Journal of the American Association for Laboratory Animal Science
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Vol 63, No 4
July 2024
Pages 412–417

Polymerase Chain Reaction on In-cage Filter Paper at Different Time Points to Detect *Helicobacter* spp.

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***Helicobacter* spp. infections in mice can have broad-ranging effects on gastrointestinal, reproductive, and immune systems. This can introduce significant confounding variables for research and may reduce scientific rigor. Screening mouse colonies for *Helicobacter* species can be accomplished via noninvasive PCR testing on filter paper placed in animal-free dirty bedding sentinel cages. In our facility, one tablespoon of dirty bedding from each cage on a rack is added to a designated sentinel cage every 3 wk at cage change, and PCR testing is performed on in-cage filter paper quarterly. We hypothesized that cages that received *Helicobacter* spp.-positive bedding at later time points would have a lower detection rate of *Helicobacter* spp. with PCR testing compared with cages that received positive bedding at earlier time points due to the filter paper becoming saturated. To determine if screening would be able to detect one positive row of cages on a rack, 9 tablespoons of *Helicobacter*-positive bedding and 71 tablespoons of negative bedding were added at the 3-, 6-, or 9-wk time points to 14 empty sentinel cages per time point. Negative bedding was added every 3 wk to cages not scheduled to receive positive bedding. Negative controls received 80 tablespoons of negative bedding and positive controls received 80 tablespoons of positive bedding at each time point. Filter paper was tested via PCR for *Helicobacter* spp. at 12 wk. All positive controls tested positive, and all negative controls tested negative. Two 3-wk cages, two 6-wk cages, and three 9-wk cages were positive, indicating no difference between time points. This resulted in a 16.7% *Helicobacter* spp. detection rate. These results indicate that PCR on in-cage filter paper may not be reliable in detecting low levels of *Helicobacter* spp. nucleic acid in dirty bedding.**

Abbreviation and Acronym: EHM, environmental health monitoring

DOI: 10.30802/AALAS-JAALAS-24-000001

Introduction

Helicobacter spp. are gram-negative spiral to curved rod-shaped bacteria spread via the fecal-oral route that colonize the gastrointestinal tracts of multiple species.[11,29] *Helicobacter* species that infect mice include *H. bilis*, *ganmani*, *hepaticus*, *muridarum*, *mastomyrinus*, *rappini*, *rodentium*, and *typhlonius*.[30] *Helicobacter* spp. infections are used experimentally in mouse models to mimic human inflammatory bowel disease (IBD) and can also cause IBD in natural infections of various mouse strains.[10,23,28] *Helicobacter* spp. infections in immunocompetent mice rarely cause overt disease but can confound research through effects on the immune and reproductive systems and impacts on tumor growth.[3,7,13,14,16,21,24-27] Infections in immunocompromised mouse strains can lead to colitis, typhlitis, rectal prolapse, and diarrhea.[10,12,19,28,29] Due to the increased use of immunocompromised mouse strains, which may experience clinical disease, as well as confounding effects on research, many research institutions have initiated efforts to exclude *Helicobacter* spp. from their facilities.[5] This has resulted in a need for quick and accurate detection of *Helicobacter* spp. infections in mouse colonies. Traditional surveillance for *Helicobacter* spp. in laboratory mouse colonies has been achieved through PCR on fecal pellets or serology of live sentinel mice exposed to dirty bedding.[18,30] More recently, PCR on in-rack or in-cage filters exposed to air or dirty bedding from colony animals has been validated for *Helicobacter* spp. detection.[15,18,20] These types of environmental health monitoring (EHM) have eliminated or reduced the need for live animal sentinels, which allows for replacement of animals with nonanimal alternatives in accordance with the 3Rs of humane animal research.[22]

Previous work has validated PCR on in-cage filter media as a viable method to screen mouse colonies for *Helicobacter* spp.[15,18,20] However, there are no published studies investigating the effect of time of testing after exposure to *Helicobacter* spp.-positive bedding on the detection rate of this test. For the purposes of this paper, detection rate is defined as the number of cages positive via PCR divided by the total number of positive cages. At our institution, bedding from each cage on a rack is added to a sentinel cage containing in-cage filter media at each change out, which occurs every 3 wk. The filters are tested quarterly. We hypothesized that filters could become saturated with non-*Helicobacter* spp. nucleic acids throughout the quarter. This would theoretically limit the ability of *Helicobacter* spp. nucleic acids to bind to the filter media and subsequently decrease the PCR detection rate. If *Helicobacter* spp.-positive bedding had been added late in the quarter, PCR would have decreased detection rate. In this study, we investigated the effect of time of adding *Helicobacter* spp.-positive bedding on PCR detection rate. Positive bedding was added at 3 different time points: 3 wk (group A), 6 wk (group B), or 9 wk (group C). *Helicobacter*

