Persistent *Helicobacter pullorum* colonization in C57BL/6NTac mice: a new mouse model for an emerging zoonosis

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Helicobacter pullorum, an enterohepatic *Helicobacter* species, is associated with gastroenteritis and hepatobiliary disease in humans and chickens. Recently, a novel *H. pullorum* outbreak in barrier-maintained rats and mice was described. In this study, persistence of infection and serological responses were further evaluated in *H. pullorum*-infected female C57BL/6NTac and C3H/HeNTac mice obtained from the barrier outbreak. C57BL/6NTac mice ($n=36$) aged 10–58 weeks were confirmed to be chronically infected with *H. pullorum* by PCR or culture of caecum, colon and faeces, with no evidence of hepatic infection; two of three C3H/HeNTac mice cleared *H. pullorum* infection by 26 weeks of age. A quantitative PCR (qPCR) assay based on the *cdtB* gene specific to *H. pullorum* demonstrated that colonization was high in the caecum and colon at 10^4 – 10^6 c.f.u. equivalents per μg host DNA, and decreased by several logs from 32 to 58 weeks of age. Infected mice were seropositive by ELISA, and *H. pullorum*-specific IgG levels decreased as colonization was lost over time in selected mice. Consistent with the lack of pathology associated with chronic infection of C57BL/6 mice with other murine enteric helicobacters, C57BL/6NTac and C3H/HeNTac mice infected with *H. pullorum* did not develop gross or histological lesions of the liver or gastrointestinal tract. The *cdtB*-based qPCR assay can be used in screening animals, food sources and environmental samples for *H. pullorum*, as this food-borne pathogen has zoonotic potential. These findings will also allow future studies in murine models to dissect potential pathogenic mechanisms for this emerging pathogen.

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INTRODUCTION

Helicobacter pullorum, an enterohepatic *Helicobacter* species (EHS), was identified as a novel species of this genus in 1994 by Stanley *et al.* (1994). *H. pullorum* is a Gram-negative, microaerophilic, slightly curved rod with monopolar, non-sheathed flagella. It was originally cultured from the caeca and livers of broiler and laying hens, and the faeces of humans (Stanley *et al.*, 1994). *H. pullorum* prevalence in avian species is poorly documented. Although most infected birds remain subclinical, *H. pullorum* infection has been linked to vibronic hepatitis and enteritis in chickens (Stanley *et al.*, 1994). In Italy, *H. pullorum* has been isolated from, or identified by PCR, in a high percentage of broiler chickens, laying hens and

guinea fowl (Nebbia *et al.*, 2007). *H. pullorum* was detected at 100 % prevalence in broilers and laying hens from 15 different farms (Zanoni *et al.*, 2007), as well as in a majority of slaughterhouse turkeys (Zanoni *et al.*, 2011). In Belgium, 110 broilers from 11 flocks were colonized with *H. pullorum* in the caecum and colon, and to a lesser degree in the liver and jejunum (Ceelen *et al.*, 2006b). *H. pullorum* has also been isolated from a diarrhoeic psittacine bird (Ceelen *et al.*, 2006a), suggesting that pet birds may be a zoonotic risk.

H. pullorum was detected by PCR in faeces from 4.3 % of 532 patients with gastroenteritis, and from 4 % of 100 clinically normal patients (Ceelen *et al.*, 2005). Importantly, as noted in several reports, *H. pullorum* has been cultured from patients with diarrhoea (Burnens *et al.*, 1994; Stanley *et al.*, 1994; Steinbrueckner *et al.*, 1997). Several EHS, including *H. pullorum* and *H. pullorum*-like organisms, have been found to cause bacteraemia and

Abbreviations: EHS, enterohepatic *Helicobacter* species; OMP, outer-membrane protein; qPCR, quantitative PCR.

A supplementary figure is available with the online version of this paper.

systemic disease in both immunocompromised (Schwarze-Zander *et al.*, 2010) and immunocompetent (Tee *et al.*, 2001) patients. *H. pullorum* infections detected by PCR assays have been associated with inflammatory bowel and hepatobiliary diseases in humans (Bascañana *et al.*, 2011; Castéra *et al.*, 2006; Fox *et al.*, 1998; Veijola *et al.*, 2007). In addition to other EHS, *H. pullorum* is considered an emerging, zoonotic human pathogen (Atabay *et al.*, 1998; Skovgaard, 2007), warranting the need to develop animal models to investigate its pathogenesis.

