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Evaluation of Therapeutic Approaches for the Treatment of *Spironucleus muris* in Mice

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***Spironucleus muris* is an intestinal protozoal pathogen that can infect various species of rodents. The infection can have a wide range of clinical presentations, from no signs of disease to death. In addition, this pathogen can adversely affect research results, especially immunologic and gastrointestinal studies. For these reasons, institutions may exclude *Spironucleus muris*. However, despite rigorous efforts to keep this pathogen out, it can be common in rodent colonies. The current recommended approach to eradicating this pathogen is by testing and culling positive animals. A similar organism, *Giardia muris*, has been effectively eliminated by using chemotherapeutics. Therefore, the objective of this study was to determine whether *S. muris* is also susceptible to chemotherapeutics. Naturally infected mice were randomized to treatment groups after confirmation of positive infection via PCR. Mice received either metronidazole, fenbendazole, a combination of metronidazole-fenbendazole, or acidified water (control) treatments for a period of 4 wk. Each week fecal testing of *S. muris* was performed via PCR to evaluate the effectiveness of the treatments. At the end of the 4 wk period, mice were euthanized via CO_2 inhalation and segments of the proximal gastrointestinal tract were submitted for histopathologic analysis. Treatment with metronidazole or fenbendazole alone or in combination, failed to clear *S. muris* infected mice. After 4 wk of treatment, none of the mice given fenbendazole via sucralose medicated gel were positive by either PCR or histopathology; however, this finding is most likely due to intermittent shedding rather than chemotherapeutic success. Therefore, the recommendation remains to test-and-cull or rederive mice as necessary to eliminate *S. muris* from laboratory animal facilities.**

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Every laboratory animal facility attempts to prevent unwanted pathogens from entering their facility. However, even with strict application of rigorous standard operating procedures, pathogens will sometimes enter facilities and infect research animals. This can have adverse effects on both animal wellbeing and research. One pathogen on the exclusion list of most laboratory animal facilities is *Spironucleus muris (S. muris)*.

S. muris (formerly *Hexamita muris*) is a protozoan parasite with a pear-shaped, multiflagellated (6 anterior and 2 posterior), binucleated, feeding trophozoite form and an infectious binucleated cyst form.[7,11,21] *S. muris* has a direct life cycle, meaning ingestion of the cyst(s) will result in infection.[38] The cysts release trophozoites that will colonize the crypts of Lieberkühn, resulting in blunting of the microvilli of the small intestines.[1,11,25,35] Blunted microvilli have also been demonstrated in infected x-irradiated male Holtzman rats.[15] Coinfection of Sha-Sha, CBA, and BALB/c mice with *Giardia muris* and *S. muris* resulted in increased gastrointestinal epithelial crypt depth, higher cell turnover rate of the villious structures of the gastrointestinal tract, and increased intraepithelial lymphocytes.[26] Although *S. muris* rarely causes mortality, it can cause severe morbidity, especially in young and immunocompromised mice. Infection can result in weight loss, runting, chronic enteritis, gas and fluid stasis in the intestines, increased enterocyte turnover, and hyperplasticity of the intestinal crypts.[1,5,12,25,35] Clinically, these effects can manifest as diarrhea, dehydration, weight loss, rough hair coat, lethargy, abdominal distension, hunched posture, or death.[1,5,12,25,35]

S. muris can also affect the immune system. In general, parasites establish a relationship with the host via effector molecules that can enhance or depress host immunologic responses, allowing the parasites to continue proliferating.[9] CBA strains of mice generate a diminished antibody response to sheep red blood cells 2 to 3 wk after infection with *S. muris*, which is the time of maximal trophozoite burden.[6] Macrophages from C57BL/6 mice heavily infected with *S. muris* can also exhibit altered metabolism and decreased reactivity to calf serum.[20]

