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List of abbreviations

AFLP	amplified fragment length polymorphism
ATCC	American Type Culture Collection
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	ATM and Rad3 related
BHI	brain heart infusion
BLAST	basic local alignment search tool
bp	base pairs
cagA	cytotoxic-associated gene A
CCUG	Culture Collection of the University of Göteborg
CDT	cytolethal distending toxin
CFU	colony forming units
CHO	Chinese hamster ovary
CLO	Centrum voor Landbouwkundig Onderzoek; huidig Instituut voor Landbouw en Visserijonderzoek (ILVO)
CPE	cytopathic effect
DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
DSBs	double strand breaks
ECACC	European Collection of Cell Cultures
EHS	enterohepatic *Helicobacter* species
EMEM	Eagle's minimum essential medium
GCT	granulating cytotoxin
HMP	heat-modifiable protein
IBD	inflammatory bowel disease
ICLS	International Council for Laboratory Standards

IR	irradiation
kDa	kilo Dalton
MIC	Minimum Inhibitory Concentration
MWCO	molecular weight cut off
NCCLS	National Committee for Clinical Laboratory Standards
NCTC	National Collection of Type Cultures
OMP	outer membrane preparation
ORF	open reading frame
PAI	pathogenicity island
PBP	penicillin-binding protein
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal RNA
SCID	severe combined immunodeficiency
SPF	specific pathogen free
spp.	species
TAE	tris-acetate-EDTA
Taq	*Thermophilus aquaticus*
TEM	transmission electron microscopy
U	unit

Scientific Background

The genus *Helicobacter* (*H.*) is a fast growing group of Gram-negative microbial organisms that are able to persistently colonize a diversity of mammalian host species and in some cases may cause major clinical disease. The clinical significance of the gastric *Helicobacter* species *H. pylori*, occurring in half of the world's human population, nowadays is becoming clear. The prevalence and clinical relevance of the enterohepatic *Helicobacter* species (EHS), however, remain to be established.

H. pullorum is an EHS which originally was isolated from the caeca of clinically healthy broiler chickens and from the liver and intestines of laying hens suffering from vibrionic hepatitis.

In human beings, *H. pullorum* is occasionally detected in faeces obtained from patients with gastroenteritis and is increasingly reported in the gallbladder from persons suffering from chronic cholecystitis and biliary cancer.

Despite the emerging character of this pathogen, hitherto, only a handful of studies have been performed involving *H. pullorum*, leaving large gaps in the knowledge about its actual prevalence and possible hazardous effects on human and animal health.

Enterohepatic *Helicobacter* species: a review of the literature

The genus *Helicobacter* nowadays includes at least 26 formally named species, with additional novel species in the process of being characterized (Fox, 1997; Whary and Fox, 2004). The genus can roughly be divided into gastric and enterohepatic *Helicobacter* species (EHS).

All gastric *Helicobacter* species have strong urease activity. They manage to survive gastric acidity by expressing urease at a level higher than that of any other known microorganism (Clyne et al., 1995; Dunn et al., 1997; Sachs et al., 2003).

EHS do not normally colonize the gastric mucosa, but do have characteristics of ultrastructure and physiology in common with the gastric *Helicobacter* species. To date, these bacterial agents have been identified in the intestinal tract and/or the liver of humans, mammals, and birds (Fox, 1997; Solnick and Schauer, 2001; Inglis et al., 2006). EHS infections are associated mostly with intestinal and hepatobiliary disease in a wide range of animals. These bacteria also may interfere with results obtained from experimental research in laboratory animals in which they are highly prevalent, and thus may lead to misinterpretation of data (Fox et al., 1994; Ward et al., 1994; Rogers and Fox, 2004; Bohr et al., 2006). Finally, members of the enterohepatic *Helicobacter* group may have zoonotic potential causing gastroenteritis, hepatitis and other disease signs in humans (Solnick and Schauer, 2001; Ljung and Wadstrom, 2002). For all these reasons, the potential importance of these emerging pathogens cannot be overlooked and undoubtedly merits further investigation.

The discovery of EHS has sparked an interest in exploring the pathogenic potential of these organisms mainly in laboratory rodents. Consequently, hitherto, most data in literature on EHS are dealing with these animal species. Hence, the following review of the literature mainly includes data on EHS associated with laboratory rats and mice.

1. Taxonomy

H. pylori, the type species of the *Helicobacter* genus, was initially described as a *Campylobacter* (*C.*) species (Marshall and Warren, 1984). Nonetheless, the 16S rRNA gene sequence was noticeably different from the latter genus, so the agent got its own genus in 1989 (Dewhirst et al., 2000; On et al., 2002; Whary and Fox, 2004). Analysis of the 16S rRNA gene sequence of more than 225 *Helicobacter* isolates from birds and mammals demonstrated the genus to be phylogenetically diverse, containing over 30 taxa of species status (Dewhirst et al., 1999).