Routine surveillance testing at a commercial rodent production facility detected a *Helicobacter* species by PCR in BN/MoTac rats and in C57BL/6NTac, C3H/HeNTac and DBA/2NTac mice (Boutin *et al.*, 2010). No clinical signs in infected rodents were observed, but minimal amounts of red-tinged peritoneal fluid were found in some mice at necropsy, although abdominal viscera appeared grossly normal. Culture of a *Helicobacter* species from mice shipped to our institution and PCR with 16S rRNA gene-based primers indicated that the isolate was *H. pullorum* (Boutin *et al.*, 2010).

Given the novel observation of natural infection with *H. pullorum* in commercially available mice and rats, we further evaluated persistence of infection, the serological response and lesion development in *H. pullorum*-infected female C57BL/6NTac and C3H/HeNTac mice obtained from the outbreak. As part of this study, we also developed a quantitative PCR (qPCR) assay to estimate *H. pullorum* colonization levels and an ELISA to evaluate seroconversion.

METHODS

Mice. Barrier-maintained 10-week-old female C57BL/6NTac ($n=25$) and C3H/HeNTac ($n=3$) mice from the *H. pullorum* outbreak were shipped to our institution for diagnostic evaluation. Additionally, 11 32-week-old female C57BL/6NTac mice distributed from the same barrier unit to another research facility were also made available for study. Thirty female *Helicobacter*-free C57BL/6NTac mice were used as age-matched controls. On arrival, the vendor report indicated that all mice were seronegative for mouse hepatitis virus, mouse rotavirus, lymphocytic choriomeningitis virus, ectromelia virus, mouse parvovirus, minute virus of mice, murine norovirus, pneumonia virus of mice, reovirus type 3, Sendai virus, Theiler's mouse encephalomyelitis virus, mouse adenovirus, K virus, polyoma virus, mouse cytomegalovirus, mouse thymic virus, lactic dehydrogenase-elevating virus, cilia-associated respiratory bacillus, *Mycoplasma pulmonis*, *Salmonella*, *Clostridium piliforme*, *Corynebacterium kutscheri*, *Citrobacter rodentium* and endo- or ectoparasites. Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility and maintained in static solid-bottomed polycarbonate microisolators on heat-treated hardwood bedding (Sanichips; PJ Murphy) and provided with a pelleted diet (RMH 3000; PMI) and water *ad libitum*. *H. pullorum*-infected and control mice were maintained in separate cubicles throughout the study. The protocol for use of these mice was approved by the Massachusetts Institute of Technology Committee on Animal Care.

Necropsy and sample collection. Eleven female C57BL/6NTac mice, aged 32 weeks, that were potentially infected with *H. pullorum* and ten age-matched control C57BL/6NTac mice were euthanized by

CO₂ on arrival. The remaining mice obtained directly from the outbreak barrier (C57BL/6NTac, $n=25$; C3H/HeNTac, $n=3$) were monitored for persistence of *H. pullorum* infection by faecal PCR, serial bleeds for antibody response, and associated clinical signs and pathology until necropsy at 38 weeks (C57BL/6NTac, $n=10$; C3H/HeNTac, $n=3$) or 58 weeks (C57BL/6NTac, $n=14$) of age along with age-matched control mice (C57BL/6NTac, $n=14$). At necropsy, blood was collected by cardiocentesis and serum was stored at -20°C . Liver, ileum, caecum and colon were removed aseptically, preserved for culture in *Brucella* broth containing 20% glycerol and stored at -80°C . Caecum and colon were collected for DNA extraction at 32 and 38 weeks of age, and, in addition, liver and ileum were collected at 58 weeks of age for DNA extraction. All samples were stored at -80°C .