Due to potential effects on rodent colony health and research reproducibility, *S. muris* should be excluded from rodent colonies. Currently, the recommendation is to test and cull, or rederive infected mice.[30] Previous studies have shown that treatment with metronidazole or combination therapy of metronidazole and fenbendazole are efficacious against *Giardia muris*, a related pathogen in the same family as *S. muris*.[4,10] Another study has shown successful treatment of unrelated flagellate species *Tritrichomonas muris* and *Tetratrichomonas microta* with metronidazole.[32] One report found that dimetridazole, a compound similar to metronidazole, may be effective in eliminating *S. muris*.[5] Metronidazole belongs to the nitroimidazole class of drugs that is thought to disrupt DNA synthesis, resulting in antibiotic and antiprotozoan properties.[3] Fenbendazole is a benzimidazole derivative that targets β-tubulin, which constitutes important cytologic structures such as mitotic spindles, the cytoskeleton, flagella, and cilia.[19] Therefore, the present study was performed to determine if fenbendazole, metronidazole, or a combination of the 2 drugs would be effective in eliminating *S. muris* infection in mice.

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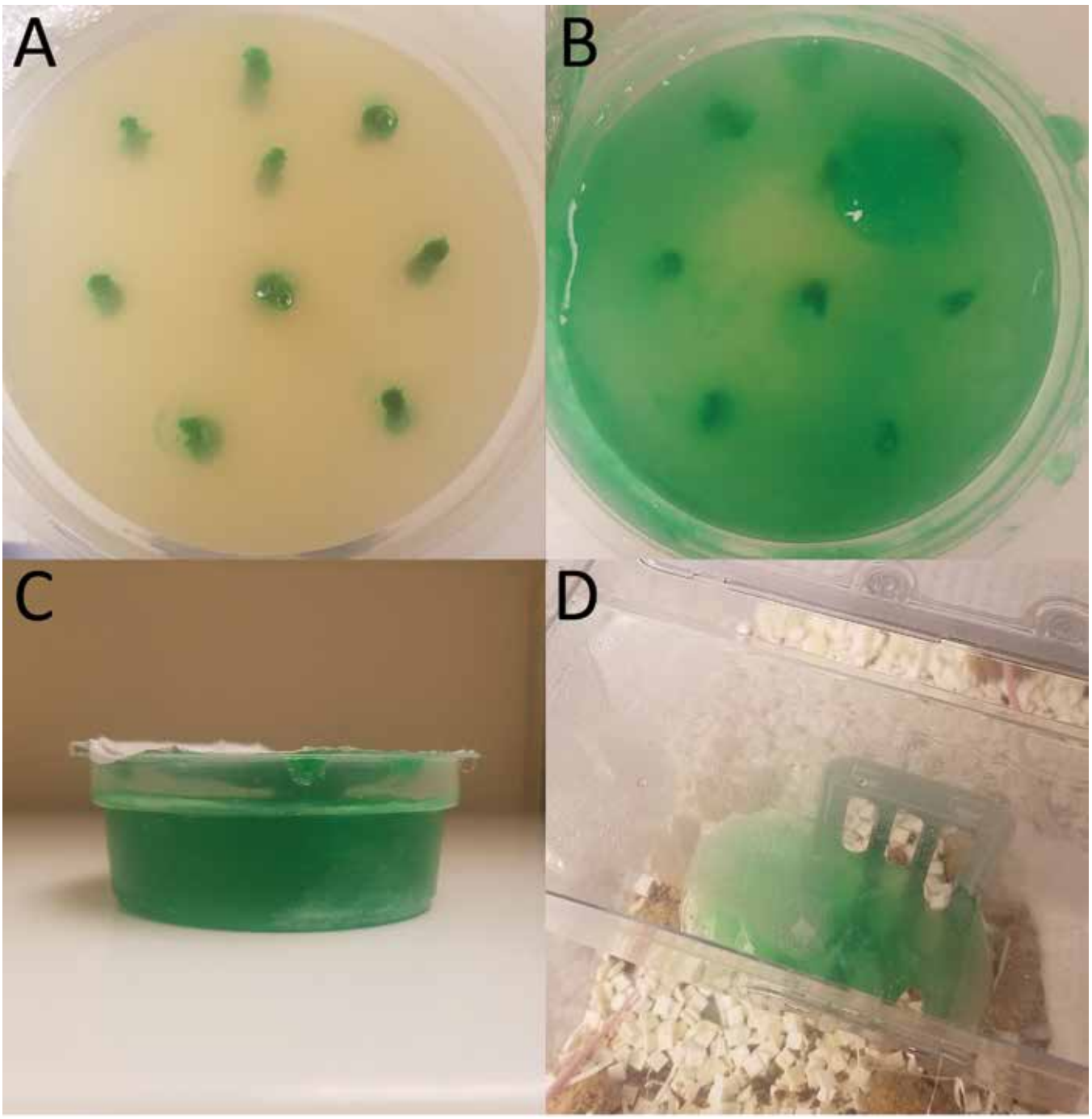