Submitted: 02 Jan 2024. Revision requested: 24 Jan 2024. Accepted: 20 Mar 2024.
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spp.-negative dirty bedding was added at all other time points, and filter media from each cage were tested at 12 wk. We hypothesized that group C would have a decreased detection rate of *Helicobacter* spp. PCR compared with groups A and B.

In addition, many of the previously published studies investigated detection rate of PCR on in-cage filter paper in populations with high prevalence or endemic *Helicobacter* spp. infection.[8,15,20] There is little evidence regarding detection rate of the test in a low prevalence population. The 1:9 *Helicobacter* spp. positive-to-negative bedding ratio used in this experiment results in dilution of *Helicobacter* spp. nucleic acids and likely leads to the filter paper being exposed to fewer *Helicobacter* spp. copy numbers compared with studies using 100% positive bedding. This could represent scenarios such as low prevalence of *Helicobacter* spp. infection on a rack, dirty bedding added from the rack containing low copy numbers due to sample selection (that is, sample not containing fecal pellets) or other factors leading to dilution of nucleic acids. At our institution, racks in rooms with active experimental manipulation of animals may contain multiple principal investigators, each with an average of one row of cages on the rack. Therefore, we added approximately 9 tablespoons of positive bedding and 71 tablespoons of negative bedding to each sentinel cage to represent approximately one row of positive cages per side of an animal housing rack. By adding *Helicobacter* spp.-positive bedding at 3-, 6-, and 9-wk time points, we sought to determine if we could detect *Helicobacter* spp.-positive cages added to a rack at different time points in relation to the time of filter media collection for PCR. This could represent scenarios such as positive animals housed on a rack for a short period of time, acute use of positive animals, or positive animals added to a rack shortly before filter paper collection, in the case of the 9-wk time point.

The present study seeks to determine the effect of time of testing on *Helicobacter* spp. PCR on in-cage filter paper exposed to low levels of *Helicobacter* spp. nucleic acids, which are both significant knowledge gaps in the field of EHM. Addressing this lack of information could improve overall animal welfare by ameliorating *Helicobacter* spp. screening practices, which can be pathogenic in immunocompromised mice. Finally, this study has the potential to increase the reproducibility of research through improved detection of a significant confounding factor, subclinical *Helicobacter* spp. infection in mice.

Materials and Methods

Sample size calculation. A power analysis was performed assuming all cages in the positive control group were positive, an α of 0.05, and an equal number of cages in each group.[9] With possible percentages of positive test cages of 20%, 40%, and 60%, we require 6, 9, and 14 cages per group, respectively, to achieve 80% power as determined by Fisher exact test. We elected to use 14 cages per group to ensure adequate power.

Soiled bedding collection and cage handling procedures. The cages in this project were bedded and handled in accordance with the University of Oklahoma Health Sciences Center standard operating procedure for sentinel health monitoring. In our facility, PCR monitoring for *Helicobacter* spp. and other pathogens is done on a quarterly basis using filter media (Allentown, Allentown, PA) placed in sentinel cages with approximately one tablespoon of dirty bedding (Biofresh; Animal Specialties and Provisions, Quakertown, PA) added from each cage on the rack at each cage change (Figure 1). Per institutional standard operating procedures, cage change outs occur every 3 wk.

For this project, dirty bedding from mouse IVC in a known *Helicobacter* spp.-positive room and dirty bedding from mouse IVCs in a *Helicobacter* spp.-negative barrier facility were collected. Both facilities housed a variety of mouse strains, sexes, and ages on a variety of experimental and breeding protocols. In rooms housing animals belonging to multiple investigators, each investigator may have a small number of cages, such as one row on a rack. Fully occupied IVC racks in our facility contain 8 to 10 cages per row, with an average of 80 cage spots per side of the rack. The median number of cages per row is 9. To determine if screening would be able to detect one positive row of cages on a rack, 9 tablespoons of *Helicobacter*-positive bedding and 71 tablespoons of negative bedding were added at the 3-, 6-, or 9-wk time points to 14 empty disposable cages (Allentown, Allentown, PA) per time point (Figure 2). This approximates 9 *Helicobacter* spp.-positive cages and 71 *Helicobacter* spp.-negative cages on a rack. Negative bedding was added every 3 wk to cages not scheduled to receive positive bedding. Fourteen negative control cages received 80 tablespoons of negative bedding and 14 positive control cages received 80 tablespoons of positive bedding at each time point.