Histological evaluation. Tissues were preserved in 10% neutral-buffered formalin, embedded in paraffin and sectioned at 4 μm for histopathological evaluation. Ileum, caecum and colon were evaluated blind by a board-certified veterinary pathologist (N.P.). In the caecum, colon and ileum, lesion scores were assigned for inflammation, oedema, epithelial defects, crypt atrophy, hyperplasia and dysplasia on an ascending scale of 0–4. In the liver, lesion scores were similarly assigned for portal, interface and lobular hepatitis, also on a scale of 0–4. A hepatitis index was then generated by combining scores for portal, interface and lobular hepatitis with the number of lobes (out of four) containing five or more inflammatory foci. Mice with a hepatitis index of ≥ 4 were defined as having hepatitis.

Sequencing of PCR products. DNA was extracted from *H. pullorum* isolated from faecal and caecal cultures and amplified using the *Helicobacter* genus-specific primers C97 (5'-GCTATGACGG-GTATC-3') and CO5 (5'-ACTTCACCCAGTCGCTG-3') to amplify a 1200 bp fragment (Fox *et al.*, 1998), which was purified using a QIAquick PCR Purification kit (Qiagen). Two randomly selected PCR products were sequenced using an ABI 3500 Genetic Analyzer (Applied Biosystems). The sequences of PCR products were compared with all *H. pullorum* 16S rRNA gene sequences available in GenBank using BLAST software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Real-time quantitative PCR of *H. pullorum*. Chromosomal DNA from a broth-grown *H. pullorum* strain MIT 98-5489 (human isolate) was prepared using a High Pure PCR Template Preparation kit according to the supplier's instructions (Roche Molecular Biochemicals). To develop a real-time qPCR assay for estimating levels of *H. pullorum* in faeces, tissues or environmental samples, cytolethal distending toxin B (*cdtB*)-based primers F1 (5'-GTCTTTTGAGTGGATTGGCT-3') and R2 (5'-CACTCCGGGTGCTTGAT-3') (Laharie *et al.*, 2009) were evaluated with SYBR Green-based qPCR for sensitivity and specificity. Sensitivity was determined by amplifying serial tenfold dilutions of known genomic copies (10^6 – 10^1) of *H. pullorum* MIT 98-5489 in a 7500 Fast Sequence Detection System (Applied Biosystems) according to the manufacturer's default setting for the Fast System, followed by a dissociation stage. The specificity of these primers for *H. pullorum* was tested using DNA (equivalent to 10^6 copies of the genomes) isolated from *Helicobacter bilis* ATCC 43879, *Helicobacter cinaedi* CCUG 18818, *Helicobacter winthamensis* ATCC BAA-430, *Helicobacter hepaticus* 3B1 and *Campylobacter jejuni* strains 81-176 and ATCC 43431. Specificity was also verified by a BLASTN search of the appropriate databases. Genomic copies of the respective bacteria were estimated based on published genome sizes of 1.95 Mb for *H. pullorum*, 2.6 Mb for *H. bilis* ATCC 43879, 2.21 Mb for *H. cinaedi* CCUG 18818, 1.69 Mb for *H. winthamensis* ATCC BAA-430 (data stored at http://www.broadinstitute.org/annotation/genome/Helicobacter_group), 1.79 Mb for *H. hepaticus* (Suerbaum *et al.*, 2003) and 1.68 Mb for *C. jejuni* 81-176 (<http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi>). Mucosal and faecal *H. pullorum* numbers calculated from standard curves were

then normalized to micrograms of murine chromosomal DNA measured by qPCR using 18S rRNA gene-based primers and probe mixture (Applied Biosystems).

***H. pullorum* and *Helicobacter* genus-specific PCR.** Nucleic acid extraction for PCR analysis from all faecal and tissue samples was carried out using a High Pure PCR Template Preparation kit (Roche) according to the manufacturer's protocol. Control mice were confirmed free of all helicobacters by PCR using *Helicobacter* genus-specific primers. All mice associated with the outbreak were assayed using *H. pullorum* *cdtB*-specific primers to amplify a 148 bp fragment (Boutin *et al.*, 2010). Extracted bacterial DNA from faecal samples was amplified using *Taq* DNA polymerase (Roche) and tissue samples with the Expand High Fidelity PCR System (Roche). The PCR conditions for the *H. pullorum* *cdtB*-specific primers have been published previously (Boutin *et al.*, 2010).