Figure 1. Preparation of medication infused sucrose gel. A) Metronidazole with green dye infused into sucralose medication gel via 1 mL syringe. B) Metronidazole with green dye 60 s after insertion and shaking to homogenize drug within sucralose medicated gel. C) Metronidazole with green dye in sucralose medication gel after overnight refrigeration. D) Metronidazole with green dye in sucralose medication gel one day post following placement in the food hopper.

Materials and Methods

Animals, Housing Conditions, and Diet. Three male mice were obtained from a colony of Six2-GFP::Cre$^{tg/+}$ (Six2^{GC}) mice on a CD1 (ICR) background infected with *S. muris*; they were also positive for *Helicobacter spp.* and MNV, as determined by fecal PCR dirty bedding sentinel testing. These 3 mice were bred with 6 wild type CRL:CD1(ICR) female mice (Strain Code 022, Charles River, Wilmington, MA) to generate offspring that were naturally infected with *S. muris* via ingestion of parental feces. Pups were weaned and separated into single sex groups. Mice were maintained in an AAALAC-accredited facility in accordance with *The Guide for the Care and Use of Laboratory Animals 8th Edition*.[17] All procedures for animal use were approved by the Tulane University IACUC.

Mice were maintained in disposable primary static enclosures (Innocage Mouse Pre-Bedded Alpha-dri, Innovive, San Diego, CA) in a quarantine facility. Complete cage changes occurred weekly. Mice were maintained on a 12:12 light-dark cycle. Temperature (20–26°C; 68–79°F) and humidity (30–70%) were maintained with standards outlined in *The Guide for the Care and Use of Laboratory Animals 8th Edition*.[17] Strict microisolation technique was used throughout the experiment to prevent any

Table 1. Summary of Positive *S. muris* PCR and Histology Results

	Pre-Treatment	Week One	Week Two	Week Three	Week Four	Histology
Met-Gel (500 mg/kg)	5/5	5/5	3/5	0/4[a]	2/4	1/4
Fen-Gel (50 mg/kg)	5/5	5/5	5/5	3/3[a]	0/3	0/3
Met (500 mg/kg) Fen (50 mg/kg) Gel	5/5	5/5	5/5	3/5	5/5	5/5
H_2O-Gel	4/4	4/4	4/4	1/4	4/4	4/4
Fen-Oral (50 mg/kg)	9/9	9/9	9/9	7/9	9/9	8/8[a]
H_2O-Oral	3/3	3/3	3/3	3/3	3/3	3/3

