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Resistance of Sprague–Dawley Rats to Infection with *Helicobacter pullorum*

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Helicobacter pullorum* is an enterohepatic *Helicobacter* spp. known to infect both chickens and humans. *H. pullorum* infection has recently been reported in both mice and rats. This study was designed to determine whether standard methods of colony health surveillance using exposure to rodent soiled bedding could detect *H. pullorum* in Sprague–Dawley rats. We exposed 8 *Helicobacter*-free Sprague–Dawley rats to bedding from *H. pullorum*-infected Brown Norway rats. Fecal samples were analyzed by PCR every 2 wk for 22 wk. Dirty bedding transfer resulted in intermittent positive fecal PCR results; however, none of the rats became persistently infected with *H. pullorum*. Select intestinal tissues collected at necropsy analyzed by PCR were negative for *H. pullorum*. To determine whether the failure to detect *H. pullorum* in Sprague–Dawley rats receiving contact bedding was due to resistance of Sprague–Dawley rats to *H. pullorum* colonization, 10 *Helicobacter*-free Sprague–Dawley rats were orally dosed with *H. pullorum*. Fecal samples were analyzed by PCR every 2 wk for 12 wk. At 2 wk after infection, 5 of 10 rats were PCR positive for *H. pullorum*. By 12 wpi, only 2 rats were persistently colonized with *H. pullorum* according to culture and PCR results. These data contrast with our previous data, which showed a high frequency of both natural and experimental *H. pullorum* infection in Brown Norway rats. Sprague–Dawley rats are resistant to experimentally induced *H. pullorum* gastrointestinal colonization when dosed orally with *H. pullorum* or exposed to bedding from *H. pullorum*-infected rats.

Abbreviations: wpi, weeks postinfection; EHS, enterohepatic *Helicobacter* species.

The genus *Helicobacter* is generally separated into 2 groups—gastric *Helicobacter* species and enterohepatic *Helicobacter* species (EHS)—dependent on the preferred site of colonization. EHS preferentially colonize the gastrointestinal tract and, in some cases, the biliary tree of their host. In 1994, the novel *Helicobacter* species, *H. pullorum*, was isolated from human feces and the intestinal contents and livers of chickens.[21] Subsequent to its original isolation and characterization, *H. pullorum* has been reported to infect humans, chickens, turkeys, guinea fowl, mice, and (most recently) Brown Norway (BN/MolTac) rats (*Rattus norvegicus*).[2,4,16,21,24]

H. pullorum is associated most often with farm-raised poultry and is suspected to cause vibrionic hepatitis in chickens.[16,21,28,29] In humans, infection with *H. pullorum* has been associated with diarrhea. In addition, PCR assays have identified this organism in patients with inflammatory bowel disease, hepatitis, cholecystitis, and hepatocellular carcinoma.[3,5,9,12,18,22,25]

We recently reported that Brown Norway rats are highly susceptible to both natural and experimental *H. pullorum* infection.[4] Another study that reported colonization of *H. pullorum* in rats detected the organism in intestinal contents by using denaturing gradient gel electrophoresis after rats were treated with a common carcinogen (4-nitroquinoline-1-Oxide). Clinical signs ascribed to *H. pullorum* infection were not noted.[30]

Transmission of EHS is known to occur through the transfer of dirty bedding in mice.[14,23,26] Sprague–Dawley (Crl:SD) rats are commonly used in surveillance programs as sentinels to ascertain disease status within the colony, and the use of dirty-bedding sentinels is a common practice in surveillance programs. The purpose of the current study was to ascertain the effectiveness of dirty bedding transfer in the detection of *H. pullorum* infection of rats. In addition, to explore their susceptibility to *H. pullorum* colonization, we orally dosed Sprague–Dawley rats with *H. pullorum*.