***H. pullorum* isolation.** Faecal samples were homogenized in 1 ml sterile PBS and passed through a 0.45 µm syringe tip filter for streaking onto sheep blood agar (Remel Laboratories) or left unfiltered for streaking onto CVA plates (BD) containing cefoperazone, vancomycin and amphotericin B. All plates were incubated at 37 °C under microaerobic conditions (80 % N₂, 10 % CO₂, 10 % H₂). Culture plates with no growth were incubated for 3 weeks to confirm their negative status.

Serum ELISA. Outer-membrane protein (OMP) of *H. pullorum* was prepared as described previously for other EHS (Whary *et al.*, 1998). Briefly, *H. pullorum* purity was confirmed by Gram staining and phase-contrast microscopy, and an *H. pullorum* pellet was washed three times in PBS and resuspended in 1 % *N*-octyl-β-glucopyranoside (Sigma). After 30 min at room temperature and ultracentrifugation at 100 000 g for 1 h, the protein layer was removed and dialysed against PBS for 24 h at 4 °C. Protein concentration was determined using a BCA protein assay (Pierce). OMP preparations of *H. hepaticus* and *H. bilis*, both endemic in mouse colonies (Taylor *et al.*, 2007), were used to examine the specificity of the *H. pullorum* ELISA. Immulon 2HB 96-well plates (Thermo Electron) were coated with 100 µl per well of OMP preparation (1 µg ml⁻¹) in carbonate buffer (pH 9.6) overnight at 4 °C. Plates were blocked with 200 µl 2 % BSA in PBS for 1 h at 37 °C. Serum was diluted 1:100 with 1 % BSA/PBS and incubated in antigen-coated wells for 2 h at 37 °C. Biotinylated goat anti-mouse IgG (Sigma) diluted 1:2000 was applied for 1 h at 37 °C, followed by incubation with ExtrAvidin-peroxidase (Sigma) for 30 min at 37 °C and ABTS substrate (Kirkegaard and Perry Laboratories) for 30 min at room temperature to allow colour development. The optical density (OD) at 405/590 nm was recorded using an ELISA plate reader (PowerWave X Microplate Spectrophotometer; Bio-Tek Instruments).

Statistical analysis. Enterohepatic lesion scores were compared by a Mann-Whitney non-parametric test. Serum IgG levels and qPCR colonization data were compared using Student's *t*-test. Seroconversion to *H. pullorum* was defined as values exceeding the mean plus 3 SD of the IgG levels obtained from *Helicobacter*-free C57BL/6 mice. Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software), with values of *P* < 0.05 considered significant.

RESULTS

Confirmation of *H. pullorum* identity by sequencing of PCR products

DNA was extracted from one faecal sample (46-week-old mouse) and caecal culture (32-week-old mouse), both

from C57BL/6NTac mice, and a 1200 bp fragment was amplified using *Helicobacter* genus-specific primers. Both products were sequenced and showed 100 % sequence similarity to the *H. pullorum* 16S rRNA gene.

Detection of *H. pullorum* from 10–10⁶ genome equivalents by a *cdtB*-based qPCR assay

The range for optimal detection of *H. pullorum* was 10–10⁶ genome equivalents with a high linear regression coefficient (*r*²=0.997) (see Supplementary Fig. S1, available in JMM Online). The BLASTN search demonstrated that primer sequences had no homology with any nucleotide sequences currently available in the databases. For testing specificity, DNA templates (equivalent to 10⁶ genome copies) from genetically related *cdtB*-positive human strains *H. bilis* ATCC 43879, *H. cinaedi* CCUG 18818, *H. winghamensis* ATCC BAA-430, *H. hepaticus* 3B1 and *Campylobacter jejuni* 81-176 and ATCC 43431 in parallel with a serial dilution of *H. pullorum* MIT 98-5489 DNA were measured in this assay. Non-*H. pullorum* DNA templates occasionally yielded low fluorescent signal (equivalent to less than ten copies); importantly, the melting temperature (*T*_m) of the detected products mismatched that of the amplicon from *H. pullorum* DNA.

