

Natural and experimental *Helicobacter pullorum* infection in Brown Norway rats

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Helicobacter pullorum is an enterohepatic *Helicobacter* species (EHS) that was recently reported as a naturally acquired infection in mice. Faecal samples from 18 out of 20 Brown Norway (BN) rats, housed in the same barrier as the *H. pullorum*-infected mice, were positive for *H. pullorum* using species-specific PCR. In addition, we determined whether *H. pullorum* was able to persistently colonize the gastrointestinal tract and/or biliary tree and elicit tissue inflammation as well as a serum IgG response in BN rats. Six (four male, two female) 6-week-old, *H. pullorum*-negative BN rats were orally dosed with 4×10^8 c.f.u. of *H. pullorum* every other day for a total of three doses. At 2 weeks post-infection, all rats were *H. pullorum*-positive by faecal PCR. Five out of the six BN rats remained *H. pullorum*-positive for the entire 30 week study. PCR analysis of tissue collected at necropsy confirmed that the colon and caecum were the primary sites of *H. pullorum* colonization. Rats that were persistently colonized by *H. pullorum* had a sustained *H. pullorum*-specific IgG response measured by ELISA. Intestinal or hepatic pathology associated with *H. pullorum* infection was not noted. To our knowledge, this is the first report documenting that rats can be persistently colonized with an EHS that also infects humans.

Received 12 January 2012

Accepted 5 May 2012

INTRODUCTION

Helicobacter species are a group of taxonomically related Gram-negative, microaerophilic bacteria, some of which are pathogenic and known to colonize the gastrointestinal and biliary tracts of many animal species. These pathogens are generally separated into two groups, gastric and enterohepatic, based on their preferred site of colonization. During the last decade, enterohepatic *Helicobacter* species (EHS) have gained recognition in the field of emerging infectious pathogens (Fox, 2002). Infection by this group of micro-organisms is generally characterized by colonization of the distal gastrointestinal tract and, in select cases, the biliary tree. As reported for the gastric pathogen *H. pylori*, gastrointestinal colonization by EHS can be associated with chronic inflammation and neoplasia (Fox *et al.*, 1996; García *et al.*, 2008; Ward *et al.*, 1994).

Helicobacter pullorum, an EHS, was first isolated by Stanley *et al.* (1994) from the faeces of diarrhoeic humans and the intestinal contents and livers of chickens. The organism is suspected to cause vibronic hepatitis in chickens. Infection

with this organism is most often associated with farm-raised birds, including chickens, turkeys and guinea fowl (Nebbia *et al.*, 2007; Stanley *et al.*, 1994). In one report, *H. pullorum* was isolated from human faeces 3 months following the patients' initial presentation with diarrhoea (Steinbrueckner *et al.*, 1997). In another case, *H. pullorum* was isolated from the faeces of a male with diarrhoea and elevated liver enzymes (Burnens *et al.*, 1994). *H. pullorum* has also been identified by PCR in humans with inflammatory bowel disease, hepatitis, cholecystitis and hepatocellular carcinoma (Castéra *et al.*, 2006; Fox *et al.*, 1998; Rocha *et al.*, 2005; Veijola *et al.*, 2007). A recent report identified an association between EHS and Crohn's disease, with *H. pullorum* being one of the most prevalent EHS identified (Laharie *et al.*, 2009).

In July 2009, routine surveillance testing detected a *Helicobacter* species in C57BL/6NTac mice and BN/MolTac Brown Norway (BN) rats at Taconic Farms, Germantown, New York. This *Helicobacter* species was later confirmed to be *H. pullorum* (Boutin *et al.*, 2010). To our knowledge, prior to this event, *H. pullorum* had not previously been reported to infect rodents either naturally or experimentally. The purpose of this report is to describe *H. pullorum* infection in naturally and experimentally infected BN rats

Abbreviations: BN, Brown Norway; EHS, enterohepatic *Helicobacter* species; qPCR, quantitative PCR; RFLP, restriction fragment length polymorphism; w.p.i., weeks post-infection.

and demonstrate that this rat strain can be persistently infected with an EHS known to infect humans.

