Journal of the American Association for Laboratory Animal Science
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Vol 53, No 6
November 2014
Pages 717–724

False-Positive Results after Environmental Pinworm PCR Testing due to Rhabditid Nematodes in Corncob Bedding

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Modern rodent colonies are housed in individually ventilated cages to protect the animals from contamination with adventitious pathogens. Standard health monitoring through soiled-bedding sentinels does not always detect infections, especially in the context of low pathogen prevalence. Recently proposed alternatives include analyzing environmental samples from the cages or rack exhaust by PCR to improve the detection of rodent pathogens but optimal sampling strategies have not yet been established for different microorganisms. Although generally very sensitive and specific, these molecular assays are not foolproof and subject to false-positive and –negative results and should always be interpreted cautiously with an overall understanding of the intrinsic controls and all the variables that may affect the results. Here, we report a limited *Aspiculuris tetraptera* outbreak in a mouse barrier facility that was detected by fecal PCR in sentinels and confirmed by fecal flotation and direct cecal examination of both sentinels and colony animals. The outbreak led to a widespread survey of all facilities for pinworms by using environmental PCR from ventilated rack exhaust plenums. Environmental PCR suggested an unexpected widespread contamination of all ventilated racks holding nonautoclaved cages, but results could not be confirmed in sentinel or colony animals by fecal flotation, cecal and colonic examination, or cage PCR testing. After additional investigation, the unexpected environmental PCR results were confirmed as false-positive findings due to the nonspecificity of the assay, leading to the amplification of rhabditid nematodes, which are not infectious in rodents but which contaminated the corncob bedding.

Rodents in modern biomedical research must remain free of adventitious pathogens that may interfere with research variables or compromise animal health or staff safety. Valuable research colonies are often housed in barrier facilities and IVC under strict biosecurity programs to prevent, contain, and eradicate potential infections.[31] In addition, colony health is monitored by health surveillance programs that rely on periodic testing of sentinels exposed to soiled bedding to detect the presence of pathogens. Over the years, comprehensive biosecurity and health surveillance programs have facilitated the eradication of several infectious agents causing clinical disease and greatly decreased the prevalence of adventitious pathogens that interfere with research variables.[3,30] In this context, the interpretation of health monitoring results can be challenging. Indeed, the predictive value of an unexpected positive result is low, and positive findings should therefore be confirmed through alternative methods before implementing costly containment and eradication measures that may encumber research programs.[31]

Health surveillance programs using soiled-bedding sentinels are not always efficient and reliable in revealing the presence of infectious agents in rodent colonies, especially those housed in microisolation cages.[5,22] New molecular techniques like PCR have been developed to improve the detection of pathogens and are now being advocated as an adjunct to traditional sentinel monitoring.[22,25,30] For some agents, PCR assays can be more sensitive than are traditional methods used to survey pathogens in sentinels and are commonly used to confirm pathogen-positive results from sentinels or detect infectious agents in mice undergoing quarantine. In addition, recent studies focusing mostly on fur mites[22] suggest that environmental PCR may be useful in assessing and managing pathogen outbreaks, but optimal environmental sampling strategies have not yet been established for different microorganisms. Using cage-specific PCR requires large sampling sizes to be representative of the entire colony and can be costly and unrewarding, especially when the expected prevalence is low. Another strategy is to take advantage of the sensitivity of PCR assays to sample areas where agent-laden particles accumulate in high concentrations, such as exhaust filters or plenums of ventilated racks, to detect pathogen contamination at the rack level.[30] However, environmental PCR tests may yield false-positive results by amplifying nonspecific DNA sequences or identifying genetic material in the absence of infectious pathogen. As such, pathogen-positive results on environmental PCR should be confirmed in the animals being assayed.

Syphacia obvelata and *Aspiculuris tetraptera* are the 2 most common pinworms in mice. Infection is normally asymptomatic in immunocompetent mice but may interfere with research by modulating the immune system as well as affecting behavioral assays and growth.[28] Both have a direct life cycle and are transmitted through the ingestion of embryonated eggs. *A. tetraptera* reside in the colon, and female worms migrate to the distal colon to deposit eggs, which are excreted in the mucus layer of feces and become infective after 5 to 8 d. Adult worms of the genus *Syphacia* reside in the cecum or anterior colon and migrate to the anus to lay eggs in the perianal region of the host. Although the persistence of pinworm eggs in the environment is controversial, they are believed to be resistant for several weeks outside their host.[15,17,28] The 'gold standard' for the diagnosis of pinworms in mice is direct examination of the cecum and colon for adult worms in necropsy, but this practice requires euthanasia of the

Received: 01 Apr 2014. Revision requested: 15 Apr 2014. Accepted: 23 Apr 2014.
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animal.[15] Antemortem diagnosis relies on the identification of typical eggs on fecal floats (*A. tetraptera*) or perianal tape tests (*Syphacia*). Although soiled bedding efficiently transfers pinworm eggs, using standard diagnostic tools may fail to detect pinworms (especially *A. tetraptera*) in sentinels. Recent studies[15] suggest that fecal PCR testing is a more sensitive method for the antemortem detection of pinworm in mice and that a combination of both PCR and direct cecal and colonic examination is the most effective screening strategy in sentinels.

