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Cross-Foster Rederivation Compared with Antibiotic Administration in the Drinking Water to Eradicate *Bordetella pseudohinzii*

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***Bordetella pseudohinzii* is a microbial agent of potential importance in mice and has confounded pulmonary research at our institution. The purpose of this study was to evaluate cross-foster rederivation and antibiotic administration in the drinking water as methods to eradicate *B. pseudohinzii*. To evaluate the efficacy of cross-foster rederivation, 29 litters representing 16 strains of mice were cross-fostered from cages positive for *B. pseudohinzii* to *B. pseudohinzii*–negative Crl:CD1-Elite surrogate dams. To evaluate antibiotic administration, sulfamethoxazole and trimethoprim (TMS; 0.66 and 0.13 mg/mL, respectively) and tetracycline (4.5 mg/mL) were administered in the drinking water. We assessed 3 antibiotic treatment groups with 12 *B. pseudohinzii*–positive cages per group (6 cages of CD1 and 6 cages of C57BL/6 mice): TMS for 4 wk, TMS for 6 wk, and tetracycline for 6 wk. Of the 29 litters that underwent cross-foster rederivation, 24 were negative for *B. pseudohinzii*. Five of the 12 cages treated with TMS for 4 wk and 1 of the 12 cages treated with TMS for 6 wk were negative for *B. pseudohinzii* at 2 wk after treatment. Three of the 12 cages treated with tetracycline were negative for *B. pseudohinzii* at 2 wk after treatment. Pearson χ^2 analysis revealed significant association between the method of eradication (cross-foster rederivation compared with antibiotic administration) and *B. pseudohinzii* infection, and an odds-ratio estimate from a logistic regression demonstrated that cross-foster rederivation was more successful. Whereas antibiotic administration in the drinking water failed to eradicate *B. pseudohinzii*, cross-foster rederivation was successful and has been used to establish a *B. pseudohinzii*–negative barrier.**

Abbreviation: TMS, sulfamethoxazole and trimethoprim

Bordetella pseudohinzii is the provisional name for a proposed novel species of *Bordetella* that is the only species with transcriptionally active clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated systems.[10] Genomic sequencing has identified that *Bordetella hinzii* strains previously identified in mice are actually *B. pseudohinzii* and are genetically distinct from the *B. hinzii* strains that have been isolated from poultry and humans.[11] At our facility, there was an association between infection with *B. pseudohinzii* and increased neutrophils in the bronchoalveolar lavage fluid of mice.[4] Infected mice did not have clinical signs, but histology demonstrated mild to moderate rhinitis.[4] *B. pseudohinzii* was cultured from the oropharynx and lung and was identified in lung tissue and feces from infected mice by using a PCR assay for *B. hinzii* that crossreacts with *B. pseudohinzii*.[4] Fecal PCR analysis is the preferred ante mortem diagnostic method to identify mice infected with *B. pseudohinzii*.[4] Infection with this organism complicated pulmonary research at our institution and made it necessary to rederive and maintain a *B. pseudohinzii*–negative mouse colony for investigators studying pulmonary disease. To eradicate *B. pseudohinzii*, cross-foster rederivation and antibiotic administration in the drinking water were evaluated.

Cross-foster rederivation has been used to eliminate murine pathogens from rodent colonies.[1,2,8,9,12,18,21] Cross-foster rederivation does not require specialized training, is less labor intensive than are other methods of rederivation, and does not require the euthanasia of donor females. At our institution, cross-foster rederivation is routinely performed and has been used to eliminate *Helicobacter* spp., murine norovirus, mouse hepatitis virus, and pinworms.[1] The disadvantages of cross-foster rederivation are that it cannot be used for pathogens that are transmitted in utero, and pups can potentially become contaminated between birth and cross-foster, either through exposure to the cage environment or from close contact with the dam. In utero infection with *Bordetella* species has not been reported, but transmission can occur from dams to offspring due to close contact.[3,16]