METHODS

Natural infection of Brown Norway rats. Faecal DNA samples from 20 BN rats were shipped to our laboratory from Taconic Farms after routine testing discovered *Helicobacter* in mice where the BN rats were co-housed in the same barrier. Samples were screened for *Helicobacter* species by PCR using 16S rRNA gene genus-specific primers and *H. pullorum* cytolethal distending toxin B (*cdtB*) gene species-specific primers (Boutin *et al.*, 2010; Fox *et al.*, 1998; Rocha *et al.*, 2005). Samples confirmed as *H. pullorum* were subjected to restriction fragment length polymorphism (RFLP) analysis following amplification using *Helicobacter* genus-specific primers.

Experimental infection of Brown Norway rats. All rats were maintained in an AAALAC accredited facility and housed singly in filter topped polycarbonate cages. Rats were fed a standard pelleted rodent diet and provided water ad libitum. BN rats were free from the following murine agents certified by the vendor: Kilham's rat virus, rat coronavirus, rat minute virus, rat parvovirus, sialodacryoadenitis virus, Toolan's H-1 parvovirus, encephalomyelitis virus, pneumonia virus of mice, Sendai virus, hantaan virus, mouse adenovirus (MAV-1, MAV-2), respiratory enteric virus III, β -haemolytic streptococcus, *Bordetella bronchiseptica*, *Clostridium piliforme*, *Corynebacterium kutscheri*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Mycoplasma Pasteurella*, *Salmonella*, *Streptobacillus moniliformis*, *Streptococcus pneumoniae*, CAR bacillus, *Helicobacter*, *Pneumocystis carinii*, endoparasites and ectoparasites. All experimental procedures were reviewed and approved by the MIT Institutional Animal Care and Use Committee.

Six *Helicobacter*-free BN weanling rats (two female, four male) were obtained from Taconic Farms. After acclimation, each rat was inoculated by oro-gastric gavage with 400 μ l of OD₆₀₀ (4×10^8 c.f.u.) of an *H. pullorum* isolate (MIT 09-6635) that was cultured from the caeca of the *H. pullorum*-infected mice identified in the initial outbreak (Boutin *et al.*, 2010). Each rat was gavaged once every other day for a total of three doses. Animals were housed singly and tested every 2 weeks [except for 12 weeks post-infection (w.p.i.)] for *H. pullorum* colonization by a species-specific faecal PCR and serum ELISA, respectively. Rats were monitored for weight loss and diarrhoea. At 30 w.p.i., animals were euthanized.

PCR. Faecal DNA extraction was performed using the High Pure PCR Template Preparation kit (Roche) or the QIAamp DNA stool mini kit (Qiagen). Pre-infection *Helicobacter*-free status was confirmed using *Helicobacter* genus-specific primers C97 (5'-GCTATGACGGGTATCC-3') and C05 (5'-ACTTCACCCAGTCGCTG-3'). The amplified 1200 bp PCR product was also used for 16S rRNA gene sequencing and RFLP analysis (see below). The *H. pullorum cdtB*-gene-specific primers (F1, 5'-GTCTTTTGAGTGGATTGGATTCT-3') and (R2, 5'-CACTCCGGGTGCTTGTGTAT-3') were used to determine infection status at all other time points (Laharie *et al.*, 2009).

Real-time quantitative PCR. The SyBr-based real-time quantitative PCR assay (qPCR) used for quantifying levels of *H. pullorum* is described elsewhere (Turk *et al.*, 2012). Briefly, a standard curve containing a serial 10-fold dilution of known genomic copies (10^6 to 10^1) of *H. pullorum* MIT 98-5489 was used to enumerate *H. pullorum* in tissue and faecal DNA samples from rats using a 7500 Fast sequence detection system (Applied Biosystems). qPCR primers were derived from the *cdtB* gene as described previously (Boutin *et al.*, 2010). Mucosal and faecal *H. pullorum* numbers calculated from standard curves were then normalized to micrograms of rat chromosomal DNA

and measured by qPCR using the 18S rRNA gene-based primers and probe mixture (Applied Biosystems).