Materials and Methods

All rats were housed in filter-topped polycarbonate cages and maintained in an AAALAC-accredited facility. Rats were fed a standard pelleted rodent diet (Prolab RMH 300, PMI International, St Louis, MO) and provided reverse-osmosis–treated water ad libitum. All Sprague–Dawley rats were certified by the vendor as negative for the following murine agents: Kilham rat virus, rat minute virus, rat parvovirus, sialodacryoadenitis virus, Toolan H1 parvovirus, pneumonia virus of mice, Sendai virus, Hantaan virus, mouse adenovirus, rat theilovirus, respiratory enteric virus, lymphocytic choriomeningitis virus, β hemolytic *Streptococcus*, *Bordetella bronchiseptica*, *Clostridium piliforme*, *Corynebacterium kutscheri*, *Klebsiella oxytoca*, *K. pneumoniae*, *Mycoplasma pulmonis*, *Pasteurella multocida*, *P. pneumotropica*, *Salmonella* spp., *Streptobacillus moniliformis*, *Streptococcus pneumoniae*, cilia-associated respiratory bacillus, *Helicobacter* spp., *Pneumocystis carinii*, and endo- and ectoparasites. All experimental procedures were reviewed and approved by the MIT Committee on Animal Care.

Dirty-bedding transfer in Sprague–Dawley rats. To determine whether *H. pullorum* would be detected by our current dirty-bedding transfer rat sentinel protocol, we transferred bedding from 5 Brown Norway rats, which were persistently infected with *H. pullorum* according to positive results by fecal culture and PCR[4], to *Helicobacter*-free Sprague–Dawley rats. Eight (4 female, 4 male) *Helicobacter*-free Sprague–Dawley rats (age, 22 to 26 d) were purchased from Charles River Laboratories

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(Charles River Laboratories, Wilmington, MA). After a 7-d acclimation period, 18 oz of dirty bedding from cages housing Brown Norway rats infected with *H. pullorum* was added to fresh bedding once weekly for 22 wk. Rats were housed in pairs and tested for *H. pullorum* colonization by species-specific fecal PCR every 2 wk for 22 wk. Liver, cecum, and colon tissue samples were collected during necropsy at 22 wk postinfection (wpi) for species-specific tissue PCR and histopathology. Persistent infection was defined as positive results in species-specific fecal PCR and positive results in at least one tissue tested by species-specific tissue PCR.

Experimental infection of Sprague–Dawley rats. To ascertain whether colonization of *H. pullorum* in rats was, in part, dependant on genetic background, Sprague–Dawley rats underwent orogastric dosing with *H. pullorum*. Ten (5 male, 5 female) *Helicobacter*-free Sprague–Dawley rats (22 to 26 d old) were obtained from a commercial vendor (Charles River Laboratories) and acclimated to the animal resource facility for 7 d. Pure cultures of *H. pullorum* obtained from the fecal cultures of infected Brown Norway rats were used as an inoculum. Each rat was dosed with 400 µL of a pure culture at OD_{600} (4×10^8 cfu) by orogastric gavage once every other day for a total of 3 doses. Rats were pair-housed, except for one male and one female rat each housed individually. Feces were collected from each rat individually and tested for *H. pullorum* colonization by species-specific PCR at 2-wk intervals for 12 wk. Liver, cecum, and colon tissue samples were collected during necropsy at 12 wpi for species-specific tissue PCR and histopathology.

PCR. Fecal DNA extraction was performed by using the QIAamp DNA stool mini kit (Qiagen, Valencia, CA). Colon, cecum, and liver DNA extractions were performed by using the High Pure PCR Template Preparation kit (Roche, Indianapolis, IN). *Helicobacter*-free status prior to experimental infection was confirmed by using *Helicobacter* genus-specific primers C97 (5′ GCT ATG ACG GGT ATC 3′) and C05 (5′ ACT TCA CCC CAG TCG CTG 3′) to amplify a 1200-bp product.[9] Primers specific for the *H. pullorum* gene cytolethal distending toxin B (F1, 5′ GTC TTT TGA GTG GAT TGG CT 3′; R2, 5′ CAC TCC GGG TGC TTG AT 3′) were used to amplify a 148-bp product and determine the infection status of rats surveyed throughout the study.[2] PCR cycling conditions were 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1.5 min, and synthesis at 72 °C for 2 min. PCR products were visualized by electrophoresis on 2% agarose gel (OmniPur Agarose, EMD Chemical, Gibbstown, NJ).