Antibiotic administration in the drinking water can be used to treat a large population of mice simultaneously, has a low labor cost, and is less stressful than are other methods of administration that require restraint or injection. *Pasteurella pneumotropica* has been eliminated from mouse colonies through the administration of enrofloxacin in the drinking water, thus demonstrating that this method of delivery can be successful against organisms that colonize the oropharynx.[5,20] Antibiotic sensitivity testing of *B. pseudohinzii* isolates from the mouse colony demonstrated susceptibility to imipenem, amikacin, gentamicin, tobramycin, marbofloxacin, tetracycline, and sulfamethoxazole and trimethoprim (TMS).[4] TMS is a bactericidal drug that is regularly administered in the drinking water to immunocompromised mice at our facility; we therefore evaluated whether this standard practice eliminates *B. pseudohinzii* infection. We also evaluated tetracycline in light of the availability of large-animal formulations that can be easily delivered in the drinking water. Although tetracycline is bacteriostatic, it is considered an effective antibiotic for intracellular bacteria,

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and most canine *Bordetella bronchiseptica* isolates are susceptible to this antibiotic.[17,19]

Materials and Methods

Facility. The Biologic Resources Laboratory is the centralized animal facility at the University of Illinois at Chicago, which has an AAALAC-accredited animal care and use program. Excluded microbial agents are *Helicobacter* spp., *Mycoplasma pulmonis*, pinworms, fur mites, murine norovirus, mouse rotavirus, mouse hepatitis virus, mouse parvoviruses, minute virus of mice, pneumonia virus of mice, reovirus 3, Sendai virus, ectromelia, lymphocytic choriomeningitis virus, murine adenovirus 1 and 2, polyomavirus, and Theiler murine encephalomyelitis virus. Mice were group-housed in autoclaved static microisolator cages with autoclaved corncob bedding, autoclaved nesting enrichment (Cotton square, Ancare, Bellmore, NY), and autoclaved municipal water bottles with weekly cage changes. All cages were opened in class II biosafety cabinets or HEPA-filtered animal transfer stations according to standard microisolation practice, which included the disinfection of gloves with 100 ppm chlorine dioxide (Quip Laboratories, Wilmington, DE) between cages. Rooms had a 14:10-h light:dark cycle, and mice had unrestricted access to an irradiated diet (Rodent Diet 7912, Envigo, Teklad, Indianapolis, IN). All animal work was performed under IACUC-approved protocols.

Animal housing. Cross-foster rederived mice were housed in a restricted-access suite, with access limited to members of the veterinary staff and selected veterinary and animal care technicians. In addition, entrance to the suite was permitted only when other animal housing or procedure rooms had not yet been entered that day. Facility procedures followed those described previously, except that the concentration of the chlorine dioxide solution used in the suite was 200 ppm. The surrogate colony and rederived mice were tested for *B. pseudohinzii* every 2 wk by using fecal-pellet PCR analysis of pooled samples (6 to 8 cages per pool). If a pooled sample was PCR positive for *B. pseudohinzii*, each cage in the pool was retested individually to identify the positive cages, which were immediately removed from the suite.

Mice treated with antibiotics in the drinking water were housed in a dedicated restricted access room that was separate from the cross-foster restricted-access suite and other facility animals. Access was limited to members of the veterinary staff and selected animal care technicians.

PCR analysis. The PCR procedure has been described previously.[4] Briefly, total nucleic acids were extracted from fecal pellets by using a commercially available platform (One-For-All Vet Kit, Qiagen, Valencia, CA). The *B. pseudohinzii* PCR assay was based on a proprietary service platform (IDEXX Laboratories, Westbrook, ME), which targets a region of the outer membrane protein A gene that is conserved among all *B. pseudohinzii* and *B. hinzii* genomic sequences deposited in GenBank. A hydrolysis probe-based real-time PCR assay targeting a housekeeping gene (18S rRNA or 16S rRNA) was used to determine the amount of genomic DNA present in the test sample, to confirm DNA integrity, and to ensure the absence of PCR inhibitors. Diagnostic real-time PCR analysis using standard primer and probe concentrations and a commercially available mastermix (LC480 ProbesMaster, Roche Applied Science, Indianapolis, IN) was performed on a commercially available real-time PCR platform (LightCycler 480, Roche).