[a]Indicates animal(s) died within group

cross-contamination between cages. Mice had free access to rodent chow (Ref number 5053 Irradiated Laboratory Rodent Diet, Purina, Richmond, IN); food pellets were placed on the cage floor and replenished every 3 d. Only mice given oral gavage of fenbendazole or acidified water had free access to acidified water (Aquavive Mouse Pre-Filled Acidified Water Bottle, Innovive, San Diego, CA). Mice receiving the drugs via sucralose medicated gel had only the gel as their source of hydration. The sucralose gel (2 oz (60 mL) MediGel Sucralose, ClearH2O, Portland, ME) was infused with metronidazole, fenbendazole, combination of metronidazole/fenbendazole, or acidified water (pH of acidified water approximately 2.8) and was provided to the mice in the food hopper,

Drug Delivery. Mice (n = 4 to 5) were divided into 4 different groups: metronidazole infused sucralose medicated gel treatment group (Met-Gel), fenbendazole infused sucralose medicated gel treatment group (Fen-Gel), metronidazole and fenbendazole infused sucralose medicated gel treatment group (Met/Fen-Gel), and acidified water infused sucralose medicated gel (H_2O-Gel). A follow-up study was performed for the fenbendazole group which showed promising results. In this study, fenbendazole (Fen-Oral; n = 9) or acidified water (H_2O-Oral; n = 3) was administered via oral gavage.

Drug infused sucralose medicated gel was prepared using the total number of milligrams for each drug necessary to achieve consumption of the targeted therapeutic dose assuming consumption of 7 mL of gel per mouse per day, a 60 mL container of Medigel and an average body weight of 30 g. Drug dosages were as follows: 0.5 mL of 250 mg/mL Metronidazole (as Benzoate) Peanut Butter Flavored Suspension (Wedgewood Village Pharmacy LLC, Swedesboro, NJ) was mixed with one drop of green food coloring dye (Mint Green, McCormick, Hunt Valley, MD; Figure 1) and then infused into the sucralose medicated gel using a 1 mL syringe (Covidien, Minneapolis, MN); 0.5 mL of 25 mg/mL Fenbendazole Peanut Butter Flavored Suspension (Wedgewood Pharmacy LLC, Swedesboro, NJ) was mixed with one drop of red food coloring dye (Dusty Rose, McCormick, Hunt Valley, MD) then infused into the sucralose medicated gel using a 1 mL syringe; 0.5 mL of metronidazole and 0.5 mL of fenbendazole were combined with one drop of blue food coloring dye (Pretty Purple, McCormick, Hunt Valley, MD) then infused into the sucralose medicated gel using a 1 mL syringe; 0.5 mL of acidified water was mixed with one drop of yellow food coloring dye (Orange Sunset, McCormick, Hunt Valley, MD) then infused into the sucralose medicated gel using a 1 mL syringe. The doses used in our study were previously identified as effective in mice for the elimination of *Giardia muris*, (500 mg/kg metronidazole and 50 mg/kg fenbendazole).[4,10] Sucralose medicated gels were shaken vigorously for 30 to 60 s. The shaken sucralose medicated gels were refrigerated overnight and administered the following day (Figure 1 A through D). Mice were acclimated to the sucralose gel infused with food coloring dye (no medication added) prior to beginning treatment to assure that the taste or coloration of the food dye was not aversive. Sucralose medicated gels were replaced every other day for a period of 4 wk. Oral gavage was performed using a 1.5 in, 20 gauge gavage needle (Poppers and Sons, New Hyde Park, NY) once daily for 4 wk. In the Fen-Oral group, 0.06 mL of the 25 mg/mL fenbendazole suspension was administered. In the H_2O-Oral group, 0.06 mL dose of acidified water was administered.

Samples Submission and Analysis. Feces were collected from each mouse prior to starting treatment, and were submitted for PCR analysis (IDEXX BioAnalytics, Columbia, MO) to confirm infection. Fecal samples were collected weekly from each individual mouse. Sample analysis was carried out via PCR (IDEXX BioAnalytics, Columbia, MO) as previously described.[18] At the end of the 4 wk treatment period, mice were euthanized using CO_2 in accordance with *The Guide for the Care and Use of Laboratory Animals 8th Edition* and *AVMA Guidelines for Euthanasia 2013 Edition*.[17,24] The distal stomach and proximal duodenum were collected at necropsy and placed in formalin. The samples were submitted for histopathologic analysis by a veterinary pathologist (IDEXX BioAnalytics, Columbia, MO). Histopathology was also performed on the stomach and proximal intestines to detect any inflammation or morphologic changes due to the presence of *S. muris*.