Here, we describe a limited *A. tetraptera* outbreak in a mouse barrier facility that was detected by fecal PCR testing of sentinels and confirmed by fecal flotation and direct cecal examination in both sentinels and colony animals. The outbreak led to a widespread survey of all facilities for pinworms by using environmental PCR testing of ventilated rack exhaust plenums. Environmental PCR results suggested an unexpected widespread contamination of all ventilated racks holding nonautoclaved cages but not in autoclaved caging racks. Positive results from nonautoclaved caging racks could not be confirmed in sentinel or colony animal by fecal flotation, cecal and colon examination, or cage PCR testing. After further investigation, the unexpected environmental PCR results were confirmed to be false-positive findings due to the nonspecificity of the PCR assay and the amplification of nematodes of the family Rhabditidae, which are not infectious in rodents but which contaminated the corncob bedding.

Materials and Methods

The Salk Institute Animal Care and Use program is AAALAC-accredited. All procedures are performed in compliance with the *Guide for the Care and Use of Laboratory Animals*[21] and approved by the Salk IACUC. The Animal Resources Department at the Salk operates 2 mouse barrier facilities (SAF and TG) and a multispecies conventional facility (CRAF), with an average daily census of approximately 14,000 mouse cages. Biosecurity, operation, husbandry, and health-monitoring programs are standardized in all facilities and regularly optimized according to contemporary standards to minimize potential outbreaks from adventitious pathogens and maintain a SPF environment. Briefly, access to the facilities is restricted and granted only after completion of a stringent training program. Traffic flow, including room and facility order, is strictly regulated and full PPE (sterile gown, shoe covers, hair bonnet, gloves, and an optional face mask) is required for entry.

The vast majority of mice are housed in microisolation cages on HEPA-filtered ventilated racks (Allentown, Allentown, NJ) with irradiated rodent diet (PicoLab Rodent Diet 20 5053, Lab Diet), automatic reverse-osmosis watering, corncob bedding (Bed-o'Cobs 1/4-in., The Andersons Inc, Maumee, OH), and Crink-l'Nest (The Andersons Inc). A minority of mice is housed in static microisolation cages in behavioral and biohazard suites. With the exception of behavioral testing (open-air procedures), all mice must be manipulated in an animal transfer station or BSL2 cabinets by using aseptic technique with Virkon-S disinfectant (DuPont, Wilmington, DE). Ventilated racks are disassembled, fully decontaminated, and autoclaved annually on a rotating basis. Cages on ventilated racks are changed every 7 to 10 d. Most cages are sanitized through a tunnel washer with 180 °F rinse water; approximately 5% of the caging (containing bedding and nesting material) is autoclaved for immunodeficient and sensitive strains. At the time of the infection, all cages were sanitized in the same cagewash area equipped with a tunnel washer (model 6000, Steris Corporation, Mentor, OH) and an automated bedding dispenser (Roe bedding dispenser, SMC Schlyer Machine, Roe, NY) linked by sealed pipes to a closed bedding reservoir in the clean storage room.

The health-monitoring program consists of a single soiled-bedding sentinel cage per half rack (maximum, 69 cages) containing 2 naïve, weanling (age, 3 to 5 wk) Swiss Webster female mice, which are replaced and serologically tested quarterly for adventitious pathogens by an outside diagnostic laboratory. Once a year, sentinels also are shipped to a reference diagnostic laboratory for comprehensive necropsy, bacteriology, and PCR testing for ecto- and endoparasites. Any pathogen-positive result is confirmed on the sentinel cohort and by a secondary method. In addition, all biologic materials used in research are tested for murine pathogens before use. Almost two-thirds of the mouse population is bred inhouse in established colonies. All imported animals coming in the barrier facility are purchased from approved commercial vendors that are free of adventitious pathogens or are rederived and tested prior to release into animal housing rooms. Mice imported into the conventional facility must undergo quarantine, testing, and treatment for endo- and ectoparasites prior to release. All transfers between animal facilities and animal-holding rooms are documented and handled by the Animal Resources Department. For the current study, registered veterinary technicians completed all inhouse diagnostic testing according to standard operating procedures, and the veterinarian confirmed the positive results.

Collection of fecal sample and fecal flotation for *A. tetraptera*. Approximately 4 to 6 fecal pellets were collected directly from the anus of mice and examined for *A. tetraptera* eggs using standard fecal flotation. Fecal samples from either individual mice or a pool of no more than 5 mice in a cage were examined. Briefly, fecal pellets were placed in a 5-ml test tube containing sodium nitrate solution (Fecasol, Vetoquinol USA, Fort Worth, TX). Pellets were softened and dispersed by using the wooden portion of a sterile cotton tip applicator to release eggs. The test tube then was filled with sodium nitrate solution and covered with a coverslip for 15 min, after which the coverslip was transferred to a glass histology slide and evaluated at a magnification of 100× or higher for the presence of eggs.