Culture. A single fecal pellet was suspended in 1.5 mL of freeze medium (*Brucella* broth with 15% glycerol); 0.3 to 0.5 mL of this suspension was collected, filtered by using a 0.45-µm syringe filter (Pall Corporation, Port Washington, NY), and streaked onto sheep blood agar plates (Remel, Lenexa, KS). An unfiltered fecal suspension was streaked on CVA plates containing cefoperazone, vancomycin, and ampohtericin B (BD, Franklin Lakes, NJ). Plates were incubated under microaerobic conditions (10% CO_2, 10% H_2, 80% N_2) and observed for growth every 2 to 4 d for 2 wk. Single colonies with visual characteristics of *H. pullorum* were replated to obtain pure cultures. Cultures were harvested and confirmed to be *H. pullorum* by using *H. pullorum*-specific PCR assays. Fecal cultures were performed at preinfection and at 4, 10, 16, and 22 wk after rats were exposed to dirty bedding. Feces of experimentally infected rats were cultured before infection and at 2, 6, and 12 wpi.

Pathology. All rats were euthanized by CO_2 inhalation. At necropsy, sections of the liver and the entire large intestine were collected from each rat. An additional 4, age-matched *Helicobacter*-free Sprague–Dawley rats (2 male, 2 female) were obtained from the same barrier at Charles River Laboratories to serve as controls. Tissues were fixed in 10% formalin, paraffin-embedded, sectioned at 5 µm, and stained with hematoxylin and eosin.

Tissue sections were evaluated by a board-certified veterinary pathologist who was blinded to sample identity. The severity of lesions in the liver and large intestine was scored according to an ascending scale from 0 to 4 based on the degree of lesion severity: 0, none; 1, mild; 2, moderate; 3, marked; and 4, severe.

For the liver, a hepatitis index was calculated by combining individual scores for lobular, portal, and interface hepatitis, as well as the number of lobes (maximum, 4) that contained 5 or more inflammatory lesions. Hepatitis was defined as having a hepatitis index equal to or greater than 4, as described previously.[20]

For the large intestine, lesions of inflammation, edema, epithelial defects, crypt atrophy, hyperplasia, and dysplasia were scored at separate locations (cecum at the ileocecocolic junction, proximal colon, and distal colon). Lesions were graded on a scale of 0 to 4 with ascending severity, as described previously.[6]

Results

Efficacy of dirty-bedding transfer for the detection of *H. pullorum* in Sprague–Dawley sentinel rats. All Sprague–Dawley rats that received dirty bedding from cages housing Brown Norway rats infected with *H. pullorum* were negative for *H. pullorum* by repeated fecal culture and PCR assays and by tissue PCR at 22 wpi. Intermittent positive fecal PCR results were noted sporadically during the 22-wk study (Table 1). Fecal cultures were negative at preinfection and at 4, 10, 16, and 22 wpi.

Resistance of Sprague–Dawley rats to experimental infection with *H. pullorum*. At 2 wpi, 5 of the 10 rats orally dosed with *H. pullorum* were positive for *H. pullorum* by species-specific fecal PCR and fecal culture. Rat 3 was euthanized after the 4-wpi time point due to an unrelated health condition. Two (SDR5 and SDR8) of the 9 remaining rats had persistent gastrointestinal colonization with *H. pullorum* (Table 2, Figure 1). Rat 5 remained positive by species-specific fecal PCR at all time points; colon tissue collected from rat 5 at necropsy was also positive for *H. pullorum* by species-specific PCR. Rat 8 was positive for *H. pullorum* by species-specific fecal PCR at 2, 10, and 12 wpi (Figure 2); in addition, cecal and colon tissue collected from this rat were positive for *H. pullorum* by species-specific PCR. Furthermore, *H. pullorum* was cultured from the feces of rats 5 and 8 at 2 and 12 wpi. Rat 9 was positive for *H. pullorum* by species-specific PCR at 6 and 8 wpi and by culture at 6 wpi but negative at all other time points during the study. Rats 2, 6, 7, and 10 were negative by species-specific fecal PCR and fecal culture at all time points. Rat 1 was positive at 2, 4, 6, and 8 wpi by species-specific fecal PCR but negative at all subsequent time points. Rat 4 was positive at 2 and 4 wpi by species-specific fecal PCR but negative at all subsequent time points. All rats not specifically mentioned otherwise were negative for *H. pullorum* by colon, cecal, and liver PCR.

Pathology. No lesions were seen in the intestinal tract of either experimentally dosed rats or those exposed to *H. pullorum*-contaminated bedding. The liver showed mild changes in rats from both groups. In the group exposed to *H. pullorum*-infected dirty bedding, there was minimal to mild portal inflammation in 6 of the 8 rats evaluated and minimal lobular inflammation in one of these rats. All 9 rats in the experimentally infected group showed minimal to mild portal inflammation and minimal to mild lobular inflammation in 4 of the 9 rats. In the experimentally

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Table 1. Fecal *H. pullorum* PCR results from Sprague–Dawley rats receiving dirty bedding over a 22-wk period.