Persistent infection of C57BL/6NTac mice with *H. pullorum* from 10 to 58 weeks of age

Ten-week-old mice shipped from the barrier where the *H. pullorum* outbreak occurred (C57BL/6NTac, *n*=25; C3H/HeNTac, *n*=3) were positive for *H. pullorum* when pooled faeces from each cage was cultured (Table 1). *H. pullorum* isolated from the faeces was confirmed by *H. pullorum*-specific PCR. At 26–27 weeks of age, the three C3H/HeNTac mice were negative for *H. pullorum* by faecal PCR and culture. Eleven 32-week-old C57BL/6NTac mice from another research facility necropsied on arrival were all positive for *H. pullorum* by PCR and/or culture of caecum and colon (Table 1); all caecal and colon samples were also positive by qPCR. Liver samples were culture negative for *H. pullorum*. Mice at 38 weeks of age were necropsied and their lower bowel assessed for *H. pullorum* colonization (Table 1). Of the ten C57BL/6NTac mice, eight of ten caecal and seven of ten colon samples were PCR positive. Only the caecum from one of three C3H/HeNTac mice, however, was *H. pullorum* PCR positive, and colon samples from all three animals were negative. Using qPCR analysis, caecal samples from all ten C57BL/6NTac mice and one C3H/HeNTac mouse were positive. At 58 weeks of age, five of 14 C57BL/6NTac mice necropsied (Tables 1 and 2) had *H. pullorum* cultured from faeces; faeces from an additional two mice were *H. pullorum* positive by end-point PCR, whereas 11 of 14 were positive for *H. pullorum* by qPCR. In the caecum, seven of 14 mice were positive by culture and eight of 14 mice were positive by PCR (Fig. 1), whereas six of 14 colon samples were positive and all livers were negative by *H. pullorum*-specific PCR (Table 1). The qPCR

Table 1. *H. pullorum* cumulative results from faecal and lower bowel tissues of female C57BL/6NTac and C3H/HeNTac mice based on *H. pullorum*-specific PCR, qPCR and culture, demonstrating persistence of infection from 10 to 58 weeks of age

B6, C57BL/6NTac mice; C3H, C3H/HeNTac mice; NA, not available.

Source	Age (weeks)	No. mice	Strain	Number of positive samples (%)										
				Faecal PCR	Faecal qPCR	Faecal culture	Caecum PCR	Caecum qPCR	Caecum culture	Colon PCR	Colon qPCR	Liver PCR	Liver culture	Total positive
Commercial barrier	10	25	B6			5 (100 %)*								5 (100 %)*
		3	C3H			2 (100 %)*								2 (100 %)*
	26	25	B6			5 (100 %)*								5 (100 %)*
		3	C3H			0 (0 %)*								0 (0 %)*
	27	3	C3H	0 (0 %)*										0 (0 %)*
	38†	10	B6	NA	NA	NA	8 (80 %)	10 (100 %)	8 (80 %)	7 (70 %)	7 (70 %)	NA	0 (0 %)	10 (100 %)
		3	C3H	NA	NA	NA	1 (33 %)	1 (33 %)	0 (0 %)	0 (0 %)	0 (0 %)	NA	0 (0 %)	1 (33 %)
	46	15	B6	9 (60 %)	14 (93 %)									14 (93 %)
	52	14‡	B6	8 (57 %)	12 (86 %)									12 (86 %)
58†	14	B6	7 (50 %)	11 (79 %)	5 (36 %)	8 (57 %)	8 (57 %)	7 (50 %)	6 (43 %)	8 (57 %)	0 (0 %)	0 (0 %)	11 (79 %)	
Research facility	32†	11	B6	NA	NA	NA	11 (100 %)	11 (100 %)	11 (100 %)	11 (100 %)	11 (100 %)	NA	0 (0 %)	11 (100 %)

*Pooled sampling by cage.

†Necropsy time point.

‡One animal euthanized for unrelated health concerns.

Table 2. Correlation of faecal and tissue PCR and qPCR testing with seroconversion status for *H. pullorum* from female C57BL/6 mice aged 46–58 weeks reported at the individual cage and mouse level

NA, Not applicable.