Perianal tape test for *Syphacia obvelata*. Perianal tape tests were performed on individual mice for the detection of *S. obvelata*.[19] Briefly, the adhesive part of clear cellophane tape was pressed against the anus and perianal skin. Tape was applied to a glass histology slide and evaluated at a magnification of 100× or higher for the presence of eggs.

Cecal and colon examination. Presence of pinworms was determined in the cecum and colon of mice that were euthanized by CO_2 asphyxiation. The abdominal cavity was opened and gastrointestinal tract excised, placed in a culture dish, macerated, and submersed in warm saline for 15 min. The sample solution was evaluated under a dissecting microscope. Pinworms were collected and mounted for microscopic identification (magnification, 100×).

Rack and cage sampling for environmental PCR testing. Environmental samples were obtained by using sterile, dry, flocked swabs (BBL Culture Swab, Becton Dickinson, Franklin Lakes, NJ) according to the standard operating procedure of the reference laboratory. Rack samples were obtained by swabbing the horizontal air-exhaust manifold on all rows of a rack. The rack blowers were turned off immediately before sampling, the exhaust plenum was opened, and all 4 sides of the rectangular shelf manifold were swabbed by using a back-and-forth streaking motion. At each testing, approximately 60 cm^2 of the manifold was swabbed to collect accumulated particles originating from the shelf cages.[22] Cage samples were obtained by swabbing the

inside perimeter of empty soiled cages at the level of the bedding. Swabs were broken, stored in sterile Eppendorf tubes at 4° C, and shipped on the day of collection by overnight courier to the reference laboratory.

Fluorogenic PCR assay and DNA sequencing. Feces and environmental samples were tested for pinworms by using validated PCR assays and standard operating procedures at the reference laboratory. For some environmental samples, the PCR signals were weak and products could not be sequenced. Therefore, an alternative PCR primer pair that targeted conserved nematode 18S rRNA gene sequences was used to amplify nematode DNA for sequence analysis from samples that tested positive in the pinworm PCR assay. Amplicons were sequenced in both directions by using Sanger methodology (Genewiz, South Plainfield, NJ), and the resulting consensus sequence was compared with GenBank sequences by using BLAST software (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/).

Case Study

The feces of 2 sentinels from 2 different rooms (2 of 262 sentinel cages tested) in the SAF barrier facility were found to be positive for *A. tetraptera* by fecal PCR testing in the June 2013 sentinel screening. The results were confirmed by fecal floats and direct cecal examination of the sentinels, their cage cohort, and colony animals. All other sentinels in the barrier (SAF, TG) and conventional (CRAF) facilities were negative for pinworms on fecal PCR testing (Table 1). The 2 positive rooms were quarantined immediately, and strict traffic flow procedures were implemented to prevent the spread of the parasite to other rooms. Considering the biology of the parasite, its potential persistence in the environment, and the collaborations and sharing of animals and procedural space between laboratories, we implemented a large testing program in all facilities to determine the extent of the infection. First, all remaining sentinel cohorts were tested by fecal flotation, perianal tape tests, and direct cecal examination to rule out false-negative results during PCR testing (260 sentinel cages tested). All results were negative for pinworms (Table 1). All transfers in and out of the 2 *A. tetraptera*-positive rooms to other rooms or facilities over the past year were reviewed. Transferred mice and their offspring or relatives still present were tested by fecal floats and perianal tape tests and were negative for pinworms (36 cages tested). In addition, environmental PCR testing of room surfaces and facility HVAC was performed in both confirmed-positive rooms as well as the behavioral testing suites and biohazard rooms where mice from pinworm-positive rooms could have been transferred to or used in open air. All results of environmental PCR testing were negative for pinworms.