Figure 1. Experimental cage with dirty bedding and filter medium. According to the University of Oklahoma Health Sciences standard operating procedures, each cage contains one tablespoon of dirty bedding from each cage on the rack and filter medium for PCR detection of infectious agents. Dirty bedding is added to the sentinel cage every 3 wk at cage change out. Filter media are tested quarterly for a list of defined excluded pathogens.

In accordance with institutional standard operating procedures, at each time point, bedding from the previous time point was dumped and any remaining dust was removed using a Kimtech Science Kimwipes (Kimberly-Clark, Roswell, GA). After dirty bedding was added, cages were thoroughly mixed using a plastic knife that was sanitized between cages with Peroxigard (Oakville, ON). Cages were then shaken 3 to 5 times with the lids closed to ensure the in-cage filter media

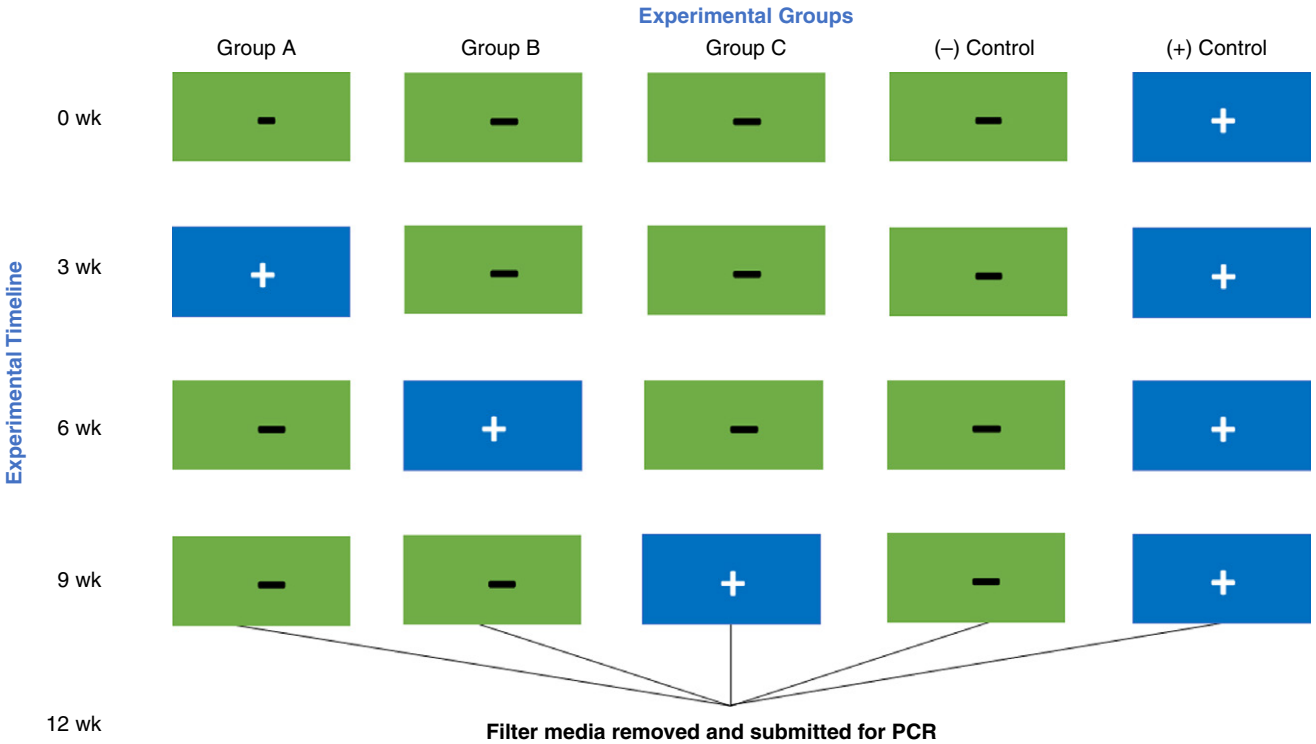

Figure 2. Experimental design and timeline. Each box represents each experimental group (5 groups, n = 14 per group) at each time point. Group A cages were exposed to *Helicobacter* spp.-positive bedding at the 3-wk time point. Group B cages were exposed to *Helicobacter* spp.-positive bedding at the 6-wk time point. Group C cages were exposed to *Helicobacter* spp.-positive bedding at the 9-wk time point. Blue boxes indicate that the cages were exposed to *Helicobacter* spp.-positive bedding. Green boxes indicate that the cages were exposed to *Helicobacter* spp.-negative bedding only. Filter media were present in each cage starting on day 0. All filter media were removed and submitted for PCR at the 12-wk time point.

were completely exposed to bedding. Cages were maintained on an IVC rack (Allentown, Allentown, PA) for the duration of the study. To minimize the risk of cross-contamination, cages containing only negative bedding were handled before cages with positive bedding. At 12 wk, filters were removed from the cages and submitted to a third-party testing company (IDEXX BioAnalytics, Columbia, MO) for PCR testing for *Helicobacter* spp.

PCR. Total nucleic acids were extracted from collection media using a commercially available platform (NucleoMag VET Kit; Macherey–Nagel, Düren, Germany). Fluorogenic real-time PCR assays were performed according to the IDEXX BioAnalytics proprietary service platform (IDEXX Laboratories, Westbrook, ME). Real-time PCR analysis was performed at IDEXX BioAnalytics (Columbia, MO) with standard primer and probe concentrations (Applied BioSystems, Waltham, MA), a commercially available master mix (LightCycler 480 Probes Master; Roche Applied Science, Indianapolis, IN), and a commercially available instrument (LightCycler 480; Roche Applied Science). Genus-wide assays were conducted to identify all *Helicobacter* spp., and species-specific assays were used to identify individual *Helicobacter* species, including *H. bilis*, *H. ganmani*, *H. hepaticus*, *H. mastomyrinus*, *H. rodentium*, and *H. typhlonius.