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Mechanical Washing Prevents Transmission of Bacterial, Viral, and Protozoal Murine Pathogens from Cages

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Infectious agents have varying susceptibilities to thermal inactivation and/or mechanical removal from cages by the use of heated, pressurized water. In this study, we tested whether 5 specific infectious organisms (*Candidatus savagella* [segmented filamentous bacterium (SFB)], *Helicobacter* sp., mouse norovirus (MNV), *Tritrichomonas* sp., and *Entamoeba muris*) could survive the cage wash process and still infect naïve mice. These 5 organisms were chosen due to their prevalence in rodent colonies, environmental stability, and/or potential to influence experimental outcomes. Cages that had housed mice shedding all 5 organisms were assigned to 1 of 3 treatment groups: 1) sanitization in a tunnel washer followed by autoclaving (121 °C [250 °F] for 20 min; *n* = 40 cages); 2) sanitization in a tunnel washer (82 °C [180 °F] for an average of 30 s; *n* = 40 cages); or 3) control (bedding change only; *n* = 40 cages). The presence of these agents in the cage was assessed by performing PCR on swabs of the empty soiled cage interior before and after the treatment. In addition, to determine if any residual nucleic acid was infectious, 2 Swiss outbred (J:ARC(S)) female mice were housed for 7 d in cages from each treatment group. The above procedures were then repeated so that every week each pair of J:ARC(S) mice (*n* = 10 pairs of mice/treatment group) were housed in another cage that underwent the same treatment; this was done for a total of 4 consecutive, 1-wk-long periods. Swabs collected from soiled cages were PCR-positive for SFB, *Helicobacter*, MNV, *Tritrichomonas*, and *Entamoeba* in 99%, 97%, 39%, 63%, and 73% of the cages tested, respectively. Cages in the tunnel wash group that were PCR-positive for SFB, *Helicobacter*, *Tritrichomonas*, and *Entamoeba* before treatment remained PCR-positive in 8%, 15%, 43%, and 10% of positive cages, respectively. None of the cages from the autoclave group were PCR-positive for any of the agents after treatment. None of the mice housed in cages in either the autoclave or tunnel wash groups became infected with any of the agents. However, 80%, 60%, and 100% of the pairs of mice housed in untreated cages were PCR-positive for SFB, MNV, and *Entamoeba*, respectively. None of the mice housed in untreated cages were positive for *Helicobacter* or *Tritrichomonas*. Our results suggest that nucleic acids from these bacterial and protozoal organisms may remain in cages after mechanical cage washing, but these nucleic acids are not infectious, and autoclaving is not necessary to prevent transmission.

Abbreviations and Acronyms: BR, bedding-removed-only treatment group (control); MNV, mouse norovirus; SFB, segmented filamentous bacteria; TW, tunnel wash treatment group; WA, wash-then-autoclaved treatment group

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Introduction

To establish and maintain specific pathogen free (SPF) rodent colonies, biosecurity protocols must be implemented to eliminate or exclude infective agents of interest. Cages are a potential fomite that could spread infective agents within rodent colonies.[13] Rodent cage components are routinely bulk autoclaved in many animal care programs after sanitization in a mechanical washer, although sterility is not maintained after autoclaving. Rodent cages, filter tops, and water bottles are manufactured from polymeric thermoplastics that degrade more rapidly when exposed to chemicals during washing and/or when subject to repeated cycles of autoclaving.[22,38,44,55] Monomeric thermoplastic components can leach during degradation; many of these are endocrine disruptors, and can confound select experimental studies.[4,8,21,31,54] Therefore, an ideal sanitation process would be mechanical washing that did not use chemicals but instead used thermal disinfection that was sufficient to eliminate excluded infective agents. The goal of this study aimed to determine whether select infective agents could be eliminated by mechanical cage washing alone or if autoclaving was also necessary. We tested 5 agents—segmented filamentous bacteria, *Helicobacter* spp., mouse norovirus, *Tritrichomonas* spp., and *Entamoeba muris*—that represent a subset of agents that are prevalent in academic rodent colonies, may affect research outcomes or animal health, and would be expected to have differing sensitivities to destruction during sanitization.

Segmented filamentous bacteria (SFB), or *Candidatus savagella* (formerly *C. arthromitus*), is a gram-positive, anaerobic, spore-forming intestinal commensal of mice and other vertebrate and invertebrate species.[19] SFB can influence Th17 cell responses that are particularly important in the NOD mouse model of

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diabetes.[16,25] SFB is prevalent in research rodents.[10] Of 113 groups of mice imported to our institution from various academic sources in 2021, 73 of 113 (64%) were PCR positive for SFB on arrival. SFB are transmitted both vertically and horizontally.[30] The mechanism of vertical transmission is unknown. Horizontal transmission most likely occurs through ingestion of spores. Cohousing mice of the same sex, but different SFB colonization status, resulted in horizontal transmission within 1 to 2 wk of cohousing.[30] The transmission of SFB by contaminated fomites has not been reported. However, because it is a spore-forming organism, autoclaving may be required to inactivate infectious SFB spores from cages.