Most ventilated rack systems, including the Allentown racks, operate similarly in positive pressure, providing each IVC with HEPA filtered air at 60 to 70 air changes hourly thus creating an effective barrier at the cage level and greatly reducing the levels of ammonia and other waste products in the cage. Air from each shelf is collected and channeled through a horizontal air exhaust manifold and then to a common terminal vertical exhaust plenum before being exhausted in the room or through the central ventilation system after HEPA filtering. PCR testing of the shelf horizontal air exhaust manifold was a very sensitive method to detect fur mites at the rack level.[22] The effectiveness of PCR to detect pinworms in air exhaust manifolds of ventilated racks has not been determined. Although the persistence of pinworms in the environment is a matter of debate,[14,17] eggs are believed to survive for weeks in the environment and are resistant to desiccation and many common disinfectants.[14,28] Pinworm eggs have been found on equipment, dust and air intake filters in conventionally housed, infected rodent colonies,[28] but environmental surveys using tape tests failed to find *Syphacia* or *A. tetraptera* eggs in infected mouse colonies housed in microisolator caging or IVC.[4,24] Although *A. tetraptera* eggs do not aerosolize as easily as do *Syphacia* eggs,[28] one might expect that the exquisite sensitivity of PCR would enable the detection of only a few eggs or of residual genetic material in the environment. As such, we speculated that environmental PCR testing of the horizontal exhaust manifolds could be a reliable and sensitive method to assess the presence of pinworm DNA and therefore a potential pinworm infection at the rack level. Consequently we tested the exhaust manifolds of all ventilated racks in positive rooms (9 racks) by PCR and, as expected, all were positive for *A. tetraptera* (Table 1). Additional PCR testing of exhaust plenums was performed on all ventilated racks in the 2 barrier (SAF and TG) and the conventional (CRAF) facilities (122 racks). Surprisingly, the vast majority of ventilated racks were positive for *A. tetraptera* despite the negative results of the corresponding sentinels on fecal PCR, fecal floats, and cecal examination. The only negative racks came from 'autoclaved' rooms (SAF 47, 48, and 54 and TG 107 and 116), in which all cages within the room were autoclaved as single units (including bedding and enrichment) before placement on the ventilated racks. We considered 3 potential scenarios to explain the results: 1) a widespread outbreak selectively affecting rooms holding nonautoclaved cages; 2) the historical presence of pinworm DNA in the exhaust manifold of racks in nonautoclaved rooms in the absence of an active infection in colony animals; and 3) false-positive environmental PCR results due to a nonspecific amplification of DNA.

A widespread outbreak that originated from an unapproved import or an enzootic infection would have been surprising in light of the negative sentinel results, the selective infection of nonautoclaved rooms, and the facility biosecurity program, which includes microisolation caging and strict standards of aseptic technique. Nonetheless, we decided to exclude this hypothesis by testing all cages containing breeding or 5- to 10-wk-old colony mice in representative autoclaved and nonautoclaved rooms (more than 210 cages on 22 different racks were tested). Breeding and young colony mice were targeted because these groups typically show an increased prevalence of *A. tetraptera*. All cages meeting these criteria were tested by pooled fecal flotation (1 flotationper cage, maximum of 5 mice per pooled sample) in 2 large nonautoclaved rooms (SAF 55 and 70) and one autoclaved room (SAF 48). In addition, pooled fecal floats and cage PCR swabs (maximum, 10 cages per pool) of more than 100 cages were collected from 2 nonautoclaved rooms (SAF 35 and 61) with the lowest crossing-point values (that is, strongest PCR signals) on exhaust manifold PCR testing. All test results were pinworm-negative, thus suggesting that exhaust manifold PCR results could not easily be correlated with fecal flotation or cage PCR results from index, high-risk colony animals (Table 2).

Alternatively, the contamination of the plenum from colony animals could have been historical. However, several racks that were positive by exhaust plenum PCR testing had been fully disassembled, processed, and autoclaved in February and March 2013, thus suggesting that the contamination occurred after those dates.

In addition, booklice of the genus *Liposcelis* were present in the exhaust plenums of several ventilated racks at the time of sampling. These booklice were collected and speciated by using standard microscopy (Figure 1). Although *Liposcelis* spp. are

Table 1. Initial assessment of pinworms in barrier and conventional facilities

Room	No. of racks	Caging type	Sentinels			IVC exhaust PCR
			PCR	Float	Tape	
SAF32	4	Nonautoclaved	—	—	—	+
SAF 35	4	Nonautoclaved	—	—	—	+
SAF 36	7	Nonautoclaved	—	—	—	+
SAF 38	7	Nonautoclaved	—	—	—	+
SAF 40	7	Nonautoclaved	—	—	—	+
SAF 42	7	Nonautoclaved	—	—	—	+
SAF 44	6	Nonautoclaved	+	+	—	+
SAF 46	4	Nonautoclaved	—	—	—	+
SAF 47	3	Autoclaved	—	—	—	—
SAF 48	3	Autoclaved	—	—	—	—
SAF 50	3	Nonautoclaved	+	+	—	+
SAF 51	5	Nonautoclaved	—	—	—	+
SAF 54	7	Autoclaved	—	—	—	—
SAF 55	7	Nonautoclaved	—	—	—	+
SAF 60	7	Nonautoclaved	—	—	—	+
SAF 61	5	Nonautoclaved	—	—	—	+
SAF 70	12	Nonautoclaved	—	—	—	+
SAF 72	12	Nonautoclaved	—	—	—	+
SAF 76	5	Nonautoclaved	—	—	—	+
CRAF22	4	Nonautoclaved	—	—	—	+
CRAF29	4	Nonautoclaved	—	—	—	+
CRAF30	4	Nonautoclaved	—	—	—	+
TG107	2	Autoclaved	—	—	—	—
TG116	2	Autoclaved	—	—	—	—

Table 2. Secondary confirmation of environmental pinworm PCR results from exhaust manifolds by fecal floats and intracage PCR on index, high-risk colony animals