Animals. Surrogate colony breeding animals were male and female CRL:CD1-Elite mice. All animals were tested on arrival and were negative for *B. pseudohinzii* by fecal PCR analysis performed every 2 wk, as described earlier. The original parents for all litters that were cross-fostered were investigators' strains and were tested for *B. pseudohinzii* by fecal PCR analysis to confirm infection-positive status prior to their inclusion in the statistical data set. All investigator strains were immunocompetent, with the exception of toll-like receptor 4 (TLR4) knockout mice.

For the antibiotic study, it was necessary to infect mice with *B. pseudohinzii*. The exact pathogenesis of *B. pseudohinzii* is unknown, but other *Bordetella* species are transmitted through infectious aerosols, close contact with infected animals, and exposure to contaminated fomites.[6] PCR-positive inhouse-bred mice were cohoused with negative vendor mice, to mimic a natural route of infection. To avoid pregnancy and aggression, only female mice were used for cohousing. Female C57BL/6J and Crl:CD1 mice (age, 6 wk) were tested for *B. pseudohinzii* by fecal PCR analysis when they arrived at the facility, and all were negative for the organism. These female mice were cohoused with inhouse-bred female C57BL/6 mice that were positive for *B. pseudohinzii* by fecal PCR analysis until all mice were PCR positive for *B. pseudohinzii* (after 4 to 6 wk of cohousing). The female C57BL/6 mice that were used for cohousing were then included in the antibiotic study. So that both sexes were represented, inhouse-bred male C57BL/6 mice that were positive for *B. pseudohinzii* by fecal PCR testing were included in the antibiotic study. Mice were group-housed by sex, with 4 animals per cage.

Cross-foster rederivation. The cross-foster rederivation procedure has been described previously.[1] Briefly, breeder cages were checked for litters daily, and those that were younger than 24 h were collected. The pups were lightly sprayed with 200 ppm chlorine dioxide and were left wet for 2 min. The surrogate mother was kept in her cage with a maximum of 2 of her own pups and the cross-fostered litter. The surrogate's remaining pups were euthanized with CO_2 followed by decapitation.

TMS study. Mice that were PCR positive for *B. pseudohinzii* were provided with TMS in the drinking water (Sulfamethoxazole and trimethoprim oral suspension, cherry flavor, HI-TECH, Amityville, NY). TMS-containing water was prepared by mixing 8 mL of the oral suspension (320 mg sulfamethoxazole and 64 mg trimethprim) into a 16-oz bottle of autoclaved water, thus yielding 0.66 mg/mL sulfamethoxazole and 0.13 mg/mL trimethoprim. Fresh bottles of TMS water were placed weekly and wrapped with aluminum foil. After either 4 wk (n = 12 cages: 6 cages of female Crl:CD1, 4 cages of female C57BL/6J, and 2 cages of inhouse-bred male C57BL/6 mice) or 6 wk (n = 12 cages: 6 cages of female Crl:CD1, 3 cages of inhouse-bred female C57BL/6, and 3 cages of inhouse-bred male C57BL/6 mice) of treatment, the TMS water was removed, and antibiotic-free water was provided. Fecal samples were collected from each cage for *B. pseudohinzii* PCR testing at the end of treatment and every 2 wk thereafter. If a cage was positive for *B. pseudohinzii*, the mice were euthanized with CO_2 followed by cervical dislocation. Animals were monitored daily for any adverse clinical signs associated with treatment. One female Crl:CD1 mouse was euthanized prior to the end of treatment due to ulcerative dermatitis.