Results

All mice were positive for *S. muris* prior to beginning treatment. All mice remained positive for *S. muris* after one week of treatment. All mice, except for 2 in the Met-Gel group, were positive for *S. muris* after 2 wk of treatment. After 3 wk of treatment, Met-Gel, Met/Fen-Gel, H_2O-Gel, and Fen-Oral groups had some mice that were negative for *S. muris* (4 of 4 mice, 2 of 5 mice, 3 of 4 mice, and 2 of 9 mice, respectively). The Met-Gel group was completely negative for *S. muris* after 3 wk of treatment, but, at 4 wk of treatment 2 mice again tested positive and 1 mouse was positive on histology. All remaining groups except for the Fen-Gel group were positive after 4 wk of treatment, as confirmed via PCR and histology. All mice in the Fen-Gel group were negative, based on PCR and histology results. Four of the 31 mice died during the course of the study (1 of 5 in the Met-Gel group and 2 of 5 in the Fen-Gel group at week 3, 1 of 9 in the Fen-Oral group at week 4). A summary of the PCR and histology results is provided in Table 1. Histopathologic analysis revealed *S. muris* on cut section primarily in the pyloric junction. *S. muris* demonstrated a preference for the gastric glands and intestinal crypts (Figure 2). No evidence of inflammation or morphologic changes were noted in association with *S. muris*,