Room	Caging type	IVC exhaust PCR	Fecal float	Cage PCR
SAF 48	Autoclaved	—	—	NA
SAF 55	Nonautoclaved	+	—	NA
SAF 70	Nonautoclaved	+	—	NA
SAF 35	Nonautoclaved	+	—	—
SAF 61	Nonautoclaved	+	—	—

NA, not applicable

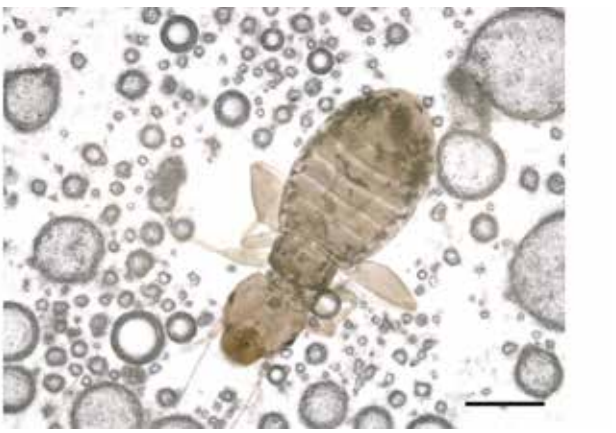

Figure 1. Booklice observed in rack plenum of both autoclaved and nonautoclaved rooms. Magnification, 4×; bar, 250 µm.

not known to harbor *A. tetraptera*, several types of nematodes can infect arthropods[32] and could have been the source of a false-positive finding due to crossreaction of the PCR primers. As such, booklice were tested for pinworm PCR by using the same primers as for the plenum PCR assays. All results were negative. In addition, booklice were present in the ventilated racks of both autoclaved and nonautoclaved rooms, suggesting that they were not the source of the pinworm-positive exhaust plenum results.

Reviewing the data revealed that the most likely source of contamination to explain a widespread contamination that specifically affected nonautoclaved rooms was a component used inside the cage throughout the facilities that is neither sanitized nor autoclaved prior to use. Only 2 components met these criteria: the corncob bedding and nesting material. We speculated that the paper-product nesting material was the least likely source of contamination given that it has poor nutritional value and is unlikely to draw infected vermin. Nonetheless, environmental swabs for pinworms were taken from packaging materials and the room in which the nesting material is stored and unpacked. Additional swabs were taken from the automatic bedding dispensing system reservoir in the clean storage room, the ducts connecting the reservoir to the dispensing unit in the clean cagewash area, and the dispensing unit in the clean cagewash area. All results were negative except for the corncob reservoir in the clean storage room, which was weakly positive for *A. tetraptera*. The PCR reaction was rerun and confirmed as a positive result, thus suggesting contamination from the corncob or the reservoir. A weak positive result was expected, considering the rapid turnover of corncob in the reservoir (at least twenty 30-lb bags daily) and the likely distribution of DNA due to rough mechanical abrasion of the bedding against the unit. Several additional swabs were taken from the corncob reservoir

more than a week after the initial swab, but all were found to be pinworm negative. The reservoir was emptied, inspected for pests (none seen), and fully decontaminated. Environmental swabs for *Aspiculuris* PCR were obtained from the distributor's storage rooms and delivery trucks where corncob bedding and rodent feed bags were stored. All results were negative, suggesting a potential contamination at the manufacturing plant.

Considering that none of the positive PCR results on exhaust plenums were confirmed in sentinel and colony animals by fecal floats and cage PCR, we started to question the specificity of the primers used for environmental assays. Plenums in autoclaved and nonautoclaved rooms were swabbed again and sent to both the original and a second reference laboratory for pinworm PCR analysis. Swabs again were positive for *A. tetraptera* at the original diagnostic laboratory in nonautoclaved rooms, but all PCR test results were negative at the second reference laboratory, thus suggesting that the primers used at the original reference laboratory were not specific and amplified another nematode (Table 3). The PCR results from samples taken from the horizontal exhaust manifolds in confirmed positive rooms (SAF 44 and 50) were also negative at the second reference laboratory, suggesting that PCR analysis of exhaust plenums is not efficient in detecting pinworms in a naturally infected mouse colony.

To test the specificity of the primers from the first reference laboratory, we sequenced the amplicons from the pinworm-positive exhaust plenum samples and obtained the following sequence:

3′ TGG ACC GTA GCG AGA CGA CCT ACA GCG AAA GCA TTT GCC AAG AAT GTC TTC GTT AAT CAA GAA CGA AAG TCA GAG GTT CGA AGG CGA TTA GAT ACC GCC GTA GTT CTG ACC GTA AAC GAT ACC ATC TAG CGA TCC GGC GGT GGT ATT ATT GCC TCG CCG GGG AGC TTC CCG GAA ACG AAA GAC TTC CGG TTC CGG GGG TAG TAT GGT TGC AAA GCT GAA ACT TAA AGG AAT TGA CGG AAT CGC ACC ACC AGG AGT GGA GCC TGC GGC TTA ATT TGA CTC AAC ACG GGA AAA CTC ACC CGG CCC GGA CAC TGT AAG GAT TGA CAG ACT GAA AGC TCT T 5′