Cage	ID	46 weeks			52 weeks			58 weeks						
		Faecal PCR	Faecal qPCR	Sera	Faecal PCR	Faecal qPCR	Sera	Faecal PCR	Faecal qPCR	Caecum PCR	Caecum qPCR	Colon PCR	Colon qPCR	Sera
1	1	–	+	+	–	–	+	–	+	–	–	–	–	+
	2	–	–	+	–	+	+	–	–	–	–	–	–	–
	3	–	+	+	–	+	+	–	–	–	–	–	–	+
	4	–	+	+	–	–	+	–	–	–	–	–	–	+
	5*	–	+	+	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	1	+	+	+	+	+	+	+	+	–	–	–	–	+
	2	+	+	+	–	+	+	+	+	+	+	–	+	+
	3	+	+	+	–	+	+	–	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	+	+	–	+	+
3	1	–	+	+	+	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	–	+	+	+	+	+	+
	4	+	+	+	+	+	+	–	+	–	–	–	–	+
	5	+	+	+	+	+	+	+	+	+	+	+	+	+
Total		9/15	14/15	15/15	8/14	12/14	14/14	7/14	11/14	8/14	8/14	6/14	8/14	13/14

*Euthanized at 50 weeks of age.

Fig. 1. PCR amplification of a 148 bp product using *H. pullorum*-specific *cdtB* primers. Lanes: M, 1 kb Plus DNA ladder (Invitrogen); 1–3 and 10, *H. pullorum*-negative caecal cultures; 4–9 and 11, bacterial DNA of *H. pullorum* caecal isolates; 12, positive control; 13, negative control.

data matched the results for end-point PCR with eight of 14 caeca and none of the 14 livers being *H. pullorum* positive; however, two additional colon samples (eight of 14) were positive by qPCR. In the C57BL/6NTac mice, *H. pullorum* colonization levels decreased significantly in the caeca of mice as they aged from 32 to 58 weeks of age (Fig. 2). At 32 and 38 weeks of age, the caeca had significantly higher levels of *H. pullorum* than the colons; however, colonization levels in the caeca and colons of the 11/14 colonized mice did not differ significantly at 58 weeks of age.

Intermittent shedding of *H. pullorum* in the faeces of some C57BL/6NTac mice

Individual faeces samples were obtained from 15 C57BL/6NTac mice to evaluate *H. pullorum* shedding between 46 and 58 weeks of age. Individual mice did not consistently shed *H. pullorum* in their faeces (Table 2), as detected by conventional PCR methods, whereas four co-housed mice were consistently negative for *H. pullorum* from 46 to

Fig. 2. Lower bowel colonization levels established by qPCR of *H. pullorum* in female C57BL/6NTac mice at 32 ($n=11$), 38 ($n=10$) and 58 ($n=8$) weeks of age. Values represent the number of copies of *H. pullorum* per μg mouse DNA using *H. pullorum*-specific *cdtB*-based primers. * $P<0.05$.

Fig. 3. Mean serum IgG response to *H. pullorum* from 27 to 58 weeks of age in female C57BL/6NTac mice. The dashed line represents the seroconversion cut-off point (0.33), defined as the mean plus 3 SD of the IgG levels from *Helicobacter*-free C57BL/6 mice ($n=8$). Numbers of mice for the *H. pullorum*-infected group were $n=25$ at 27 and 38 weeks and $n=14$ at 46–58 weeks. * $P<0.05$.

58 weeks of age by faecal PCR. Co-housed mice in two other cages were intermittently faeces-positive for *H. pullorum*. Analysis of these faeces samples using qPCR revealed *H. pullorum* positivity in 14 of 15 mice at 46 weeks, 12 of 14 mice at 52 weeks and 11 of 14 mice at 58 weeks. The intermittent *H. pullorum* faecal shedding, detected by qPCR, occurred in mice housed in cage 1 and reflected the low quantity of faecal *H. pullorum* in these mice. All mice in cages 2 and 3, when analysed by qPCR, remained infected over the 46–58-week period.

Most infected mice are seropositive for *H. pullorum* with minimal IgG cross-reactivity with *H. hepaticus* and *H. bilis*

The majority of mice maintained an IgG response throughout infection, with 93–100% seropositivity observed from 27 to 58 weeks of age (Table 2). Two mice that did not seroconvert (38 and 58 weeks of age) were negative for *H. pullorum* by PCR/qPCR in all faecal and tissue samples tested. Conversely, some seropositive mice were faecal or tissue negative for *H. pullorum* by qPCR at 46–58 weeks (Table 2), suggesting clearance of infection. IgG titres decreased from 27 to 58 weeks of age, particularly between 27 and 38 weeks of age ($P=0.0045$, Fig. 3). Over the 31 weeks that serum IgG antibody levels were monitored, a 42.7% decrease in titres was observed, despite positive seroconversion status being retained and the majority of mice remaining positive by faecal or tissue analysis (Table 1). Only one and two of 28 *H. pullorum*-positive serum samples met the criteria for seroconversion to *H. hepaticus* and *H. bilis* antigens, respectively (data not shown).