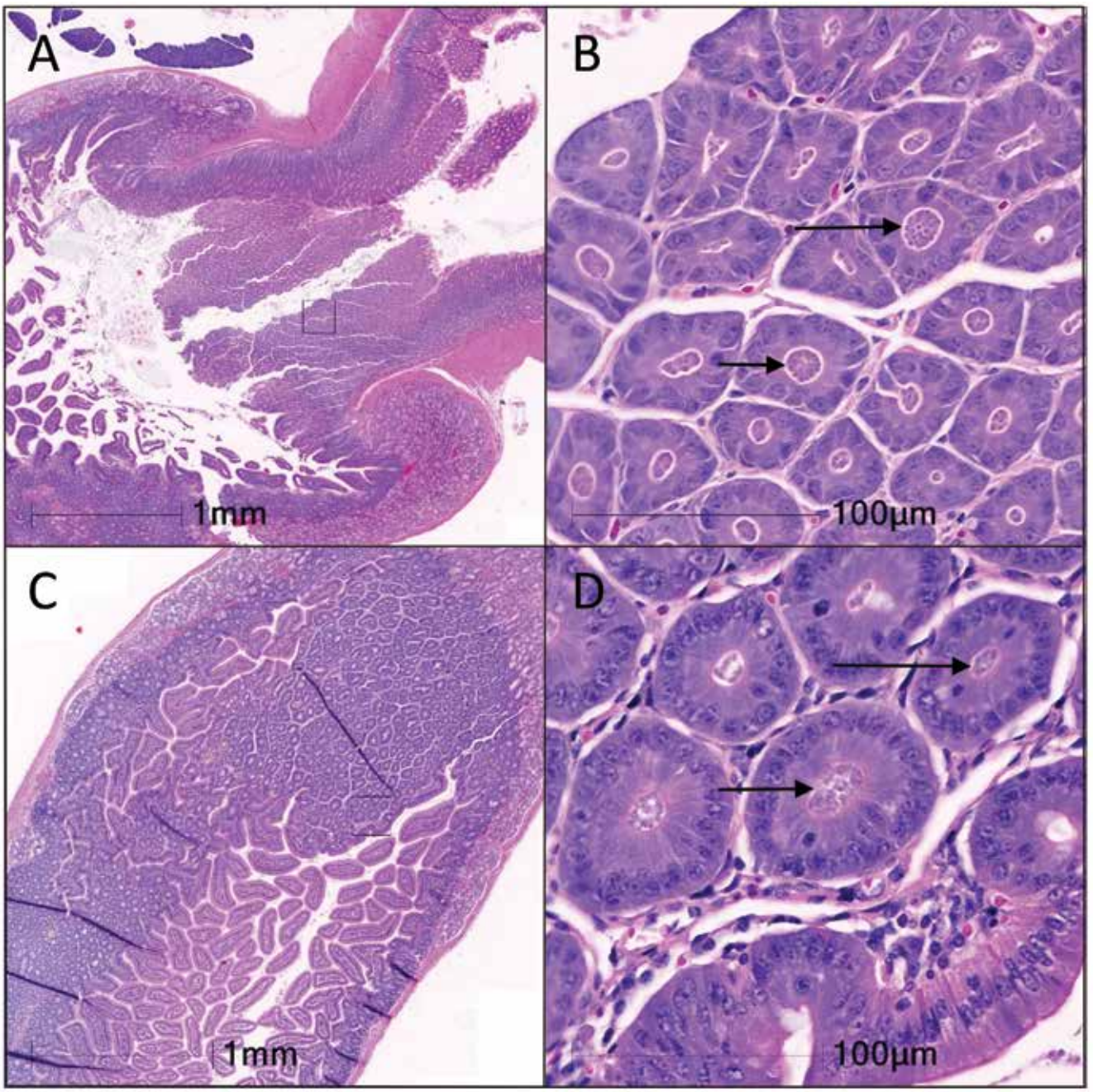

Figure 2. Histopathology of *S. muris*. Organisms were present in the gastric glands of the pylorus (A and B) and less frequently in the crypts of Lieberkühn of the duodenum (C and D). The black boxes in A and C indicate the regions shown in B and D. The arrows in B and D highlight *S. muris* organisms.

although *Kazachstania sp.*, a gastric yeast, was identified in the stomach of several mice.

Discussion

Maintaining a specific pathogen-free facility is important for animal health, and experimental reproducibility. We evaluated 3 potential therapeutics for the treatment of *S. muris*, which can cause morbidity, rare mortality, and altered research outcomes, especially in gastrointestinal and immunologic studies. The current prevalence of *S. muris* in laboratory animal facilities in the United States is unknown. Previous studies have reported a 58% to 98% prevalence of *S. muris* in laboratory rodents in the UK and Brazil.[14,37] This prevalence was higher than that found in wild rodents surrounding a laboratory animal facility in another study.[28] In these previous studies, parasitic infection was confirmed by either direct observation of trophozoites from the small and large intestines by wet mount light microscopy or by fecal flotation. The specificity of these methods is unknown, but sensitivity for direct visualization by light microscopy of the intestinal contents is 71%.[30] The sensitivity for detection by fecal floatation is unknown. The lower sensitivity of these diagnostic techniques may have underestimated the actual prevalence, as determined by more recently developed PCR methods.[13,18]

In this study we investigated the effectiveness of fenbendazole and metronidazole against *S.muris*. These drugs are effective treatments against *Giardia* in mice, a protozoan parasite of the same family as *S. muris*.[4,10] In addition, metronidazole has