Tetracycline study. Mice that were PCR positive for *B. pseudohinzii* were provided with tetracycline (TetraMed 324 HCA, Bimeda, Le Sueur, MN) in the drinking water. Tetracycline water was prepared by mixing 3 g of powder (2140 mg of tetracycline) into a 16-oz bottle of autoclaved water, thus yielding 4.5 mg/mL tetracycline.[7] Fresh bottles of tetracycline water were placed weekly and wrapped with aluminum foil. After 6 wk of

treatment (n = 12 cages: 6 cages of female Crl:CD1, 4 cages of female C57BL/6J, and 2 cages of inhouse-bred male C57BL/6 mice), the tetracycline water was removed, and antibiotic-free water was provided. Fecal samples were collected from each cage for *B. pseudohinzii* PCR testing at the end of treatment and every 2 wk thereafter. If a cage was positive for *B. pseudohinzii*, the mice were euthanized with CO_2 followed by cervical dislocation. Mice were monitored daily, and no adverse clinical signs were observed throughout the study.

Statistical analysis. Statistical analysis was performed by using SAS Enterprise Guide (version 4.3, SAS Institute, Cary, NC). The 3 antibiotic-treatment groups were pooled for a total of 36 cages, and Pearson χ^2 testing was used to determine the association between the method of eradication (cross-foster rederivation compared with antibiotic administration in the drinking water) and *B. pseudohinzii* infection. An odds ratio was estimated from a logistic regression to determine the effectiveness of cross-foster rederivation compared with antibiotic administration. Logistic regression was also performed to detect whether the type of antibiotic, duration of treatment, sex, or strain was significantly associated with *B. pseudohinzii* infection. A P value less than 0.05 was considered to be significant.

Results

Cross-foster rederivation study. Of the 29 litters cross-fostered into the barrier suite from breeding cages that were PCR positive for *B. pseudohinzii*, 24 of them were negative for the organism at all testing time points. In the 5 litters that were positive for *B. pseudohinzii*, the organism was detected at their first fecal PCR analysis, when the pups were 2 to 4 wk old.

TMS study. After 4 wk of TMS treatment, 10 of 12 cages were negative via fecal PCR for *B. pseudohinzii*, but only 5 of 12 cages were negative 2 wk after treatment and only 3 of the 12 cages were negative 4 wk after the treatment (Table 1). All 12 cages were negative for *B. pseudohinzii* immediately after 6 wk of TMS treatment, but only 1 of the 12 cages was still negative for *B. pseudohinzii* at 2 wk after treatment (Table 2).

Tetracycline study. A 6-wk treatment with tetracycline rendered 9 of 12 cages negative for *B. pseudohinzii*. However, at 2 wk after treatment, only 3 of the 12 cages were still negative for the organism (Table 3).

Statistical analysis. Pearson χ^2 testing demonstrated that there was a strong association ($P < 0.0001$) between the method of eradication (cross-foster rederivation compared with antibiotic administration in the drinking water) and *B. pseudohinzii* infection (Table 4). The odds-ratio estimate from a logistic regression indicated that cross-foster rederivation was 19.883 times (95% confidence interval, 5.591 to 70.705) more effective than was antibiotic administration for the eradication of *B. pseudohinzii*. None of the other variables (type of antibiotic, $P = 0.21$; duration of treatment, $P = 0.21$; sex, $P = 0.07$; strain, $P = 0.49$) was significantly associated with eradication of *B. pseudohinzii*.

Table 1. Fecal PCR results for cages of mice treated with TMS in the drinking water for 4 wk

	Sex	End of treatment	2 wk after treatment	4 wk after treatment
Crl:CD1	Female	4 of 6	3 of 4	1 of 3
C57BL/6J	Female	4 of 4	0 of 4	0 of 0
C57BL/6	Male	2 of 2	2 of 2	2 of 2
	Total	10 of 12	5 of 10	3 of 5

Data are given as the number of cages negative for *B. pseudohinzii* among the total number of cages tested.

Table 2. Fecal PCR results for cages of mice treated with TMS in the drinking water for 6 wk

	Sex	End of treatment	2 wk after treatment
Crl:CD1	Female	6 of 6	1 of 6
C57BL/6	Female	3 of 3	0 of 3
C57BL/6	Male	3 of 3	0 of 3
	Total	12 of 12	1 of 12

Data are given as the number of cages negative for *B. pseudohinzii* among the total number of cages tested.

