negative colony were operated for 1 wk for baseline testing. The absence of residual *Pp* DNA in the washed and autoclaved IVC racks, their pipes, and fresh IVC prefilters as well as the negative infection status of the negative colony was verified by testing gauze pieces and prefilter material at the end of the baseline week. All samples taken for baseline testing were negative at all times. After the baseline tests, the experimental rack was populated with the positive colony and the sentinels. Gauze pieces attached to the prefilter were tested weekly and prefilter samples were tested biweekly, to detect *Pp* DNA in the EAD. In all 6 rounds, *Pp* could be detected after 1 wk; positive results were confirmed during the subsequent week in all 6 rounds. For SBS monitoring, a cage occupied by 3 sentinel mice was added to racks A through F after baseline testing in each experimental round. Care was taken that all sentinels were continuously exposed to soiled bedding from the experimental colony with a known prevalence of *Pp* over 12 wk. In addition, all sentinels were exposed to *Pp*-positive soiled bedding when kept in rack X during baseline testing. All 3 SBS in a cage were tested after 6 and 12 wk of exposure by using oral swabs and single-animal fecal samples. All sentinel mice tested negative at all times. To confirm these results, *Pp* serology, bacterial culture of an oral swab, and complete FELASA health monitoring were performed for 2 SBS in each round at the end of the exposure time. In total, 3 animals tested positive for *Helicobacter hepaticus*, but all other animals were negative for all FELASA-listed organisms. PCR analysis of oral swabs and feces from the *Pp*-positive colony showed continuous colonization of the oral cavity and excretion in the feces, respectively, for 6 mo. The blood samples taken at the end of the study showed that all 12 of these mice were seronegative.

Minimal prevalence of *Pp*-infected mice for reliable detection by EAD PCR analysis. After successful detection of *Pp* by EAD PCR at a prevalence of 7 of 63 cages in a rack within 1 wk of exposure, we investigated the minimal prevalence necessary for reliable detection. The experimental setup was the same as that in the previous experiment (except without sentinel mice), but the number of cages containing *Pp*-positive mice was reduced from 4 to 2 to 1 during 3 rounds of testing. Again, a new round was started after 2 consecutive positive EAD results were obtained. In all instances, even with a prevalence of just 1 in 63 cages per rack, the presence of *Pp*-positive mice was detected during the first week and confirmed in the second week. Again, the *Pp*-negative colony, remained negative according to oral swab PCR testing throughout the entire experimental period.

Discussion

Accurate data on the health status of laboratory animals is crucial for their use in research projects. Undetected subclinical infection by unwanted organisms represents an uncontrolled variable that might interfere with the results of animal experiments.[2,8,31] As discussed previously, the standard procedures for the detection of *Pp* in mouse colonies can be nonspecific or produce false-negative results. As a consequence, *Pp* infections are frequently overlooked or reported incorrectly.

Analysis of EAD samples repeatedly and reliably detected *Pp* infections even at the very low prevalence of one cage of infected mice in an IVC rack. In contrast, the use of SBS failed to detect *Pp* infections even at a prevalence of 7 cages in a rack. None of the sentinel mice in our study became infected with *Pp* at any time. None of the methods used to test sentinels—real-time PCR of oral swabs and fecal samples, bacterial culture of oral swabs, and serology—detected infection of the sentinel mice. Although mice easily become infected with *Pp* by direct contact and display continuous colonization of the oral cavity and gut, as shown in the current study, exposure to soiled bedding seems insufficient to infect sentinel mice. Because all pooled fecal samples from cages of infected mice showed positive results over a period of 6 mo, infected mice likely continuously shed bacterial nucleic acids with their feces. Given that the viability of *Pasteurellaceae* on wood bedding is limited to 30 min,[29] live bacteria likely never reached the sentinel cage, because cage changing and the preparation of soiled bedding takes more than 30 min. Alternatively transferred bacteria might have lost their infectivity or an infectious dose may not have been transferred, resulting in lack of infection of the sentinel animals with *Pp.*

The *Pp*-negative colony was tested by using pooled fecal samples from each cage as well as oral swabs collected from one mouse per cage at the beginning of the experiment, between rounds 1 and 6 and after round 6 for a period of 6 mo. Testing only one animal per cage (as a representative of all cage inhabitants) by oral swab seemed reasonable given that we demonstrated that cagemates became infected within 3 to 12 d of cohabitation.