Members of the *Helicobacter* genus are gram-negative, microaerophilic bacteria that have important rodent health and research implications. Many species of *Helicobacter* have been isolated from mice, some of which may cause hepatic and GI pathology and reduce reproductive efficiency.[15,18,39,51] Even *Helicobacter* species that do not produce clinical signs in colonized animals can affect research outcomes, particularly in studies evaluating neoplasia or immune responses.[11] This bacterial genus is highly prevalent in research mouse colonies, with rates in excess of 80%.[5,35] *Helicobacter* spp. are primarily transmitted fecal-orally, although species differences have been reported in the ability of soiled bedding to transmit rodent *Helicobacter* to sentinel animals.[15,39,52]

Murine noroviruses (MNV) are nonenveloped, positive-strand RNA viruses that belong to the *Norovirus* genus in the Caliciviridae family. They are the most prevalent infectious viral agent of research mice, with a reported sample prevalence of approximately 30% in North America.[20,43] MNV infection can affect research outcomes in some models.[28,33,47] The high prevalence of this virus in mouse research facilities, as well as its potential to confound research outcomes, warrants considering it for exclusion from certain colonies. However, whether standard cage wash practices without autoclaving are sufficient to eliminate MNV from cage surfaces and prevent transmission to naïve mice remains unknown.

Tritrichomonas species, including *T. muris* and *T. musculus*, are triflagellate, single-celled protozoa that reside in the gastrointestinal tract of mice and other rodents. *Tritrichomonas* spp. have historically been viewed as benign commensals, but recent evidence shows that infection can exacerbate experimentally induced colitis and alter baseline T-cell homeostasis toward a proinflammatory mucosal environment.[17] The current prevalence of *Tritrichomonas* spp. in research mouse colonies is unknown but has been historically reported to be between 8% and 54% and is considered to be one of the most prevalent protozoa in research mice.[2,43] Of 113 groups of mice imported to our institution from various academic sources in 2021, 48 (42%) were PCR-positive for *Tritrichomonas* spp. on arrival. This protozoan is considered to be highly transmissible with a minimal infectious dose of only 5 pseudocysts.[45] After a prepatent period of 3 to 10 d, infected mice shed pseudocysts in the feces.[45] The ability of *Tritrichomonas* spp. pseudocysts to survive harsh environmental conditions has not been extensively evaluated; in moist conditions, pseudocysts can survive 1 to 3 wk but they appear to be susceptible to inactivation in a dry environment and at high temperatures.[29] Little information is available on the horizontal transmission of *Tritrichomonas* spp. by fomites or soiled bedding. One study reported that *Tritrichomonas* spp. was poorly transmitted to sentinel mice exposed to soiled bedding from infected mice.[13]

The nonflagellate enteric protozoan *Entamoeba muris* is also generally considered nonpathogenic.[1] Infection occurs after the ingestion of environmentally hardy cysts.[41] This organism is prevalent in academic rodent colonies; *Entamoeba muris* was detected in 8% of mouse fecal samples submitted to a major diagnostic laboratory.[43] This prevalence, based on individual sample submission, likely underestimates institutional prevalence. As *E. muris* is traditionally tolerated within academic research institutions, testing for the agent may be limited. Of 178 groups of mice imported to our institution in 2021, 22 (12%) were positive for *E. muris*. The impact of colonization with *E. muris* on research outcomes has not been well studied.

The goal of this study was to evaluate the transmissibility of these selected agents via contaminated cages, and to determine whether cage wash alone was sufficient to prevent their transmission to naïve mice or the additional step of autoclaving is necessary.

Materials and Methods

Experimental design. Cages housing both Swiss Webster (SW; Taconic Biosciences, Germantown, NY) and NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG; Jackson Laboratories, Bar Harbor, ME) female mice that were shedding SFB, *Helicobacter* spp., MNV, *Tritrichomonas* spp., and *Entamoeba muris* (confirmed by PCR), changed weekly, were used to generate contaminated cages (n = 120) over a period of 4 wk (30 cages per week). After soiled bedding was removed from contaminated cages, a cotton applicator (Daiso, Hiroshima, Japan) was used to swab the interior perimeter of the cage bottom and then from corner to corner in an "X" pattern. Swabs were stored at −80 °C (−112 °F) before conducting PCR on an extract from each swab. Each contaminated cage was then randomly assigned to 1 of 3 groups: 1) WA: sanitization in a tunnel washer followed by autoclaving (n = 40); 2) TW: sanitization in a tunnel washer (n = 40); or 3) BR: bedding removed with no further cage processing (n = 40; Figure 1). Cages assigned to the BR group were not sanitized further. Autoclaved bedding, enrichment, food, water, wire bar lid, and filter top, as described below, were added to each cage together with 2 female outbred Swiss (J:ARC(S); Jackson Laboratories, Bar Harbor, ME) mice, resulting in 30 pairs of mice, with 10 pairs per group. Mice were rotated through 4 test cages within a given group for 4 consecutive 1-wk periods.