Table 3. Fecal PCR results for cages of mice treated with tetracycline in the drinking water for 6 wk

	Sex	End of treatment	2 wk after treatment
Crl:CD1	Female	4 of 6	1 of 4
C57BL/6J	Female	3 of 4	1 of 3
C57BL/6	Male	2 of 2	1 of 2
	Total	9 of 12	3 of 9

Data are given as the number of cages negative for *B. pseudohinzii* among the total number of cages tested.

Table 4. Pearson χ^2 analysis ($P < 0.0001$)

	Method of eradication	
	Cross-foster rederivation (no. of litters)	Antibiotic administration (no. of cages)
B. pseudohinzii infection status		
Not infected	24	7
Infected	5	29
Total	29	36

Discussion

This study demonstrated that cross-foster rederivation is superior to antibiotic administration in the drinking water for the eradication of *B. pseudohinzii* from mice and for the long-term maintenance of PCR-negative animals. The overall success rate was 82.75% for cross-foster rederivation compared with 19.44% for antibiotic administration in the drinking water. The difference in efficacy is likely due to the inherent difference in how the organism is eliminated by each treatment. Cross-foster rederivation prevents infection by removing litters from a contaminated dam and cage environment, whereas the administration of antibiotics in the drinking water is designed to eliminate the microbial agent from animals with an established infection and does not address the carrier state or cage contamination. Antibiotic administration in the drinking water decreases microbial burden, but when antibiotic administration is discontinued, recrudescence of a carrier state or reinfection from contamination in the cage can occur. The presence of a carrier state is not surprising, given that *Bordetella* species are able to evade the immune system within phagocytes, and *B. bronchiseptica* can form biofilms in the nasal cavity of mice.[6] Cross-foster rederivation can prevent the initial infection of

mice and removes them from a contaminated environment, making this method a more successful and preferred practice for the eradication of *B. pseudohinzii*.

Cross-foster rederivation had an overall success rate of 82.75% in this study. Two observations from this initial study have led to changes that have markedly improved the success rate. The first observation was that of the 5 litters that were positive after cross-foster rederivation, 3 of them were cross-fostered on a Monday. After the results of the current study were analyzed, our procedure was modified such that mice are no longer cross-fostered on Mondays due to the concern that litters may be older than 24 h (that is, 24 to 36 h old, given the weekend work schedule). Because *Bordetella* species colonize the oropharynx,[6] close contact between the dam and litter through postpartum cleaning of pups between birth and cross-fostering might place litters at risk of becoming infected. This study and our subsequent experience with cross-foster rederivation to eliminate *B. pseudohinzii* have demonstrated that strict adherence to cross-fostering of litters younger than 24 h is imperative. The second observation was that 2 of the 5 positive litters were TLR4 knockout mice. This strain has increased susceptibility to infection with *B. bronchiseptica* and therefore perhaps *B. pseudohinzii* as well.[13] TLR4 knockout mice were successfully cross-fostered by pretreating breeding pairs with TMS in the drinking water at the concentration used in the current study. TMS treatment might decrease the number of *B. pseudohinzii* organisms to which the litter potentially was exposed prior to cross-fostering. As a result of strict adherence to the 24-h policy and pretreatment of TLR4 breeding animals with TMS, no other litters have been positive for *B. pseudohinzii* after cross-fostering.