No observation of significant gross or histological lesions attributable to *H. pullorum*

At all necropsy time points, with the exception of spontaneous lymphomas, the liver, ileum, caecum and colon appeared grossly normal, and no *Helicobacter*-associated lesions were seen histologically. Lesion scores (for inflammation, oedema, epithelial defects, crypt atrophy, hyperplasia and dysplasia) in the caecum, colon and ileum ranged from 0 to 1 on an ascending scale of 0–4 (data not shown). Hepatitis indices ranged from 0.5 to 6.5 and did not differ significantly from control mice (data not shown). The only mild abnormality was neutrophilic infiltration of the liver in two 32-week-old *H. pullorum*-infected mice. One *H. pullorum*-infected mouse at 50 weeks of age was euthanized due to loss of body condition, and necropsy confirmed multicentric lymphoma. A significant number of control C57BL/6NTac mice (up to 58 weeks old) also developed lymphoma with involvement of the liver.

DISCUSSION

Persistent *H. pullorum* infection was documented in C57BL/6NTac mice from 10 to 58 weeks of age. Only one of three C3H/HeNTac mice remained persistently infected at 26 weeks. The extent of resistance to *H. pullorum* infection in C3H/HeNTac mice could not be fully appreciated, given the small number of mice from this inbred strain available for the study. These preliminary results, however, suggest that susceptibility to *H. pullorum* infection varies among different mouse strains. Although pooled faeces collected from individual cages at the initiation of the study were positive for *H. pullorum*, faecal shedding of *H. pullorum* was intermittent based on faecal qPCR of individual C57BL/6NTac mice sampled from 46 to 58 weeks of age. Intermittent faecal shedding and/or clearing of *H. hepaticus* from the lower bowel have also been reported for BALB/c interleukin-10 (IL-10)-deficient mice infected with *H. hepaticus* (Nagamine *et al.*, 2008). Faeces from one cage of mice remained negative from 46 to 58 weeks, suggesting that mice in this cage spontaneously cleared the *H. pullorum* infection.

A 16S rRNA gene-based qPCR assay for *H. pullorum* was reported by González *et al.* (2008). However, the primers used in this previously published assay were identical to the corresponding sequences of the *Helicobacter canadensis* 16S rRNA gene, an emerging human pathogen closely related to *H. pullorum* (Fox *et al.*, 2000). In addition, there is only a single nucleotide difference in the amplicons produced by these primers from the 16S rRNA gene between *H. pullorum* and *Helicobacter mastomyrinus* (Shen *et al.*, 2005). In this study, we developed a qPCR assay with high specificity in detecting *H. pullorum* in environmental and clinical samples. The *cdtB* gene was used as a qPCR target for the following two reasons. First, *cdtB* encodes a protein that functions as an active subunit of bacterial cytolethal distending toxin (Cdt), a virulence factor produced by

several pathogenic bacteria (Smith & Bayles, 2006). Secondly, this gene is absent from *H. canadensis*. Using the BLASTN search, the qPCR primers we selected had no sequence homology with any sequences currently available in the database. Our qPCR assay did not amplify a valid fluorescent signal from chromosomal DNA (equivalent to 10⁶ copies) from genetically related *cdtB*-positive bacterial species including *H. bilis* ATCC 43879, *H. cinaedi* CCUG 18818, *H. winghamensis* ATCC BAA-430, *H. hepaticus* 3B1 and *Campylobacter jejuni* 81-176 and ATCC 43431, despite the recognition that the *H. pullorum* CdtB displays significant amino acid sequence similarity (52–74%) to CdtBs identified in these organisms.