This sequence was used as a query sequence in a BLAST search of GenBank and yielded a 100% match for 3 nematodes of the family Rhabditidae: *Mesorhabditis longespiculosa*, *M. miotki* and *M. spiculigera*. This family is very complex and diverse, with some species living free in the environment whereas others infect animals, plants, or arthropods, but none is known to infect mice.[32] Using BLAST, we then compared the obtained rhabditid amplicon sequence with the known sequence of *A. tetraptera*. As expected, we found a 91% sequence identity with the *A. tetraptera* 18S rRNA gene and a 100% match in the region of the primer. We therefore considered the so-called *A. tetraptera*-positive results from the exhaust plenums of ventilated racks in nonautoclaved rooms to be false-positive findings due to nonspecific primers. Because the mitochondrial genome of mesozoa, nematodes, rotifers, and lice can be fairly conserved,[36] we compared the sequence of the rhabditid amplicon to the known *Liposcelis* spp. genome to confirm that the false-positive results was not due to the presence of booklice in the plenums. We found only 87% sequence identity to *Liposcelis* spp. 18s rRNA, with a very poor match in the primer region. Considering the negative PCR results on the booklice samples, the presence of booklice in both autoclaved and nonautoclaved rooms, and the poor sequence identity between the amplicon and the booklice genome, we excluded the possibility that the false-positive results were due to booklice or the infection of these lice with a rhabditid nematode. The positive result from the corncob reservoir rather suggested historical contamination of the bedding with the nematode that affected all facilities with caging receiving nonautoclaved bedding.

Table 3. Comparison of environmental pinworm PCR results from exhaust manifolds between 2 reference laboratories

Room	Caging type	Fecal float or fecal PCR	Environmental PCR	
			Lab 1	Lab 2
SAF 35	Nonautoclaved	—	+	—
SAF 44	Nonautoclaved	+	+	—
SAF 50	Nonautoclaved	+	+	—
SAF 61	Nonautoclaved	—	+	—
SAF 70	Nonautoclaved	—	+	—
SAF 47	Autoclaved	—	—	—
SAF 48	Autoclaved	—	—	—
SAF 54	Autoclaved	—	—	—

The situation and risk assessment was explained to our investigators. Based on these data, mice in the 2 original rooms that were confirmed to be positive for *A. tetraptera* by both fecal PCR and flotation analysis as well as on direct cecal examination were treated with fenbendazole-containing feed (PicoLab Rodent Diet 5001 with 150 ppm fenbendazole, Lab Diet) for 5 wk. Racks within the room were relocated to quarantine during the last week of treatment, and rooms were fully decontaminated by manually wiping down all surfaces with 10% bleach followed by fogging with 5% hydrogen peroxide (Sanosil S010 Disinfectant, Sanosil, Hombrechtikon, Switzerland). All mice were transferred into clean cages and housed on a new autoclaved rack before being returned to their original room for the last week of treatment. Extensive follow-up testing by both fecal PCR assays and fecal flotation on sentinels and colony mice was performed in all rooms for 9 mo. All results were negative for pinworms, confirming that the treatment was successful and that the outbreak was limited to these 2 rooms.

Discussion

Molecular techniques like PCR analysis have been developed to improve the detection of pathogens in both human and veterinary medicine. PCR-based testing is now advocated as an adjunct to traditional health-monitoring programs, especially for airborne pathogens such as Sendai virus,[2,13] which are not easily transmitted to sentinels by soiled bedding.[22,25,30] For some agents, PCR testing can be more sensitive than traditional methods used to survey pathogens in sentinels. Recent studies suggest that environmental PCR testing from exhaust filters or plenum of ventilated racks[30] may be useful in assessing and managing pathogen outbreaks, but optimal environmental sampling strategies have not yet been established for different microorganisms.[22] This strategy has been proven useful in detecting MHV and Sendai virus in ventilated racks operating in both positive and negative pressure, but the results were less conclusive and more variable for MPV and *Helicobacter*.[10] Importantly, these results were obtained from experimentally infected mice, which are expected to shed many more infectious particles than are naturally infected mice. PCR samples taken from shelf exhaust manifolds of IVC racks operated in positive pressure were more reliable than were standard soiled bedding sentinels for the detection of fur mites in a naturally infested mouse colony.[22] The effectiveness of PCR testing to detect pinworms in air-exhaust manifolds of ventilated racks has not been determined. The persistence of pinworms in the environment is a topic of debate,[14,17] but eggs are believed to survive for weeks in the environment and are resistant to desiccation and

many common disinfectants.[14,28] Nonetheless, very few studies have specifically examined the persistence of *A. tetraptera* eggs in the environment.[15,20,28,33] Pinworm eggs have been found on equipment, dust, and air-intake filters in conventionally housed rodent colonies,[28] but environmental surveys using tape tests failed to find *Syphacia* or *A. tetraptera* eggs in mouse colonies housed in microisolator or IVC caging.[4,24] Although *A. tetraptera* eggs do not aerosolize as easily as *Syphacia*,[28] one may expect that the exquisite sensitivity of PCR would allow the detection of only a few eggs or residual genetic material in the environment. We therefore speculated that environmental PCR testing of samples taken from the horizontal exhaust manifolds of our positive-pressure racks would be a reliable and sensitive method to assess the retrospective presence of pinworm DNA and pinworm infection at the rack level and survey, on a large scale, the health status of our facilities. The initial results suggested a widespread infection throughout our facilities in racks holding nonautoclaved cages.