After bedding removal, cages assigned to the WA and TW groups were sanitized without chemicals in a tunnel washer (Basil 6300, Steris, Mentor, OH) operating with a belt speed of 6 linear ft/min (1.8 m/min). A data logger (OM-CP-HITemp140, Omega Engineering, Bridgeport, NJ) was secured to the interior bottom of a nonexperimental cage and processed through the tunnel washer to confirm temperatures as the cage progressed through the washer before the experimental cages were washed. The washer sump set points were as follows: 40-s cold water prewash, 40-s wash at 88 °C (190 °F), 30-s rinse at 88 °C (190 °F), 20-s final rinse at 88 °C (190 °F), and drying with an air knife blow-off system at 2,200 cubic feet per minute (128 m/s) air flow at 88 °C (190 °F) for 1 min. The maximum temperature to which the cages were subjected was confirmed using temperature-sensitive tape (Thermostrip, Cole-Parmer, Vernon Hills, IL) placed on the flat surface of a wire-bar lid immediately before the experimental cages were processed through the tunnel washer. Real time operational parameters displayed on the washer's operator interface screen were evaluated during the processing.

After washing, cages in the TW group were opened in a Class II, type A2 biologic safety cabinet (LabGard S602-500, Nuaire, Plymouth, MN) and swabbed for infectious agents as described above. Then autoclaved bedding, enrichment, wire bar lid, food,

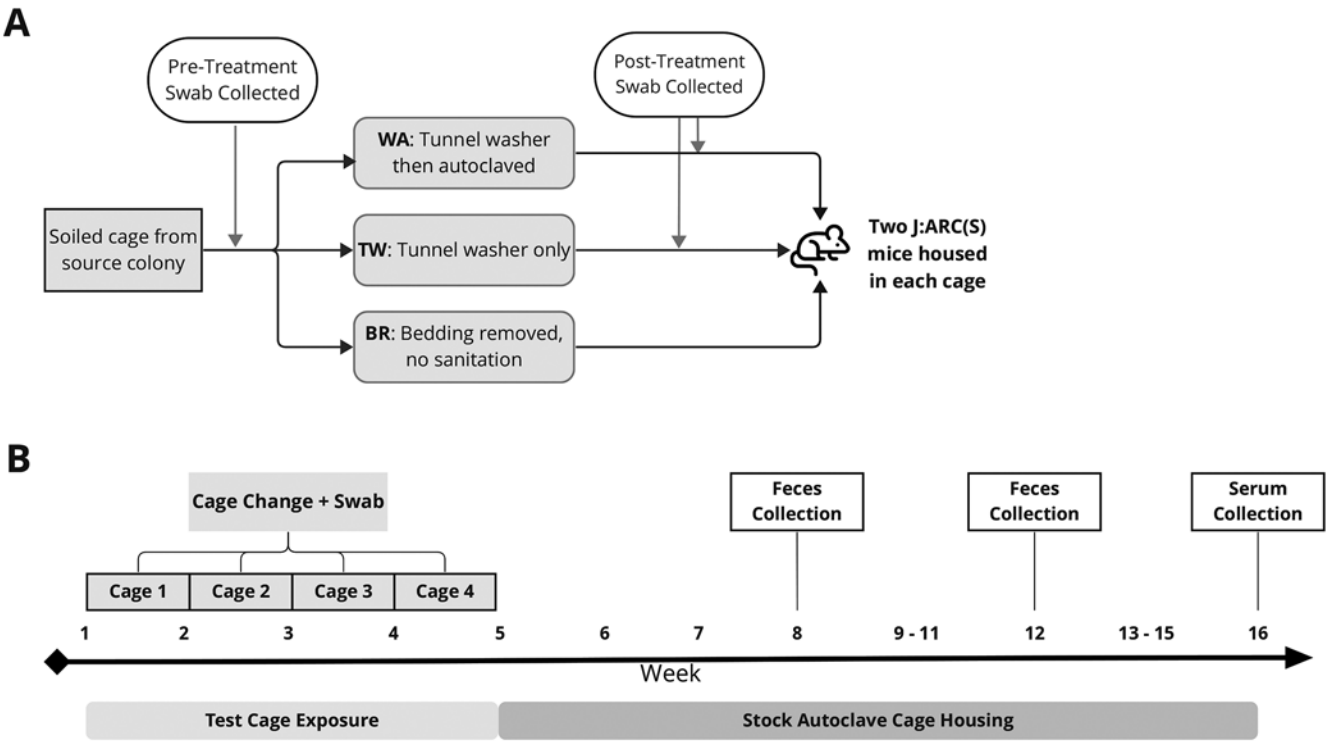

Figure 1. Schematic of the experimental design, including: (A) sampling and treatment of soiled caging and (B) timeline of exposure of J:ARC(S) pairs to treated test cages. The schematic represents housing of a single pair of mice from a treatment/control group for 4 consecutive 1-week periods.

and water, processed as described below, were added to each cage together with 2 J:ARC(S) mice.