Restriction fragment length polymorphism. RFLP was performed using previously described methods (Shen *et al.*, 2000). Briefly, the amplified 1200 bp *H. pullorum*, *H. bilis*, *H. muridarum* and *H. troglontum* DNA samples were digested using *AluI* and *HhaI* in a 37 °C water bath for 3 h. Digested samples were subsequently electrophoresed on a 3% agarose gel and stained with ethidium bromide.

Culture. One faecal pellet was suspended in 1.5 ml of freeze medium (20% glycerol in Brucella broth); 0.3 ml to 0.5 ml of suspended faeces was collected and filtered using a 0.45 μ m syringe filter (Pall Corporation) and streaked onto sheep blood agar plates (Remel). An unfiltered faecal suspension was streaked on CVA plates containing cefoperazone, vancomycin and amphotericin B (Becton Dickinson). Plates were incubated under microaerobic conditions (10% CO₂, 10% H₂, 80% N₂) and observed for growth every 2–4 days. Single colonies with visual and physical characteristics of *H. pullorum* were replated to obtain a pure culture. Cultures were harvested and confirmed to be *H. pullorum* using an *H. pullorum*-specific PCR. Faecal cultures from BN rats were performed at both the pre-infection and final end points as well as intermittently throughout the 30-week study.

ELISA. *H. pullorum* outer-membrane protein (OMP) antigen was prepared by methods described previously (Whary *et al.*, 1998). The OMP concentration was measured using a BCA Protein Assay kit (Pierce). Immunolon 2HB 96-well plates (Thermo Electron Corporation) were coated with 100 μ l OMP in carbonate buffer (pH 9.6) at a concentration of 1 μ g ml⁻¹ per well and incubated overnight at 4 °C. All wells were blocked by incubating with 200 μ l 2% BSA (BSA) for 1 h. Serum was assayed at a dilution of 1:100 with 1% BSA-PBS. Biotinylated goat anti-rat secondary antibodies (Southern Biotech) at a concentration of 1:1000 were used to detect *H. pullorum*-specific IgG. Plates were then incubated with extravidin peroxidase (Sigma) followed by 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonate) (ABTS) (Kirkegaard & Perry Laboratories) for colour development. The optical density (OD) of each sample was measured and recorded at both 405 and 590 nm at 30 min by a Powerwave X ELISA plate reader (Bio-Tek Instruments). All samples were run in triplicate. Seroconversion, as determined by the presence of *H. pullorum*-specific IgG, was defined as OD values exceeding the mean plus 2 \times SD of the OD of the samples at the pre-infection time point. The Student's *t*-test was used to determine significance at $P < 0.05$.

Pathology. All rats were euthanized by CO₂ inhalation and blood was collected to assess specific IgG response to *H. pullorum*. At necropsy, the liver and entire large intestine were fixed in 10% formalin, paraffin-embedded, sectioned at 5 μ m and stained with haematoxylin 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, using an ascending scale from 0 to 4, based on the degree of lesion severity: 0, absent; 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 (out of a total of four) that contained five or more inflammatory lesions. Hepatitis was defined by a hepatitis index equal to or greater than 4 (Rogers *et al.*, 2007).