Like any other large-scale diagnostic methodology, PCR assays are subject to false-positive and -negative results. As such, any results must be interpreted cautiously with an overall understanding of the PCR procedure, the intrinsic controls, and all other variables that may affect results including primer design and specificity as well as manipulation and processing of the sample in the home facility, during transport, and at the reference laboratory. Validating a PCR assay for diagnostic purposes is challenging[11] in both human and veterinary medicine. The first step is to design primers by computer modeling to optimize PCR conditions, specificity, and sensitivity. Diagnostic laboratories typically use primers that target ubiquitous housekeeping genes or ribosomal RNA common to several species to identify pathogens at the genus level, whereas primers complimentary to specific elements within the genome are used for organism speciation. The specificity of the chosen primers must then be compared with all available sequences in genome databases, such as GenBank. The in vitro sensitivity of the assay then is verified by using 10-fold dilutions of the target pathogen, whereas specificity is evaluated by using DNA extracted from a large bank of pathogens of the same family. Finally, the in vivo specificity and sensitivity are validated by testing known clinical samples in the presence of positive and negative controls.

In our case, the PCR assay targeted a region of the 28S rRNA gene common to *S. muris*, *S. obvelata*, and *A. tetraptera.* According to the first reference laboratory, the analytical validation of the assays confirmed a reproducible 10-copy detection limit for all 3 pinworms. The performance of the assay was evaluated in vivo by using more than 200 known positive and negative clinical samples. The results of the validation showed that the assay has 100% sensitivity and 100% specificity in detecting *A. tetraptera*, *S. obvelata*, and *S. muris* in clinical samples, with select positive samples being confirmed with sequence analysis.

Results from this case report suggest that the PCR assay was not specific despite the stringent validation performed by the first reference laboratory. One possible explanation is that the databases used to design the primers were flawed or incomplete at the time of the validation process. Indeed, genome databases such as GenBank are known to contain unverified, incomplete, and inaccurate sequences[8] as well as entries from genetically different organisms under the same species name.[9] In addition, large numbers of microorganisms are discovered every year and are classified phylogenetically according to the genome sequence. The nematode phylum is especially rich in species and is biologically diverse, with plant and animal parasites as well as free-living organisms. Recent estimates suggest that there are between 1 and 10 million existing nematode species, but only 25,000 have actually been described in the literature, and only a few have been fully sequenced genomically.[12] As such, reference laboratories are confronted with the ongoing discovery of new microorganisms and the characterization of their genomes and must continue to validate PCR assays over time and by using different databases to ensure the specificity of their primers. Although the validation process of a PCR assay designed to test for mouse-specific pathogens in a biologic sample taken from the animal (for example, feces, lymph nodes) might look straightforward, the same does not hold true for environmental PCR. Indeed, validation of these assays should take into account the wide variety of ubiquitous microorganisms present in the environment that are not infectious to mice but that may be a source of false-positive results. Similarly, the interpretation of PCR results obtained from environmental samples by using assays developed for mouse biologic materials should take into account those caveats and be performed cautiously.

The family Rhabditidae is one of the richest groups of nematodes and likely contains thousands of members, many of which remain to be discovered.[32] Their classification is extremely complex and remains a subject of debate. These nematodes are free-living, saprophagous parasites that can be found in almost every terrestrial habitat but predominantly in soils and sediments. They play an important ecologic role by feeding on decaying organic matter, including plant tissues and bacteria. Rhabditid nematodes are very resistant in the environment and can survive under the harshest conditions.[1] None of the 3 rhabditid species that matched the query DNA sequence are pathogenic for mice. Although some rhabditids can parasite insects, we could not isolate them from the booklice present in our plenum.[32] In addition, these mites also were present in the plenums of autoclaved racks, which tested negative by PCR. After extensive environmental PCR testing of samples from inside the positive rooms and within the facilities, the only pinworm-positive result was found in the corncob bedding feeder, thus suggesting contamination of the product. Contamination of the corncob bedding would explain the widespread, nonspecific positive results of pinworm PCR assays from all racks holding nonautoclaved cages and the negative results from racks holding autoclaved cages, because heat would have denatured the DNA present in the corncob bedding during the autoclaving process. The signal from the PCR reaction was weak but was confirmed through a second, albeit weaker reaction. This weaker signal was expected, considering the rapid turnover of corncob bedding in the reservoir (more than twenty 30-lb bags daily).