The *H. pullorum* ELISA developed in this study was considered highly sensitive, as all faecal qPCR *H. pullorum*-positive mice seroconverted. Both mice that were seronegative were also faecal- and tissue-qPCR-negative. Specificity was also high, as there was minimal cross-reactivity with the selected common mouse helicobacters *H. hepaticus* and *H. bilis*. This high degree of sensitivity and specificity is consistent with ELISA data reported for *H. hepaticus*, *Helicobacter rodentium* and *H. bilis* using OMP preparations (Whary *et al.*, 2000). The persistence of antibody titres to *H. pullorum* in the mice that were negative by faecal or tissue qPCR indicated that they were initially infected, but cleared *H. pullorum* infection over time. It is known that antibody titres to *H. pylori* can persist in humans for months, as demonstrated in patients who have successfully eradicated *H. pylori* from their stomachs when treated with antibiotics (Berger *et al.*, 2003).

H. pullorum prevalence studies in both chickens and humans suggest that *H. pullorum* infection may be subclinical in nature and its diagnosis under-represented (Ceelen *et al.*, 2005, 2006b; Manfreda *et al.*, 2011; Nebbia *et al.*, 2007; Zanoni *et al.*, 2007). *H. pullorum* isolation and identification can be challenging due to a lack of easily identifiable phenotypic, biochemical and antibiotic susceptibility markers (e.g. sheathed flagella and resistance to polymyxin B) common to other *Helicobacter* species, shared markers with *Campylobacter* species and fastidious growth requirements (Atabay *et al.*, 1998; Melito *et al.*, 2000; Wainø *et al.*, 2003). Now that an *H. pullorum* qPCR assay is available, rapid and sensitive testing can be employed to better assess *H. pullorum* prevalence in humans, avian species, rodents and other possible reservoirs, including wild rodents.

H. pullorum was consistently detected by PCR or culture in the caecum and colon but was not detected in the liver of any mice sampled. No significant gross or histological lesions, other than hepatic lymphomas (found commonly in aged C57BL/6 mice) in infected and control mice, were noted in the liver, ileum, caecum or colon in the two inbred strains of *H. pullorum*-infected mice examined at 32, 38 and 58 weeks of age. These results are consistent with earlier studies in C57BL/6NCr mice, demonstrating that persistent infection with *H. hepaticus* does not elicit

inflammation in the gastrointestinal tract or liver (Ward *et al.*, 1994; Whary *et al.*, 2001). An exception to these findings was a substrain of C57BL/6 mice infected with *H. hepaticus* that developed hepatitis and liver tumours when examined at 20 months of age (García *et al.*, 2008). In earlier studies, 1-day-old broilers were inoculated with various strains of *H. pullorum* isolated from humans or poultry (Ceelen *et al.*, 2007). The birds remained asymptomatic despite swollen caeca with foamy contents and mild to moderate inflammation; minimal to no gross or histological lesions were seen in the liver, colon and jejunum. All faecal samples and the majority of caecum and colon samples in all birds, irrespective of inoculum strain, were positive for *H. pullorum* by PCR and/or culture at 42 days post-inoculation. *H. pullorum* PCR detection in the jejunum and liver was less common, and *H. pullorum* could not be cultured from the liver. Persistent colonization and faecal shedding until the chickens reached slaughter age led the authors to conclude that chickens represent a risk to humans for *H. pullorum*-associated food-borne infections (Ceelen *et al.*, 2007). It will be important to explore whether *H. pullorum* strains isolated from chickens can colonize mice and to determine whether the strains are pathogenic.

In summary, *H. pullorum* probably represents a food-borne pathogen with a risk of acquired zoonotic infection, similar to *Campylobacter jejuni*, occurring via consumption of undercooked poultry (Atabay *et al.*, 1998; De Groote *et al.*, 2000; Skovgaard, 2007). The reported association between *H. pullorum* infection and gastroenteritis and hepatitis in humans and chickens, and the significance of *H. pullorum* in clinical disease, require further studies to validate causation. Our results indicate that C57BL/6NTac mice can be persistently infected with *H. pullorum*, and provide an opportunity to use a variety of mouse models to study *H. pullorum* pathogenesis. For example, our preliminary results indicate that *H. pullorum* infection in B6.129P2-IL-10^{tm1Cgn} mice will be useful in studying the immunobiology of inflammatory bowel disease (unpublished data).

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