* In addition to positive and negative controls for each real-time PCR assay, a multiplexed hydrolysis probe-based real-time PCR assay targeting a mammalian gene (18S rRNA) and a bacterial gene (16S rRNA) was performed for all samples to assess the recovery of amplifiable nucleic acid and confirm the absence of PCR inhibition. For the purposes of this study, any result with a copy number greater than zero was considered a positive cage.

Statistical analysis. All analyses were performed using Prism 9 for Windows (GraphPad Software, Boston, MA). Fisher exact tests were used to determine if there was a statistically significant difference between the numbers of positive and negative cages between groups A and B, A and C, and B and C. P less than or equal to 0.05 was considered significant. Linear correlations were quantified using a Pearson correlation coefficient, and P less than or equal to 0.05 was considered significant.

Results

Of the 14 cages in group A, 2 were positive for *Helicobacter* spp. on PCR at 12 wk. Two cages out of 14 in group B tested positive and 3 cages in group C tested positive. All 14 positive control cages tested positive, and all 14 negative control cages tested negative (Figure 3). The total genus-wide *Helicobacter* spp. copy numbers detected for positive and negative control groups were 67,657 and 0, respectively (Table 1). The total genus-wide *Helicobacter* spp. copy number detected for group A was 824, for group B was 374, and for group C was 545 (Table 1). *Helicobacter typhlonius*, *H. mastomyrinus*, *H. bilis*, *H. hepaticus*, and *H. ganmani* were detected in one or more samples and *H. rodentium* was not detected in any of the samples (Table 1). Overall detection rate of *Helicobacter* spp. PCR on group A, B, and C cages was 16.7% (7/42) (Table 2). The detection rate for groups A and B was 14.3% (2/14) (Table 2). The detection rate for group C was 21.4% (3/14) (Table 2).

There was no statistically significant difference between the number of positive and negative cages between groups A and B, B and C, or A and C (P = 1.0 for all comparisons). This suggests that the length of time (3, 6, or 9 wk) between introduction of infected bedding and PCR testing does not impact the detection rate of *Helicobacter* spp. PCR on in-cage filter media. There was no correlation between detection rate and *Helicobacter* spp. PCR copy number (r = –0.14, P = 0.9) for groups A, B, or C.