To our knowledge, this report is the first to describe the contamination of corncob bedding with a free-living nematode. Corncob is a common and cost-effective bedding substrate for rodents, due to its absorbency, biodegradability, and ability to control intracage ammonia levels.[16] It is produced from the central core of the maize plant. Raw stalks are passed through different hammer and roller mills to produce fractions, which are heat-dried to approximately 200 °F. Particles then are sized and separated through a sifting process, and dust is removed by aspiration prior to packaging.[18] Despite these harsh production conditions, corncob bedding can contain high levels of enterobacterias, including coliforms, yeast, mold, and spores, as well as endotoxins and endocrine disruptors that can interfere with research variables or even cause clinical disease.[16,26,29,34,35] Theoretically, contamination with the rhabditid parasite could have occurred at any of several points during the manufacturing process, transport, unpacking, and storage inside our facilities. The extent of the contamination, which affected more than 114

ventilated racks in our different facilities, suggests the contamination of the product at its source and within multiple corncob bags. In addition, the pinworm-positive results were limited to the inside of the corncob hopper, where bags are dumped after initial decontamination and unloading in our facilities. All other environmental PCR tests from samples taken in our storage room, the distributor's storage rooms, delivery trucks, and on the outside of corncob bags were negative for pinworm. Despite the facts that the discovered parasite was not pathogenic and that detection of genomic material by PCR does not necessarily imply the presence of infectious particles, this outbreak investigation does reveal a potential risk of contamination from nonsterilized corncob bedding.

Some authors have advocated the sterilization of corncob bedding by ionizing irradiation or autoclaving prior to its use in barrier facilities.[16,35] Ionizing irradiation is a relatively efficient sterilization process for feed and bedding but is costly and does not ensure product sterility. Indeed, bacterial spores and viruses may survive irradiation even at the high doses (40 to 50 kGy), which are recommended for sterilizing diets intended for gnotobiotics and germ-free animals.[6] Sterilization is particularly dependent on the environmental conditions during the ionizing process.[23] Furthermore, despite the precautions to prevent contamination during packaging, risks of contamination during transport and storage in the facility remain.[23] The autoclaving process uses a combination of steam and high pressure to sterilize bedding. Autoclaving on site may kill bacteria, viruses, fungi, and parasites, but it is a time-consuming and costly process that may decrease the absorptive capacity of the bedding material.[16] In addition, the autoclaving process is not foolproof, and validation can be difficult and prone to inconsistencies in obtaining disseminated target temperature if one assesses multiple sites in the load.

The lice observed in our rack plenum belong to the genus *Liposcelis*, also known as booklice. These organisms attack a wide variety of raw materials and foodstuffs. Under favorable conditions, they can be prolific breeders and form large, gregarious populations.[27] Booklice typically find their way into lab animal facilities on paper products, such as the exterior packaging of feed bags, and are resistant to many common insecticides.[7] Although *Liposcelis* spp. are not known to harbor *A. tetraptera*, several types of nematodes can infect arthropods and could have been the source of false-positive results due to crossreaction of the PCR primer. In addition, the mitochondrial genome of mesozoa, nematodes, rotifers, and lice are well conserved[36] and could have explained the false-positive result. However, considering the negative PCR results from samples of the booklice themselves, the presence of booklice in both autoclaved and nonautoclaved rooms, and the poor sequence identity between the amplicon and the booklice genome, we excluded the possibility that the false-positive results arose from these lice or their infection with a rhabditid nematode.

Another potential consideration is that the false positives observed in this case study were due to contamination of the samples with exogenous DNA during processing at the reference laboratory. Contamination can either be vertical (for example, from tubes, pipettes, laboratory surfaces, technicians, or previous amplicons in the laboratory) or horizontal (cross-contamination from one sample to another or carryover). However, reference laboratories have very strict operating procedures and internal controls to prevent such cross-contamination. In addition, the confirmation of the results on samples obtained and processed at different time points, the negative results from the second reference laboratory, and the sequencing of the amplicons all suggest that the false-positive findings were indeed due to the nonspecificity of the assay.

Overall, these results show that some PCR tests can yield false-positive results despite all safeguards by reference laboratories to ensure the specificity of their assays. The validation of environmental PCR assays is especially challenging considering the wide variety of ubiquitous microorganisms present in the environment that are not infectious to rodents but that may result in false-positive results. In addition, even when the PCR assay is specific, it may identify genetic material in the absence of any infectious pathogens. Importantly, the shelf exhaust samples from racks in confirmed positive rooms and analyzed by the second laboratory were negative for pinworms, thus suggesting that PCR testing of exhaust samples is not efficient in detecting pinworms in a naturally infected mouse colony. Additional studies such as the one in fur mites[22] are warranted to determine the sensitivity of PCR testing of exhaust-manifold samples in detecting pinworms throughout large, naturally infected mouse colonies. Nonetheless, the results of this case study emphasize the potential risks of environmental PCR assays and the need to confirm results by testing colony animals before initiating costly and potentially disruptive containment and eradication measures in the face of a pinworm outbreak.

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