been shown to reduce the growth and organismal load of other *Spironucleus* species in parrots and fish.[8,31,33] The first part of this experiment used sucralose medicated gel to deliver the drugs. The Fen-Gel showed promising efficacy against *S. muris*, with all remaining animals of that treatment group testing negative on histology and PCR. However, concern for false negatives prompted further investigation, so a follow-up oral gavage treatment experiment was added to the study. Metronidazole was also potentially effective, with only one mouse positive by histology/PCR and one mouse positive by PCR alone. However, because not all mice were negative in this group, we considered the possibility that these mice had become reinfected after a 4 wk treatment regimen. Contrary to a previous study showing the effectiveness of combined metronidazole and fenbendazole therapy for the treatment of *Giardia* in mice,[4] our combination therapy did not show the same effect, and all mice remained positive in this group. Four of the 31 mice died during the experiment (1 of 5 in the Met-Gel group and 2 of 5 in the Fen-Gel group at week 3, 1 of 9 in the Fen-Oral group at week 4) We did not identify a cause for these deaths based on gross necropsy; however, the parasite itself has previously been reported to cause death in mice.[1,5,12,25]

Fecal PCR did not detect *S. muris* in several treated and control animals. Most of these false negatives occurred during the third week of treatment. This could be due to low or undetectable parasitic load (less than 1/µL),[18] from effective treatment followed by reinfection, the ability of the immune system to suppress the organism, the number of fecal pellets submitted per animal, or the intermittent shedding of cysts. Failure to detect organisms should not be mistaken for eradication of the organism. Therefore, repeat testing is important, as no test is 100% specific or 100% sensitive for *S. muris*, and the potential for false negatives is high for several testing modalities. Furthermore, this study could be improved by individually housing mice to minimize chances of possible reinfection. Another concern regarding this study was the effectiveness of metronidazole and fenbendazole to have complete equilibrating solubility in the sucralose gel. Poor equilibration could result in under-dosing or over-dosing of medication, which could explain discrepancies between the effectiveness of these drugs in treating other pathogens and the discrepancy between the efficacy of combination therapy as compared with treatment with a single drug. We did not measure the amount of gel consumed. However, consumption of metronidazole may have been lower due to its bitter taste, even when masked with peanut butter flavor and sucralose.

Because of a concern that the sucralose medicated gel delivery system did not achieve intake of the correct daily dosage, a follow-up experiment with oral gavage drug administration was performed to ensure accurate dosing of each individual mouse. Metronidazole was not used as an oral gavage treatment because given the necessary high concentration, the solution was too viscous to deliver via a standard oral gavage needle. A more dilute compounded solution would require a volume larger than the capacity of the mouse stomach to reach the targeted dose. The concentration of the intravenous formulation of metronidazole was also inappropriate for this study. In addition, limited information is available on bioavailability of the intravenous metronidazole formulation when administered orally. After 4 wk of daily oral gavage treatment with fenbendazole, all mice remained positive as confirmed by PCR and histology.

Our data indicate that fenbendazole, metronidazole, or a combination of metronidazole and fenbendazole are not efficacious for eradication of *S. muris* in mice at the tested doses and routes of administration. However, this study may present potential therapeutic options for reducing the *S. muris* burden in individual mice. Our results are consistent with a previous published in vitro study showing that albendazole, a related benzimidazole, was an ineffective treatment for *S. muris*.[29] However, our results do not agree with the previous study regarding efficacy of metronidazole against *S. muris*.[29] This difference in results could be a consequence of the complexity of in vivo experiments, which cannot be accurately conducted in vitro. Therefore, the recommendation for eradication of *S. muris* from an infected colony is to test, cull, and rederive mice as necessary. Proper decontamination of rooms and equipment is also necessary to fully eradicate the pathogen from a facility. When applied for an appropriate contact time, most disinfectants, as well as high temperatures (above 45 °C), are sufficient for desiccation of *S. muris*.[23] Further studies will be necessary to assess the efficacy of these drugs at higher therapeutic doses, their pharmacokinetics and toxic effects, the efficacy of other similar drugs, and more effective mechanisms of drug delivery. In addition, studies should be performed to validate drug delivery systems and to ensure adequate mixing of drug solutions used in medicated gels. Studies could also use other strains and species that can harbor *S. muris*, most notably the rat and hamster, as the transmission of *S. muris* and host immunologic response to *S. muris* is different among these animals.[2,5,11,16,22,27,34,36]

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