	SD1	SD2	SD3	SD4	SD5	SD6	SD7	SD8
preinfection	–	–	–	–	–	–	–	–
2 wpi	–	+	–	–	–	–	+	–
4 wpi	–	–	+	–	–	+	+	+
6 wpi	–	–	–	–	–	–	–	–
8 wpi	–	–	–	–	–	–	–	–
10 wpi	–	–	–	–	–	–	–	–
12 wpi	–	–	–	–	+	+	+	–
14 wpi	–	–	–	–	–	–	+	–
16 wpi	+	–	–	–	–	–	–	–
18 wpi	–	–	–	–	–	–	–	–
20 wpi	–	–	–	–	–	–	–	–
22 wpi	–	–	–	–	–	–	–	–

–, negative; +, positive

Table 2. *H. pullorum* PCR and culture results from feces of experimentally infected Sprague–Dawley rats

		SDR1	SDR2	SDR3	SDR4	SDR5	SDR6	SDR7	SDR8	SDR9	SDR10
preinfection	PCR	–	–	–	–	–	–	–	–	–	–
	culture	–	–	–	–	–	–	–	–	–	–
2 wpi	PCR	+	–	+	+	+	–	–	+	–	–
	culture	+	–	+	+	+	–	–	+	–	–
4 wpi	PCR	+	–	–	+	+	–	–	–	–	–
6 wpi	PCR	+	–	na	–	+	–	–	–	+	–
	culture	+	–	na	–	+	–	–	–	+	–
8 wpi	PCR	+	–	na	–	+	–	–	–	+	–
10 wpi	PCR	–	–	na	–	+	–	–	+	–	–
12 wpi	PCR	–	–	na	–	+	–	–	+	–	–
	culture	–	–	na	–	+	–	–	+	–	–

–, negative; +, positive; na, not applicable

infected group, 4 of the 9 rats had a hepatitis index greater than 3, and 2 of 9 rats had a hepatitis index greater than 4. Although hepatitis is defined by a hepatitis index equal to or greater than 4, the hepatitis scores in these rats were not significantly different from those of controls. No correlation existed between portal or lobular liver inflammation and fecal or tissue PCR results.

Discussion

Transmission of EHS in mice can occur by cohousing and transfer of dirty bedding but, these bacteria also can infect rodents through aerosol transmission when polycarbonate filter tops are not used.[1,26] Colony surveillance by using dirty-bedding transfer protocols has been shown to be an effective method for detection of EHS in mice.[14,26] Although no literature is available that describes the mode of transmission for EHS among rats, it is reasonable to assume that the mode of transmission in rats is similar to transmission patterns of EHS in mice. Unexpectedly, transfer of *H. pullorum*-contaminated bedding failed to transmit *H. pullorum* to Sprague–Dawley sentinel rats in the current study. The failure of *H. pullorum* to infect Sprague–Dawley sentinel rats may reflect insufficient numbers of viable *H. pullorum* in the dirty bedding. Alternatively, as the results of our experimental *H. pullorum* infection study suggest, Sprague–Dawley rats may not be appropriate as sentinels to detect the presence of EHS in rats maintained for research purposes.

The apparent resistance of Sprague–Dawley rats to natural and experimental *H. pullorum* infections is consistent with the paucity of reports describing EHS infection in rats and suggests that rats, in general, are more resistant to EHS infections than mice. Indeed, prior to documentation of *H. pullorum* infections in Brown Norway rats, only 3 EHS—*H. bilis*, *H. trogontum*, and *H. muridarum*—were reported to naturally infect rats.[11,13,15,17] In natural infections with *H. trogontum*, the organisms were isolated from clinically normal Wistar rats, whereas *H. muridarum* was isolated from both Holtzman and Wistar rats.[13,15,17] *H. bilis* was isolated from a nude rat (Cr:NIH-rnu) with histologic evidence of proliferative colitis.[11] In addition, bacteria with "*H. bilis*-like" morphology have been observed in the livers of Wistar rats experimentally infected with the liver fluke *Fasciola hepatica* and in the bile ducts of rats originating from the United Kingdom.[8] However, despite the infrequent reports of EHS in rats, EHS have been isolated from rats located on several continents:

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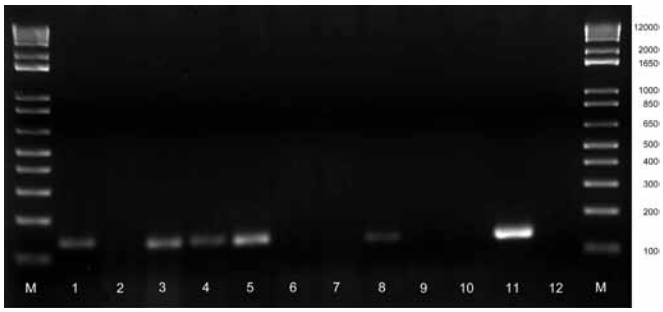

Figure 1. Electrophoresis gel showing *H. pullorum*-specific PCR results of orally dosed Sprague–Dawley rats at 2 wpi. M, 1-kb plus marker (Invitrogen); lanes 1 through 10, rats 1 through 10; lane 11, positive control; lane 12, negative control.

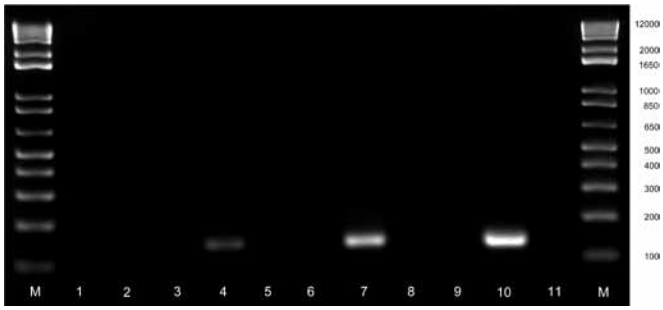

Figure 2. Electrophoresis gel showing *H. pullorum*-specific PCR results of orally dosed Sprague–Dawley rats at 12 wpi. M, 1-kb plus marker (Invitrogen); lanes 1 through 9, rats 1, 2, and 4 through 10; lane 10, positive control; lane 11, negative control.

H. trogontum was isolated from clinically normal rats surveyed in South America, *H. muridarum* was cultured from rats sampled in Australia, and nude rats infected with *H. bilis* were housed in a research facility in North America.[11] More recently, molecular identification of *H. pullorum* in the intestinal contents of F344 rats housed in a research facility resulted from studies conducted in China.[30] These observations, although indirect, combined with the lack of previous publications regarding *H. pullorum* or other EHS infections in the large number of Sprague–Dawley rats used in testing and research, may indicate that for unknown reasons, Sprague–Dawley rats are resistant to EHS enteric infections. Indeed, only 2 orally dosed Sprague–Dawley rats in the current study became persistently infected with *H. pullorum*; with an additional 4 of 10 rats being transiently positive by fecal PCR and fecal culture analysis.

In contrast, our earlier studies indicated that *H. pullorum* efficiently infected Brown Norway rats.[4] In addition, nearly all Brown Norway rats and C57BL/6 mice surveyed during an outbreak of *H. pullorum* in a commercial rodent barrier were infected with *H. pullorum* according to PCR and culture results.[4,24] Consistent with our previous findings in *H. pullorum*-infected Brown Norway rats and C57BL mice and what is known about EHS infection in most strains of immunocompetent mice and rats, no significant clinical signs or intestinal lesions were seen in *H. pullorum* experimentally infected Sprague–Dawley rats.[4]

Although infection with most EHS do not cause significant histopathologic changes in immunocompetent rats or mice, persistence of *H. pullorum* and other EHS in rat or mouse colonies may confound in-vivo–based research.[27] Importantly, genetically susceptible or immunocompromised rats or mice commonly used in research may develop histologic evidence of typhlocolitis when infected with EHS.[10,11] In addition, hepatocellular carcinoma or colon carcinoma can develop in immune-deficient mice infected with *H. hepaticus* or *H. bilis*.[7,10,19,27] *H. pullorum* is a zoonotic agent and has been associated with human disease.[21,22] The recent molecular detection of *H. pullorum* in the intestinal microflora of rats exposed to a carcinogen may reflect its presence in a wider population of commercially available rats.[30] Further investigative and epidemiologic studies of *H. pullorum* are warranted.[2,4,24]

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