At the beginning of the cross-foster rederivation study, a previously described 4-, 8-, and 12-wk testing scheme was used.[1] The first *B. pseudohinzii*–positive litter was identified at the 4-wk PCR test. This result prompted us to revise the testing time points to 2, 4, and 8 wk. With this change in testing, positive litters were detected at the 2-wk fecal PCR test. At this time point, the fecal sample is predominantly from the surrogate dam, suggesting that the contaminated litter was capable of transmitting *B. pseudohinzii* to the surrogate dam soon after cross-fostering. All *B. pseudohinzii*–positive litters were identified at the first PCR test; conversely, all litters that were negative at the first *B. pseudohinzii* fecal PCR test remained negative. Currently, we perform 2-, 4-, and 8-wk testing; after a negative 8-wk test, the litter is moved into a separate *B. pseudohinzii*–free colony room in the barrier suite. This room is tested monthly through pooled fecal PCR. The rederivation of a *B. pseudohinzii*–negative colony started in June 2015. Aside from the 5 positive litters that were identified in the initial study, the *B. pseudohinzii* barrier has remained negative (current census, approximately 230 cages). This success is attributed to allowing only sterilized supplies into the suite of rooms, restricting animal care and investigator personnel access, maintaining strict room entry order (the *B. pseudohinzii*–negative suite is entered prior to any other room that house rodents or where rodent procedures are performed), and testing according to the described paradigm. These practices have enabled us to maintain a *B. pseudohinzii*–negative colony within a facility where *B. pseudohinzii* is enzootic.

CRL:CD1-Elite mice were used as surrogates because they breed well and are good mothers; anecdotally CD1 mice may be more susceptible than are C57BL/6 mice to infection with *B. pseudohinzii*. In the rederivation arm of the study CRL:CD1-Elite surrogate dams became infected from contaminated litters within 2 wk of cross-fostering. During cohousing for the antibiotic arm of the study, the CRL:CD1 mice became *B. pseudohinzii* positive faster than did the C57BL/6J mice. Moreover, CRL:CD1 dirty-bedding sentinel mice in general colony rooms have been fecal-PCR positive for *B. pseudohinzii*.[4] Until more is known about strain susceptibility to *B. pseudohinzii*, we recommended the use of CD1 mice as surrogate mothers in light of their natural maternal behavior and because they appear to be an effective contact sentinel that can be fecal-PCR positive for *B. pseudohinzii* within 2 wk of exposure to a contaminated litter.

Antibiotic administration in the drinking water was evaluated because it has been successfully used to eliminate *Pasteurella pneumotropica* and because it allows for the treatment of large numbers of mice simultaneously.[5,20] The disadvantage of using this method as a form of eradication is that it can be difficult to administer therapeutic doses that reach clinically significant plasma concentrations (for example, due to precipitation in the water, degradation, and poor palatability).[14,15] The initial high rate of negative fecal-PCR tests for *B. pseudohinzii* was promising; however mice quickly became PCR-positive for *B. pseudohinzii* during the 2- to 4-wk posttreatment period. The few cages that were negative at the final time point might also have become positive for *B. pseudohinzii* if further testing had been performed. *Bordetella* species are able to form biofilms in the nasal cavity and can live within phagocytic cells, both of these features make animals susceptible to becoming carriers.[6] We surmise that antibiotic administration decreased or temporarily stopped *B. pseudohinzii* shedding so that it could not be detected by fecal PCR; however animals were still colonized. Increased doses or an extended duration of treatment might prove to be efficacious for eradicating *B. pseudohinzii*; however this requires further investigation. The provision of antibiotics in the drinking water may also decrease the number of *B. pseudohinzii* organisms and associated pulmonary inflammation and should be investigated with the understanding that antibiotic administration is a nonexperimental variable that might influence other aspects of pulmonary research.

B. pseudohinzii has the potential to confound pulmonary research, and its eradication should be considered for colonies of mice used for the study of pulmonary disease. The current study demonstrated that cross-foster rederivation of litters younger than 24 h can be used successfully to eradicate *B. pseudohinzii*. Sterilized supplies, restricted room access, strict room entry order, and PCR testing as early as 2 wk after cross-fostering are critical to maintaining a *B. pseudohinzii*–negative mouse colony after cross-foster rederivation. Although additional studies need to be conducted to determine effectiveness, the provision of TMS in the drinking water of immunocompromised donor dams may decrease the bioburden to which litters are exposed prior to cross-fostering.

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