For the large intestine, lesions or inflammation, oedema, epithelial defects, crypt atrophy, hyperplasia and dysplasia were scored at

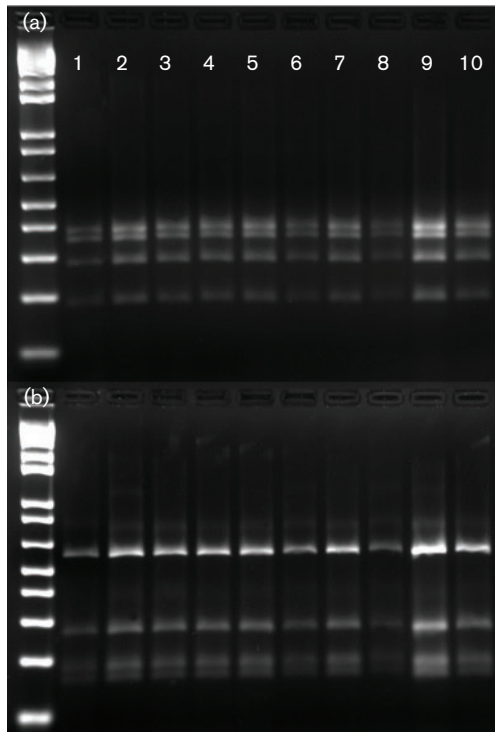


Fig. 1. RFLP using *HhaI* (a) and *AluI* (b) digestion of 1200 bp PCR products from 10 rat caecal DNA samples from the initial outbreak.

separate locations (caecum at the ileocecolic junction, proximal colon and distal colon) (Turk *et al.*, 2012).

RESULTS

Molecular analyses indicated a high prevalence of naturally acquired *H. pullorum* infection in Brown Norway rats

Eighteen out of the 20 faecal DNA samples collected from BN rats housed in the barrier where the *H. pullorum* outbreak was first detected in mice were positive by PCR using both *Helicobacter* genus-specific and *H. pullorum*-specific primers. Ten of the 1200 bp helicobacter PCR products were subjected to RFLP analysis using *AluI* and *HhaI* restriction enzymes and yielded patterns identical to *H. pullorum* MIT 09-6635. Using *HhaI* digestion, *H. pullorum* had the same pattern as *H. bilis*, but a different pattern from those of *H. muridarum* and *H. trogontum*. When analysing *AluI* restriction digestion patterns, *H. bilis* and *H. trogontum* had similar patterns, but all three rat *Helicobacter* species (*H. bilis*, *H. muridarum*, *H. trogontum*) were different from the restriction pattern of *H. pullorum* (Figs 1 and 2). 16S rRNA analysis of the 1200 bp products from faeces of two rats and the *H. pullorum* mouse isolate (MIT 09-6635) indicated 99% sequence identity with *H. pullorum* ATCC 51801 isolated from humans.

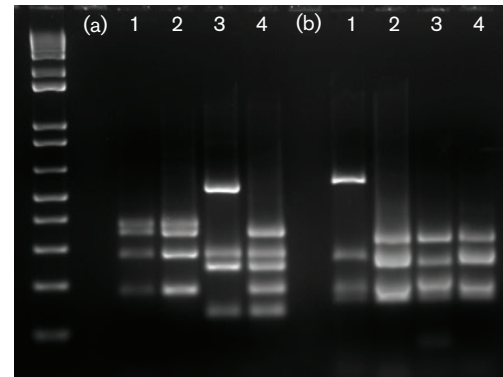


Fig. 2. RFLP using *HhaI* (a) and *AluI* (b) digestion of 1200 bp PCR products from *H. pullorum* (MIT 09-6635) from a BN rat. For comparison, lanes 2, 3 and 4 are PCR products from *H. bilis*, *H. muridarum* and *H. trogontum*, respectively.