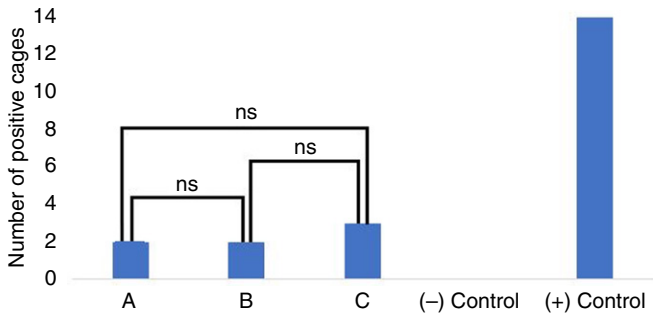

Figure 3. Number of *Helicobacter* spp.-positive cages per experimental group. Group A cages were exposed to *Helicobacter* spp.-positive bedding at the 3-wk time point, group B cages were exposed to *Helicobacter* spp.-positive bedding at the 6-wk time point, and group C cages were exposed to *Helicobacter* spp.-positive bedding at the 9-wk time point. In-cage filter paper from each cage was submitted for PCR at the 12-wk time point. Any result with a copy number greater than zero was considered a positive cage. All positive control cages were positive on PCR. Groups A, B, and C had few *Helicobacter* spp.-positive cages compared with the positive control group. ns, not significant ($P > 0.05$).

Discussion

Because the rate of cage positivity is not correlated with PCR copy number ($r = -0.14$, $P = 0.9$), this suggests that there was no relationship between time point of exposure to *Helicobacter* spp.-positive bedding (3, 6, or 9 wk) and copy number. It is therefore unlikely that *Helicobacter* spp.-positive bedding added later in the quarter would have a higher risk of going undetected compared with positive bedding added at earlier time points.

Overall detection rate among the 3 experimental groups was 16.7% and group A, B, and C detection rates were 14.3%, 14.3%, and 21.4%, respectively (Table 2). Previous studies on detection rates of *Helicobacter* spp. PCR on in-cage filter paper have used higher amounts of positive bedding, which resulted in 100% detection of *Helicobacter* spp.[15,20] The results of these previous studies are consistent with the 100% detection rate in the control group in the present study. An approximately 1:9 ratio of *Helicobacter* spp. positive-to-negative bedding was used for groups A, B, and C in the present study, whereas one previous study used a 1:4 ratio of dirty *Helicobacter* spp.-positive

Table 1. Estimated *Helicobacter* spp. copy number detected per cage

Experimental group	Cage ID number	*Helicobacter* spp.	Sum of *Helicobacter* spp. copy numbers	*H. bilis*	*H. ganmani*	*H. hepaticus*	*H. mastomyrinus*	*H. rodentium*	*H. typhlonius*
Group A	38	175	824	0	0	0	37	0	53
	42	649		0	19	9	92	0	108
	All other cages (n = 12)	0		0	0	0	0	0	0
Group B	46	150	374	0	0	0	18	0	21
	47	174		0	0	0	37	0	31
	All other cages (n = 12)	0		0	0	0	0	0	0
Group C	57	213	545	0	0	0	22	0	49
	62	158		0	0	0	31	0	86
	69	174		0	0	0	15	0	104
	All other cages (n = 11)	0		0	0	0	0	0	0
(–) Control	All cages (n = 14)	0	0	0	0	0	0	0	0
(+) Control	1	8,781	67,657	8	102	108	914	0	1,875
	2	2,438		0	39	30	297	0	383
	3	8,995		14	303	106	1,148	0	1,941
	4	5,123		11	112	42	600	0	1,102
	5	7,701		11	121	55	638	0	1,901
	6	7,088		0	0	77	908	0	1,738
	7	6,281		0	199	60	728	0	1,148
	8	14,241		11	18	66	508	0	5,433
	9	630		0	19	0	70	0	102
	10	568		0	15	9	53	0	123
	11	2,102		0	16	14	251	0	191
	12	874		0	11	10	138	0	244
	13	2,380		0	23	15	333	0	541
	14	455		0	0	0	61	0	118

Group A cages were exposed to *Helicobacter* spp.-positive bedding at the 3-wk time point, group B cages were exposed to *Helicobacter* spp.-positive bedding at the 6-wk time point, and group C cages were exposed to *Helicobacter* spp.-positive bedding at the 9-wk time point. In-cage filter paper from each cage was submitted for PCR at the 12-wk time point. Any result with a copy number greater than zero was considered a positive cage. *Helicobacter rodentium* was not detected in any sample. *Helicobacter bilis*, *H. ganmani*, *H. hepaticus*, *H. mastomyrinus*, and *H. typhlonius* were detected in one or more samples.