WA group cages were fitted with an autoclaved filtertop after retrieval from the tunnel washer. The filtertop was secured in place with heat sensitive autoclave tape (Medline, Mundelein, IL), and the cage was autoclaved (Century SLH Scientific, Steris, Mentor, OH) as described below. After autoclaving, cages in the WA group were opened and swabbed for infectious agents as described above, and autoclaved bedding, enrichment, wire bar lid, food, and water, as described below, were added to each cage followed by 2 J:ARC(S) mice. The time interval from removal of soiled bedding to placement of J:ARC(S) mice was approximately 12 h for TW and WA cages.

Thirty contaminated cages were processed each week as described above. Thus, the cage wash and housing processes for each of the 3 groups were repeated so that each pair of J:ARC(S) mice was housed in a different cage from the same treatment group for 4 consecutive 1-wk exposure periods, resulting in testing a total of 120 contaminated cages (40 cages/treatment group). After the fourth and final 1-wk exposure period, mice were housed in autoclaved cages for the remainder of the study (Figure 2). Feces were collected from each group of J:ARC(S) mice at 8 and 12 wk after the first exposure period, stored at −80 °C (−112 °F), and tested for infectious agents by PCR. Sixteen weeks after the first exposure period, mice were euthanized by carbon dioxide asphyxiation, and blood was collected via postmortem cardiac puncture and submitted for anti-MNV antibodies.

Animals. Four- to 6-wk-old female Swiss Outbred (J:ARC(S); Jackson Laboratories, Bar Harbor, ME) mice (n = 60) were used as test animals. An existing colony of Swiss Webster (SW; Taconic Biosciences, Germantown, NY) and NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG; Jackson Laboratories, Bar Harbor, ME) female mice of varying ages known to be naturally infected with SFB, MNV, *Tritrichomonas* spp., and *E. muris* were used to create the contaminated cages. All cages of cohoused NSG and SW mice were confirmed to be positive for all agents of interest via PCR of feces pooled at the level of the cage prior to study onset. All mice were free of mouse hepatitis virus, Sendai virus, mouse parvovirus, minute virus of mice, murine astrovirus 2, pneumonia virus of mice, Theiler meningoencephalitis virus, epizootic diarrhea of infant mice (mouse rotavirus), ectromelia virus, reovirus type 3, lymphocytic choriomeningitis virus, K virus, mouse adenovirus 1 and 2, polyomavirus, murine cytomegalovirus, mouse thymic virus, Hantaan virus, mouse kidney parvovirus, *Mycoplasma pulmonis*, *Citrobacter rodentium*, *Salmonella* spp., *Filobacterium rodentium*, *Clostridium piliforme*, *Corynebacterium bovis*, pinworms (*Syphacia* spp. and *Aspiculuris* spp.), and *Encephalitozoon cuniculi*, as determined by testing naïve outbred SW mice exposed repetitively to soiled bedding from cages housing mice in the colony. J:ARC(S) mice were also negative for SFB, *Helicobacter* spp., MNV, *Tritrichomonas* spp., and *E. muris* prior to study initiation, as determined by PCR of pooled feces from shipping containers and vendor colony health reports. Vendor colony health reports were based on direct sampling of colony mice.

Husbandry and housing. Mice were maintained in individually ventilated polysulfone cages with stainless-steel wire-bar lids and filter tops (number 19, Thoren Caging Systems, Hazelton, PA) on aspen chip bedding (PWI Industries, Quebec, Canada) at a density of 3 to 5 mice per cage. Each cage was provided with a bag constructed of Glatfelter paper containing 6 g of crinkled paper strips (EnviroPak, WF Fisher and Son, Branchburg, NJ) and a 2-inch square of pulped virgin cotton fiber (Nestlet, Ancare, Bellmore, NY) for enrichment. Mice were fed a natural ingredient, closed source, gamma irradiated, autoclavable diet (5KA1, LabDiet, Richmond, VA) and provided reverse osmosis acidified (pH 2.5 to 2.8 with hydrochloric acid) water in

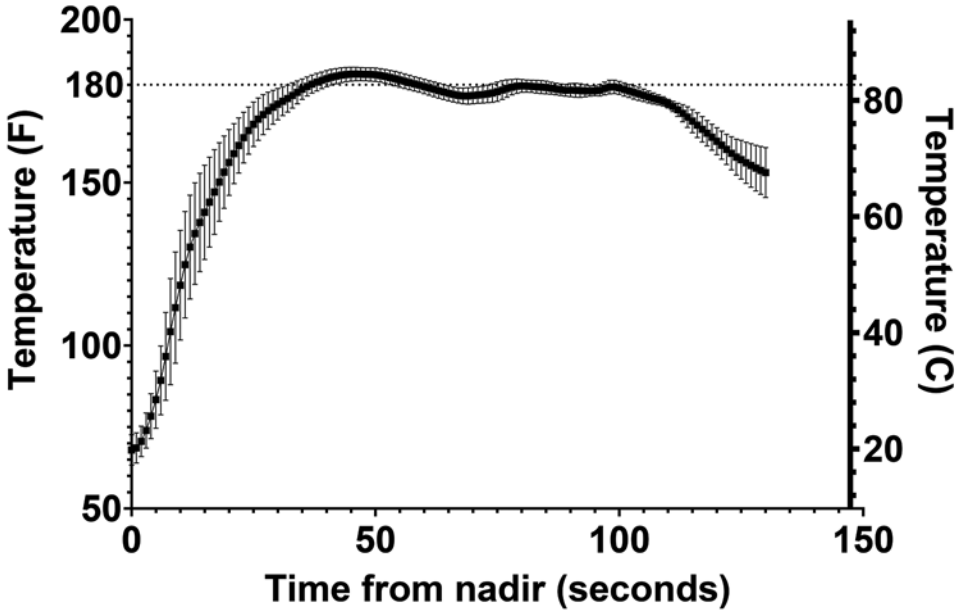

Figure 2. Temperature recorded by data logger run through the tunnel washer (n = 8 runs). Data presented as mean ± SD.