Experimentally infected brown Norway rats were persistently infected with *H. pullorum* and developed an *H. pullorum* IgG immune response

Pre-inoculation faecal samples analysed by PCR were negative for *Helicobacter* genus-specific and *H. pullorum*-specific DNA. All rats were confirmed as *H. pullorum*-positive at 2 w.p.i. by both species-specific faecal PCR and faecal culture. Positive faecal cultures were confirmed by *H. pullorum*-specific PCR. At 4 w.p.i. and continuing through 30 w.p.i., BN rats #2–6 tested positive (except at 6 w.p.i. and 20 w.p.i.) by either species-specific faecal PCR or faecal culture. BN rat #1 was positive for *H. pullorum* by species-specific PCR and faecal culture at 2 weeks and PCR positive at 4 w.p.i. but negative at all subsequent time points (Table 1). *H. pullorum* was detected at necropsy in 4/6 caecal samples and 0/6 liver samples by PCR and in 5/6 caecal samples and 1/6 liver samples by qPCR. qPCR detected quantities of *H. pullorum* ranging from 11 to 558 684 bacteria mg^{-1} host DNA in faecal samples collected at necropsy. IgG seroconversion to *H. pullorum* was apparent in 4/6 BN rats by 2 w.p.i. and in all five colonized rats by 12 w.p.i. and was maintained through 24 w.p.i. (See Fig. 3). During the 30-week experiment, all rats were clinically normal with no signs of diarrhoea.

Pathology

No significant lesions in the intestine were observed in the experimentally infected group. All six BN rats had minimal portal liver inflammation and one rat had minimal lobular inflammation.

DISCUSSION

Eighteen of 20 BN rats surveyed from the natural outbreak were infected with *H. pullorum* based on *H. pullorum*-specific PCR and RFLP analyses, which was confirmed by

Table 1. *H. pullorum* PCR and culture results in experimentally infected BN rats over the 30 w.p.i.

Values are presented as PCR/culture results, respectively. Single values represent PCR results where cultures were not taken. +, Positive; -, negative.

W.p.i.	BN1	BN2	BN3	BN4	BN5	BN6
0 (pre-dosing)	-/-	-/-	-/-	-/-	-/-	-/-
2	+/+	+/+	+/+	+/+	+/+	+/+
4	+/-	+/+	+/+	+/+	+/+	+/+
6	-	-	-	+	+	+
8	-	+	+	+	+	+
10	-	+	+	+	-	+
14	-/-	+/+	+/+	+/+	-/+	+/+
16	-	+	+	+	+	+
18	-	+	+	+	+	+
20	-	+	-	+	-	+
22	-	+	+	+	+	+
24	-/-	+/+	+/+	+/+	+/+	+/+
26	-/-	+/+	+/+	+/+	+/+	+/+
28	-	+	+	+	+	+
30	-/-	+/+	+/+	+/+	+/+	+/+

16S rRNA gene sequencing. In experimentally infected BN rats, 5/6 rats became persistently colonized for the 30 week duration of the experiment. The remaining rat was negative by PCR and culture after 4 w.p.i. PCR results for BN2 and BN3 at 6 w.p.i. and BN3 and BN5 at 20 w.p.i. were negative using our detection methods, which most likely indicates intermittent shedding of *H. pullorum*. ELISA data measuring IgG response to *H. pullorum* in BN rats indicated seroconversion in four out of the six rats by 2 w.p.i. and five out of the six rats by 12 w.p.i. These data are most likely representative of the persistent *H. pullorum* infection we noted in the natural *H. pullorum* outbreak in BN rats and indicate that serological testing may be a useful screening tool to determine natural exposure or infection with *H. pullorum*. Similar to EHS infections in mice, and *H. pylori* infections in humans and mice, seroconversion denotes a

persistent infection and indicates that serum antibodies to these helicobacters are not protective to the host.

Gastrointestinal colonization with *H. pullorum* has been confirmed in humans, poultry and, more recently, mice (Boutin *et al.*, 2010; Stanley *et al.*, 1994; Veijola *et al.*, 2007). Our results confirmed the ability of *H. pullorum* to successfully colonize the gastrointestinal tract of BN rats. However, *H. pullorum* infection did not elicit clinical signs or cause gastrointestinal pathology in rats. This finding is consistent with the lack of gastrointestinal lesions in C57BL/6NTac mice persistently infected with *H. pullorum* (Boutin *et al.*, 2010; Turk *et al.*, 2012).