Table 2. Detection rate of *Helicobacter* spp. PCR on in-cage filter media at different time points

Experimental group	Number of positive cages/ total number of cages	Detection rate (%)
Group A	2/14	14.3
Group B	2/14	14.3
Group C	3/14	21.4
Total	7/42	16.7

Group A cages were exposed to *Helicobacter* spp.-positive bedding at the 3-wk time point, group B cages were exposed to *Helicobacter* spp.-positive bedding at the 6-wk time point, and group C cages were exposed to *Helicobacter* spp.-positive bedding at the 9-wk time point. Any result with a copy number greater than zero was considered a positive cage.

bedding to unused bedding[20] and another study used only positive bedding.[15] These results suggest that situations such as low prevalence of *Helicobacter* spp.-infected mice, infected mice present on a rack for short time periods, or addition of infected mice to a rack shortly (≤3 wk) before filter paper collection may result in decreased detection rates of *Helicobacter* spp. using PCR on in-cage filter paper.

To combat the low detection rate of *Helicobacter* spp. PCR on in-cage filter paper in populations with low prevalence or other sources of dilution of *Helicobacter* spp. nucleic acids, a multifaceted approach should be used for *Helicobacter* spp. detection in mouse colonies.[6] Combining a direct colony sampling method, such as fecal pellet *Helicobacter* spp. PCR, with in-cage filter paper or another EHM method, has the potential to increase *Helicobacter* spp. detection rates. PCR on fecal pellets has 100% diagnostic sensitivity and feces can be noninvasively collected from animals or animal cages.[2] However, relying solely on direct testing for colony health monitoring has several drawbacks, including increased staff labor and an increased risk of missing positive animals with random sampling tactics.

One limitation of the present study was the inclusion of bedding from cages with food-grinding behavior. Food grinding in mice is a behavior in which the mice fragment feed into small particles, often referred to as "orts," which then accumulate in the cage bottom.[4,17] Accumulation of orts results in an increase in particulate matter inside the cage. If in-cage filter media are exposed to bedding containing orts, the orts could potentially coat the filter media and prevent other nucleic acids from adhering to the filter, possibly resulting in a decreased PCR detection rate. In addition, the presence of orts in dirty bedding could further dilute the *Helicobacter* spp. nucleic acid needed for positive detection. Future work is necessary to elucidate the effects of food grinding on the detection rate of *Helicobacter* spp. PCR.

In addition, the present study was limited to the evaluation of *Helicobacter* spp. PCR on in-cage filter paper in disposable cages. Caging type may influence the PCR detection rate for pathogen detection. Different forms of EHM, such as swabs and exhaust dust testing, have also been shown to result in varying detection rates[1,8,18] As a result, the detection rate of *Helicobacter* spp. PCR may vary depending on the caging type and collection methods used. When designing a pathogen surveillance program, animal facility personnel may consider validating their testing methods internally, as a methodology from the literature may not correlate with an individual facility's caging and testing methods and could significantly affect testing results.

In conclusion, our study revealed low detection rates of *Helicobacter* spp. using PCR on in-cage filter paper when the filter paper was exposed to low concentration of *Helicobacter* spp. infected dirty bedding at the 3-, 6-, and 9-wk time points. Factors contributing to the decreased detection rate could include a lower *Helicobacter* spp. positive bedding-to-negative bedding ratio, short (3 wk) duration of filter paper exposure to infected bedding, presence of food grinding, and caging and collection media types. Multimodal EHM programs may help increase overall *Helicobacter* spp. detection rates. Further studies are needed to determine minimum duration of *Helicobacter* spp. infected bedding exposure to in-cage filter paper, minimum detectable concentration of infected bedding, and threshold of nucleic acid adsorption by the filter media to fully understand the potential limitations of the use of this EHM method to detect *Helicobacter* spp. in research facilities.

Acknowledgments

We thank the University of Oklahoma Health Sciences Center Department of Comparative Medicine for the time and financial resources needed to complete this project. We also thank Dr. Lori Garman for her assistance with power analysis and sample size calculations and Dr. Matlock Jeffries for his help with statistical analysis. We also thank Dr. Marcus Crim at IDEXX BioAnalytics for his assistance with the PCR component of the Materials and Methods section.

Conflict of Interest

The authors have no conflict of interest to declare.

Funding

This work was internally funded.

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