polyphenylsulfone bottles with stainless-steel caps and sipper tubes (Tecniplast, West Chester, PA) ad libitum.

Autoclaved cages were subjected to a pulsed vacuum cycle of 4 pulses at a maximum pressure of 12.0 psig (6.9 kPa), with sterilization temperature of 121 °C (250 °F) for 20 min, and a 10.0 in Hg vacuum dry (3.4 kPa). Sterilization was confirmed by autoclave tape color change and post hoc verification of cycle chamber operating conditions. In addition, autoclave performance was verified weekly by using biologic indicators (Attest Biologic Indicators, 3M, Saint Paul, MN). Water bottles were autoclaved at a temperature of 121 °C (250 °F) for 45 min with a purge time of 10 min. Cages were changed every 7 d in a Class II, type A2 biologic safety cabinet (BSC; LabGard S602-500, Nuaire, Plymouth, MN). The rooms were maintained on a 12:12-h light:dark cycle (on at 0600, off at 1800), relative humidity of 30% to 70%, and room temperature of 22 ± 1 °C (72 ± 2 °F). The animal care and use program at the Memorial Sloan Kettering Cancer Center (MSK) is accredited by AAALAC, and all animals are maintained in accordance with the recommendations provided in the Guide.[24] All animal use in this investigation was approved by MSK's IACUC in agreement with AALAS' position statements on the Humane Care and Use of Laboratory Animals and Alleviating Pain and Distress in Laboratory Animals.

PCR. DNA and RNA were copurified from feces or cage swab samples using the Qiagen DNeasy 96 blood and tissue kit (Qiagen, Hilden, Germany). Nucleic acid extraction was performed using the manufacturer's recommended protocol, "Purification of Total DNA from Animal Tissues", with the following buffer volume modifications: 300µL of Buffer ATL + Proteinase K, 600µL of buffer AL + EtOH, and 600µL of lysate were added to individual wells of the extraction plate. Washes were performed with 600µL of buffers AW1 and AW2. Final elution volume was 150µLof buffer AE.

Real time (qPCR) PCR was carried out using a BioRad CFX machine (Bio-Rad, Hercules, CA). PCR assays targeted conserved regions for each agent. Probes were either labeled with FAM or HEX and quenched with ZEN and Iowa Black FQ (IDT, Coralville, IA). Multiplex assays targeting *Candidatus savagella* (segmented filamentous bacterium [SFB]) and *Helicobacter* sp. or *Tritrichomonas* sp., and *Entamoeba muris* were run using Qiagen's Quantifast Multiplex PCR+R Kit (Qiagen, Hilden, Germany) using the kit's recommended concentrations and cycling conditions.

RT-qPCR for mouse norovirus (MNV) was run using QuantaBio qScript^{tm} XLT One-Step RT-qPCR ToughMix (Quantabio, Beverly, MA) using the kit's recommended concentrations and cycling conditions.

Serology. Blood was collected from individual mice by cardiac puncture after euthanasia, pooled at the level of the cage, and submitted to a commercial diagnostic laboratory for measurement of serum antibodies to MNV (Charles River Research Animal Diagnostic Services, Wilmington, MA).

Statistical analysis. The incidence proportion of PCR positive cages for each agent was compared between treatment groups for initial assignment, and between pre- and posttreatment cages for each agent in the WA and TW groups using Fisher's exact test. All analyses were performed using statistics software (Graph Pad Prism 9.1.0, La Jolla, CA). A P value of less than or equal to 0.05 denoted statistical significance. Tunnel washer data are presented as mean ± standard deviation (SD).

Results

Cage wash temperature. Figure 2 shows the temperature data for all tunnel washer runs (n = 8). The data logger recorded a temperature of at least 82 °C (180 °F) in all wash cycles run. The mean maximum temperature was 84 ± 1 °C (183 ± 2 °F). The mean amount of time recorded at greater than or equal to 82 °C (180 °F) was 31 ± 20 s.

PCR results. One hundred nineteen (99%), 116 (97%), 47 (39%), 75 (63%), and 87 (73%) of the soiled cages (n = 120) were PCR positive for SFB, *Helicobacter* spp., MNV, *Tritrichomonas* spp., and *E. muris* respectively (Table 1). There were no significant differences in the proportion of soiled cages PCR positive for SFB, *Helicobacter*, or MNV assigned to each of the 3 treatment groups. After PCR analysis it was determined that the WA group had been assigned a significantly greater proportion of *E. muris* PCR positive cages than the BR group (P = 0.023) and a significantly greater proportion of *Tritrichomonas* spp. PCR positive cages than the TW group (P = 0.034).