Faeces from wild rodents and geese around the exterior of the barrier structure where the *H. pullorum* outbreak occurred were collected and tested for the presence of *Helicobacter* species by genus-specific PCR. Although *H. pullorum* was not identified, another avian *Helicobacter* species was identified; *H. brantae* naturally colonizes geese and is 97% similar to *H. pullorum* by 16S rRNA gene analysis (Boutin *et al.*, 2010; Fox *et al.*, 2006). The source of the *H. pullorum* outbreak in the commercially received mice and rats remains unknown; however, the *H. pullorum* strain isolated (MIT 09-6635) was 99% similar to human *H. pullorum* isolate NCTC 12826 by 16S rRNA gene analysis, indicating that an infected human may have been responsible for initiating the *H. pullorum* outbreak in the rodent barrier (Boutin *et al.*, 2010). In humans, *H. pullorum* has been cultured from diarrhoeic patients and identified by PCR in patients with inflammatory bowel disease and hepatobiliary disease (Castéra *et al.*, 2006; Fox, 2002; Fox *et al.*, 1998). EHS including *H. pullorum* have also been identified by PCR in patients with hepatobiliary tumours (Rocha *et al.*, 2005).

Prior to this study, only three EHS, *H. bilis*, *H. trogontum* and *H. muridarum*, were known to naturally colonize rats. Of the aforementioned, only *H. bilis* has been shown to

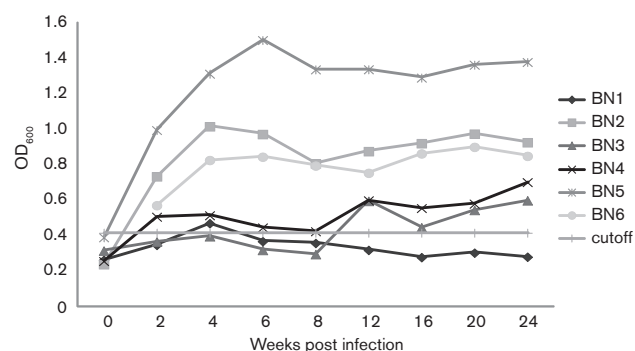


Fig. 3. IgG response to experimental infection with *H. pullorum*. Rats 2–6 were persistently infected with *H. pullorum* and developed moderate to robust *H. pullorum* IgG responses. Rat 1 did not colonize with *H. pullorum* and did not seroconvert.

cause clinical disease in rats, causing colitis in naturally and experimentally infected *nu/nu* rats (Haines *et al.*, 1998; Lee *et al.*, 1992; Mendes *et al.*, 1996). Our finding of natural *H. pullorum* infection in rats and the ability of *H. pullorum* to experimentally infect rats warrants further studies to determine whether wild rats are infected with *H. pullorum* and serve as reservoirs capable of contaminating food stuffs used for poultry or human consumption.

Isolation of *H. pullorum* from naturally infected rats and mice also supports the rationale that this bacterium should be included in the differential diagnosis when *Helicobacter* genus-specific PCR results are positive. Additionally, the ability of *H. pullorum* to colonize multiple species, including humans, and its demonstrated persistent shedding in the faeces of rats and mice pose a zoonotic risk to personnel exposed to *H. pullorum*-infected animals (Turk *et al.*, 2012). To our knowledge, this is the first report confirming that rats, like mice, can be persistently colonized by an EHS known to colonize humans. Further studies on the epidemiology and pathogenesis of *H. pullorum* will be facilitated by the availability of the *H. pullorum* genome (<http://www.ncbi.nlm.nih.gov/genome/?term=helicobacter%20pullorum>).

ACKNOWLEDGEMENTS

Grant support was provided by grants from the National Institutes of Health (nos R01RR032307, R01AI50952, T32RR070036 and P30-ES02109). Partial funding for this project was also provided by Taconic Farms Inc., Germantown, NY.

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