Contrary to our findings, a previous study[25] failed to detect the presence of *Pp* on swab samples taken from a ventilated rack housing infected animals. In addition, analysis of swab samples taken from cages and laminar flow hoods, as well as analysis of the inner surface of the prefilter, failed to detect bacterial DNA.[25] Several reasons might explain these differences. In contrast to the cited study, we used a specific and highly sensitive real-time PCR method that allows detection of *Pp* nucleic acids at a much lower copy number. No detailed information about the rack model or air handling unit and air changes per hour was provided in the previous study;[25] perhaps the prefilter was not the optimal sampling site in that particular rack system. Moreover, the analysis of gauze pieces or prefilter material itself might result in a higher copy number than swab samples, due to the higher amount of dust. Differences in the preparation of DNA from dust might also be a reason for lower sensitivity in the previous study.[25]

One of the key factors in EAD analysis is choosing an optimal sampling site. In our experiments, the dust was collected by using gauze pieces attached to the 'dirty side' of the exhaust-air prefilter directly above the exhaust-air hose opening. Thus, the collection gauze was in an optimal position to collect dust particles potentially contaminated with *Pp* nucleic acids. In

addition, we analyzed prefilter samples collected from the same position. At this location, soiled prefilters showed visible quantities of dust evenly distributed over the surface at the end of the regular exchange interval of 4 wk. For the IVC system used in our studies, we therefore regard the exhaust air prefilter as an appropriate sampling site. However, the preferred position for EAD sampling within a rack must be determined for each different IVC rack system, because airflow direction within the rack can vary. The terminal vertical exhaust plenum in another rack system, for example, was demonstrated to be an unreliable sampling site for the detection of fur mites, whereas sampling at the shelf exhaust manifold allowed detection of a single infested cage.[16] When the number of infected animals is low, as is often the case in endemic infection with agents of low transmissibility, the effectiveness of EAD analysis of gauze pieces attached to the rack prefilter is likely to be highly dependent on the uniformity of airflow within the rack. Unbalanced airflow might lead to inaccurate sampling of dust from cages occupied by infected rodents.[7]

The presence of residual DNA from the previous experimental round within the rack, tubes, or air handling unit, which would lead to false-positive results, was excluded by baseline testing. A fresh, cleaned, and autoclaved rack was used for each round of experimentation. When using EAD PCR for hygienic monitoring, residual nucleic acids, which pose a risk of generating false-positive results, might constitute a major drawback. Discriminating between previous and current infections might not be possible. We regard EAD testing as a highly sensitive screening method that permits surveillance of agent-free colonies. Therefore, a positive result, when obtained for the first time, would cause concern and prompt further confirmation and possibly an elimination strategy. A possible strategy might include pooled samples (for example, oral swabs, fecal samples) to trace infections stepwise down to individual cages or involve removing mice from positive cages and repeatedly retesting. Once a colony has an established agent-positive status, verifying the absence of the unwanted agent after an elimination attempt requires careful sanitizing of the equipment to avoid contamination with residual living or dead infectious agents or residual nucleic acids. We recommend washing and autoclaving the contaminated rack and then running it for several days before performing a baseline test and adding any cages to the system. In our studies, the infectious status of the *Pp*-negative colony had already been verified, and therefore these cages were included in the baseline test. Changing the prefilter only might not ensure complete elimination of residual contamination and could lead to false-positive results. When using EAD samples for health monitoring of ongoing endemic infections, IVC racks should be washed (for example, in a rack washer) and autoclaved on a regular basis.

Use of EAD PCR for screening makes it possible to monitor entire experimental animal facilities efficiently by using reasonable sample numbers. Because the exhaust-air prefilter in IVC systems must be changed regularly, collection of prefilter material for PCR analysis requires only minimal additional work. Recently, 2 suppliers offer updated IVC rack systems that provide system-optimized sampling devices to enable contamination-free recovery of EAD samples from the optimal location; other suppliers likely will follow soon.

EAD sampling has the potential to constitute a key contribution to the basic 3Rs principles regarding the use of laboratory animals. The associated improvement in *Pp* detection leads to increased knowledge regarding animal health status, might reduce the number of experimental animals needed, and represents a technologic refinement given that mice no longer have to be manipulated for oral swabbing or disturbed for fecal collection. This simple and low-cost sampling method has great potential to become a useful tool for primary health monitoring and surveillance of rodent populations. Further experiments are needed to investigate whether EAD analysis can be adapted for use for other agents, as it has been for mouse hepatitis virus, mouse parvovirus, *Helicobacter muridarum*,[7] mouse norovirus,[33] and murine fur mites.[16]

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