Table 1. PCR results from cage swabs and test animal feces. "Before" = positive cage swab samples out of total number of cage swab samples collected prior to treatment. "After" = positive cage swab samples collected after treatment out of positive pretreatment cages. "Feces" = J:ARC(S) pairs with at least 1 positive fecal sample at the 8- or 12-wk collection times. "Serology" = J:ARC(S) pairs with positive serology results. "-" = not performed.

		SFB	*Helicobacter*	MNV	Tritrichomonas	Entamoeba
Autoclave	**Before**	100% (40 of 40)	98% (39 of 40)	40% (16 of 40)	78% (31 of 40)[a]	85% (34 of 40)[a]
	After	0% (0 of 40)[b]	0% (0 of 39)[b]	0% (0 of 16)[b]	0% (0 of 31)[b]	0% (0 of 34)[b]
	Feces	0% (0 of 10)	0% (0 of 10)	0% (0 of 10)	0% (0 of 10)	0% (0 of 10)
	Serology	—	—	0% (0 of 10)	—	—
Cage Wash	**Before**	100% (40 of 40)	98% (39 of 40)	40% (16 of 40)	53% (21 of 40)[a]	73% (29 of 40)
	After	8% (3 of 40)[b]	15% (6 of 39)[b]	0% (0 of 16)[b]	43% (9 of 21)[b]	10% (3 of 29)[b]
	Feces	0% (0 of 10)	0% (0 of 10)	0% (0 of 10)	0% (0 of 10)	0% (0 of 10)
	Serology	—	—	0% (0 of 10)	—	—
Bedding Dump	**Before**	98% (39 of 40)	95% (38 of 40)	38% (15 of 40)	58% (23 of 40)	60% (24 of 40)[a]
	Feces	80% (8 of 10)	0% (0 of 10)	60% (6 of 10)	0% (0 of 10)	90% (9 of 10)
	Serology	—	—	70% (7 of 10)	—	—

[a] $P < 0.05$ in incidence of infectious agent between treatment groups
[b] $P < 0.0001$ in incidence of infectious agent between pre- and posttreatment samples

None of the treated cages in the WA group were PCR positive for any of the agents of interest (Table 1). In the TW group, 7% (3/40), 15% (6/39), 0% (0/16), 43% (9/21), and 10% (3/29) of the cages that were initially positive for SFB, *Helicobacter* spp., MNV, *Tritrichomonas* spp., and *E. muris*, respectively, remained PCR positive for these agents after washing (Table 1). The decrease in the incidence of PCR positive cages between pretreatment and posttreatment samples was significant ($P < 0.0001$) for all agents in the WA and TW groups. No fecal samples from mice in the WA group were PCR positive at either time point.

Despite treated cages in the TW group remaining PCR positive for SFB, *Helicobacter* spp., MNV, *Tritrichomonas* spp., and *E. muris*, none of the mice housed in these cages tested positive for any agent via fecal PCR 8- and 12-wk after exposure. In contrast, 80% (8/10), 60% (6/10), and 90% (9/10) of the 10 pairs of BR mice housed in untreated cages tested positive for SFB, MNV, and *E. muris*, respectively, by 12-wk after exposure (Table 1). None of the BR mice housed in untreated cages were shedding *Helicobacter* spp. or *Tritrichomonas* spp., as determined by fecal PCR, at 8- or 12- wk after exposure.

Serology results. No groups of mice in the WA or TW groups had seroconverted to MNV by 15-wk after the initial exposure, while 7/10 (70%) groups of mice in the BR group were seropositive at the same time point (Table 1). Serology results were generally consistent with fecal PCR results from mice in the same cage except for 1 cage which was MNV negative via fecal PCR at 8- and 12-wk post exposure but was seropositive at 15-wk after exposure.

Discussion

Washing soiled cages in a tunnel washer that exposed cages to at least 82 °C (180 °F) wash water for an average of 30 s prevented the transmission of all 5 infectious agents to naïve immunocompetent mice. Washing alone did not remove or destroy all nucleic acid on the cages, whereas none were detected in cages that were autoclaved after washing (Table 1). These results suggest that autoclaving is not necessary to prevent transmission of these agents to mice, and that while nucleic acid persists on cages after washing, this material does not appear to be infectious to an immunocompetent mouse or, if viable, is below the minimal infectious dose of each agent. Furthermore, *Helicobacter* spp. and *Tritrichomonas* spp. were not transmitted to mice exposed to soiled caging (the BR group), suggesting that certain species of each genus may be poorly transmitted by fomites.

The temperature of the water reaching the surface of the cage, as recorded by the data logger affixed to the cage interior, was notably lower than the sump temperature set point. Despite all regions of the tunnel washer, aside from the cold-water prerinse, being set at 88 °C (190 °F), the water reaching the cage reached or exceeded 82 °C (180 °F) for an average of only about 30 out of 90 s per run in the heated water (wash and rinse) sections of the tunnel washer. Similar results were reported in a rack style washer that also demonstrated average and maximum temperatures lower than the setpoint when the wash and rinse temperature was set to 82 °C (180 °F).[48] This thermal loss between the sump temperature and the water reaching the cage is to be expected, as heat transfer between the water and cooler washer chamber environment will occur once the water leaves the heated sump where the temperature sensor is located and is released as a spray at the manifold from which the water is ejected. Facility staff should be mindful of this disparity when establishing washer sump temperature set points, as published data on time and temperature combinations are typically meant to indicate the values that reach the organisms.[50]

Despite the difference between the sump temperature setpoint and the actual water spray temperature reaching the cage surface, cage wash alone was still effective at preventing transmission of all 5 agents to naïve mice. However, the nucleic acid of the selected infectious agents was still detectable on cages after washing based on PCR positive cages detected after processing in the tunnel washer. These results most likely reflect the presence of noninfectious nucleic acid remnants. Cage wash eliminates microorganisms by both thermal inactivation and the physical removal of organic material by water. Residual organic material is still subject to thermal degradation. Although temperatures of 82 °C (180 °F) for 30 s exceed the thermal inactivation threshold for most bacteria and viruses, complete DNA degradation requires temperatures above 100 °C (212 °F).[27,49] Nucleic acid remaining on sanitized surfaces most likely represents nonviable organisms, as none of the mice in the TW group became infected with any agents, despite exposure to multiple PCR positive cages. PCR can detect nonviable bacteria and viruses.[3,26] Alternatively, residual nucleic acid could

have been from viable organisms, but the remaining infectious material was below the minimal infectious dose. Because all exposed animals were immunocompetent, an effective innate immune response to a low number of viable organisms could have prevented colonization. Immunocompromised mice were not used in the present study to allow assessment of serologic outcomes. However, if the minimal infectious dose for these organisms is lower in immunocompromised mice, active infection could have resulted from exposing them to PCR positive cages.

Our finding that residual nucleic acid may persist after cage washing is not novel. A study evaluating sanitation of IVC racks contaminated with *Corynebacterium bovis* found that DNA remained detectable via PCR after washing with detergent with a final rinse temperature of 82 °C (180 °F).[37] Autoclaving after washing rendered these racks PCR negative. A separate study assessing cage wash efficacy for removal of *C. bovis* from cages found that a tunnel washer was effective in generating *C. bovis* culture-negative cages; however, that study did not use PCR assessment.[9] These results, interpreted in concert, suggest that residual *C. bovis* nucleic acid remaining after tunnel washing is likely not infectious, which is consistent with our current results. Other publications have demonstrated that cage wash temperatures as low as 110 °F (43 °C) can prevent transmission of murine pathogens in a tunnel washer in conjunction with chemical detergents.[12] That study demonstrated that cage sanitization using a tunnel washer at both 180 °F and 110 °F prevented transmission of MPV, MHV, and *Syphacia oblevata* to sentinel mice, while mice exposed to unwashed cages became infected.[12] A rack washer set to 140 °F (60 °C) that used chemical detergent rendered heavily soiled cages visually clean; they were considered sanitized as determined by ATP monitoring and RODAC sampling, although transmission of infectious organisms was not assessed.[48] In the current study, we were able to prevent transmission without the use of chemical detergents, as had been used in the referenced studies. Our institution does not use chemical detergents, as alkaline solutions are reported to contribute to thermoplastic degradation, particularly at higher temperatures.[23,40]

MNV was the only agent that was not detected after tunnel washing, as no cages were PCR positive after treatment. However, MNV was detected in only about 40% of soiled cages before washing. Therefore, PCR positive cages may have been detected after washing had the number of positive cages been greater before treatment. Given the reported thermal inactivation threshold for MNV, had any residual nucleic acid been detected, it would have likely been noninfectious nucleic acid.[6] In addition, mouse parvovirus, which has greater thermal resistance than MNV, did not infect sentinel mice exposed to washed cages as assessed serologically.[13]

Mice that were exposed to untreated soiled cages became infected with SFB, MNV, and *E. muris* as evidenced by PCR-positive feces from mice housed in these cages. Despite multiple exposures to PCR positive cages, none of the mice in the BR group tested positive for *Helicobacter* spp. or tritrichomonads. Intermittent shedding of organisms could have resulted in false negative PCR results from exposed mice. However, because fecal samples were collected at multiple time points, this was unlikely. Despite having a low minimal infectious dose, tritrichomonads have been reported to be poorly transmitted to soiled bedding sentinels, although in our facilities we frequently find soiled-bedding sentinels that are positive for *Tritrichomonas* spp.[7] The basis for this difference has not been elucidated. Nucleic acid was readily detected on the surface of soiled cages; therefore mice in the BR group were likely exposed to *Tritrichomonas* spp. pseudocysts. Therefore, pseudocysts or trophozoites present on the cage either were no longer infectious or were no longer adherent to the cage surface. *Tritrichomonas muris* pseudocysts are known to be infective for up to 7 d in a moist environment.[29] Similarly, *Spironucleus muris* is reported to be poorly transmitted to soiled-bedding sentinels, despite a reportedly low minimal infectious dose and cysts that remain infectious in the environment for 2 wk after shedding.[32,42,45] Mice were housed in BR cages immediately after emptying, so cyst desiccation was unlikely to contribute to the lack of infectivity. *E. muris* is also a cyst-forming protozoan but was readily transmissible via contaminated caging, suggesting prolonged infectivity of cysts shed from the host. *E. muris* has been detected by PCR in the feces of soiled-bedding sentinels, although at a lower rate than colony prevalence.[14]

Nearly all soiled cages were PCR positive for *Helicobacter* spp., suggesting the presence of bacteria on the cage surface. Despite this, none of the mice in the BR group became infected with *Helicobacter* spp. after exposure to the unwashed cages. Mice reportedly shed *Helicobacter* spp. persistently once the infection has been established, so spontaneous clearance of the organism is unlikely.[53] We did not identify the *Helicobacter* spp. present in this study, so the *Helicobacter* spp. present in our source colony could be one that is poorly transmitted on fomites. Species of *Helicobacter* that have been isolated from mice include *H. hepaticus* and *H. bilis* which can cause pathology in the liver and GI tract, *H. typhlonius, H. mastomyrinus,* and *H. apodemus* which may cause GI pathology and/or impaired reproductive efficiency in certain mouse strains, and *H. muridarum, H. rappini, and H. rodentium* which are typically nonpathogenic but could induce disease in highly immunocompromised strains.[15,18,51] Several *Helicobacter* spp. are inconsistently transmitted to soiled-bedding sentinels, and these species would likely be poorly transmitted via contaminated caging.[12,39,52] *H. hepaticus*, but not *H. muridarum*, was transmitted to soiled-bedding sentinels.[11,34] All species of *Helicobacter* described above have been detected in sentinel mice at our institution, but the species (single or multiple) detected in the present study was not identified. The species present likely was one that transmits poorly on fomites or to soiled-bedding sentinels, given the lack of transmission in the BR group. Given the number of cages that were PCR positive in the BR group, we likely would have seen infection of the mice in that group if a species of *Helicobacter* that is effectively transmitted via soiled caging had been present.

One pair of mice in the BR group was repeatedly PCR-negative but seropositive for MNV. While this could have been a false positive result, given the history of repeated exposure to soiled cages containing MNV, a more likely possibility is that fecal PCR analysis failed to detect infection. The immunocompetent exposed mice likely cleared the infection and stopped shedding virus prior to the first fecal PCR sample taken at 8 wk after exposure. Depending on the mouse and MNV strains, fecal shedding may cease prior to 60 d, and as quickly as 7 d after experimental inoculation.[36,46]

The time interval between collecting cage swabs and receiving PCR results required us to randomly assign cages to treatment groups, as compared with knowing the status of the cage and assigning equal numbers of PCR positive cages for each agent to each group. This resulted in the unequal distribution of PCR positive cages for *E. muris* and *Tritrichomonas* spp. between some of the treatment groups. This distribution likely did not impact interpretation of the data because in all instances the WA group received the greatest number of PCR positive cages, so the greater number of posttreatment positive cages in the TW group

could not have been due to the disproportionate assignment of PCR positive cages. In addition, although all source cages housed mice that were PCR positive for all 5 agents of interest, not all soiled cages tested positive for each organism. This could reflect intermittent fecal shedding, variability in the transfer of the agent from feces and soiled bedding to the surface of the cage, or variability when swabbing the cage. Finally, although all source cages were determined to be positive for all agents of interest, housing density varied from 3 to 5 mice per cage, and PCR copy number was not quantified. This likely resulted in varying numbers of organisms present on each cage; however, because we were not able to assign cages to a treatment group based on PCR result due to the lag time between cage assignment and receiving PCR results, we could not control for this variable throughout the study. Furthermore, our study design represents real-world conditions in which mice are exposed to cages housing varying numbers of mice of differing infection status.

This study suggests that autoclaving of cages after washing is unnecessary to prevent transmission of SFB, *Helicobacter* spp., MNV, *Tritrichomonas* spp., or *E. muris* to J:ARC(S)mice from cages that previously housed mice shedding these agents if cages were washed at the time and temperature used in the present study. Although we did not directly assess other materials that are treated similarly, such as wire bar lids and filter tops, they would be expected to be adequately sanitized when treated the same way. Avoiding the routine, bulk autoclaving of plastic caging will decrease thermal degradation of plastic, which can reduce the release of endocrine disruptors such as BPA, extend cage life, and eliminate the costs associated with this additional processing step. However, the residual nucleic acid detected could create challenges for institutions using PCR on racks and cages in lieu of, or in addition to, the use of sentinel mice in their colony health monitoring program. These results may also vary for other organisms and mice of differing immunologic competencies.

Because the use of lower water temperatures would significantly reduce energy costs, additional studies are needed to determine whether lower cage wash temperatures would also prevent the transmission of these and other agents. The present study adds to the body of evidence that cage washing alone is effective to prevent the transmission of a variety of infectious agents from contaminated soiled caging to naïve mice.

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