Currently, the *Helicobacter* genus is assigned to the rRNA superfamily VI which additionally includes the genera *Campylobacter* and *Arcobacter* and a number of other taxa. For simplicity, all of these agents may be referred to as Campylobacteria, a term that reveals their morphological resemblances on the whole (On et al, 1996). The most common EHS are presented in Table 1.

Table 1 The most common enterohepatic *Helicobacter* species and their hosts

Enterohepatic *Helicobacter* species			
Species	**Host**	**Species**	**Host**
H. bilis	mouse, human	*H. pullorum*	chicken, human
H. canis	dog, cat, human	*H. canadensis*	human, bird, pig
H. cinaedi	hamster, human	*H. rodentium*	mouse
H. cholecystus	hamster	*H. trogontum*	rat
H. fennelliae	human	*H. typhlonius*	mouse
H. hepaticus	mouse, gerbil, human	*H. mesocricetorum*	hamster
H. muridarum	mouse, rat	*H. aurati*	hamster
H. pametensis	bird, swine	*Helicobacter* sp. flexispira taxon 5	sheep, dog, human, mouse
H. ganmani	mouse		

2. Morphology and *in vitro* culture

Members of the EHS group are Gram-negative, non-spore forming, spiral, curved or fusiform rods of 0.3-0.6 μm width and 1-5 μm length. Early transmission electron microscopic studies described two types of EHS. A first group resembles C*ampylobacter* spp., though they are longer and have one or more polar flagella at each end. In most species, these flagella are sheathed. A second group, including the Lockhard 1 type organism, possesses periplasmic fibrils and bipolar tufts of sheathed flagella. This group of organisms constitutes one of the three assemblies of spiral bacteria detected in thin sections of gastric mucosa from dogs and represents straight cylinders with a fibril tightly coiled around their bodies (Lockhard and Boler, 1970).

To date, only motile helicobacters are known. However, in old cultures or under certain circumstances, the organisms may loose their motility (Dewhirst et al., 2000). The spiral morphology and flagella of these bacteria could enhance their speed in viscous surroundings such as methylcellulose solution (Jung et al., 1997).

The bacteria may transform into coccoid or spherical cells, particularly in older cultures or upon exposure to air (Dewhirst et al., 2000). The actual phenomenon of transformation from a bacillary form with a spiral or helical shape into a coccoid form has been investigated in *H. pylori* but not in other *Helicobacter* spp. Several factors may influence the spiral to coccoid conversion of *H. pylori*, such as acid pH, stress, oxygen, temperature, nutritional starvation. In comparison to spiral forms, coccoid shapes of *H. pylori* have been demonstrated to possess damaged genomic DNA, less total amounts of DNA and RNA, a loss of membrane potential and considerably diminished levels of intracellular ATP, indicative of a metabolic state of cellular degeneration (Taneera et al., 2002).

In vitro culture of EHS is difficult and may be hampered by the fastidious growth requirements of these species. Their fastidious nature requires nutrient-rich complex media and long incubation times. Therefore the frequency of occurrence of infections with these microorganisms probably is underestimated (Shames et al., 1995; Taneera et al., 2002). Additionally, the phenotypic similarity between member species of the genera *Helicobacter* and *Campylobacter* may result in misidentification (Nilsson et al., 2000a).

Agar-grown EHS usually present as swarming or single pointed colonies (Euzéby, 2000; 2002).

Columbia, Trypticase Soy and Brucella agar supplemented with 5 % sheep or horse blood and occasionally TVP (trimethoprim, vancomycin, polymyxin) are mainly used as cultivation agar media. EHS associated with laboratory rodents and other mammals grow best in a microaerobic environment at 37°C. Several species also may grow at 42°C, but not at 25°C. *H. ganmani* is unusual in that this species grows anaerobically at 37°C, but cannot be cultivated under microaerobic conditions. Isolation can take place using nylon or cellulose acetate filters with a pore size of 0.45 µm or 0.65 µm which may reduce contamination by other bacteria (Figure 1) (Steele and McDermott, 1984; Fox et al., 1994; 1995; 1996a,b; Franklin et al., 1996; Mendes et al., 1996; Livingston et al., 1997; Atabay et al., 1998; Foltz et al., 1998; Whary et al., 1998; Chien et al., 2000; Euzéby, 2000; Franklin et al., 2001; Robertson et al., 2001; Euzéby, 2002; Garcia et al., 2002). Brucella broth supplemented with 5 % fetal calf serum also can be adopted for isolation.

Corry and Atabay (1997) mentioned the growth of two *H. pullorum* strains both on the selective CAT (cefoperazone amphotericin teicoplanin) and to a lesser extent mCCDA (modified charcoal cefoperazone deoxycholate) medium. No growth was however seen on ISTBA (iso-sensitest) agar medium containing 5 % lysed horse blood either with or without cefoperazone for inexplicable reasons (Corry and Atabay, 1997). Taneera et al. (2002) suggested that activated charcoal enhances growth of several EHS including *H. pullorum*, probably due to the capacity of this supplement to remove toxic compounds in culture media. In contrast to the former conclusions, Atabay et al. (1998) reported that *H. pullorum* was not able to grow on CAT medium neither on mCCDA medium in their experiment.

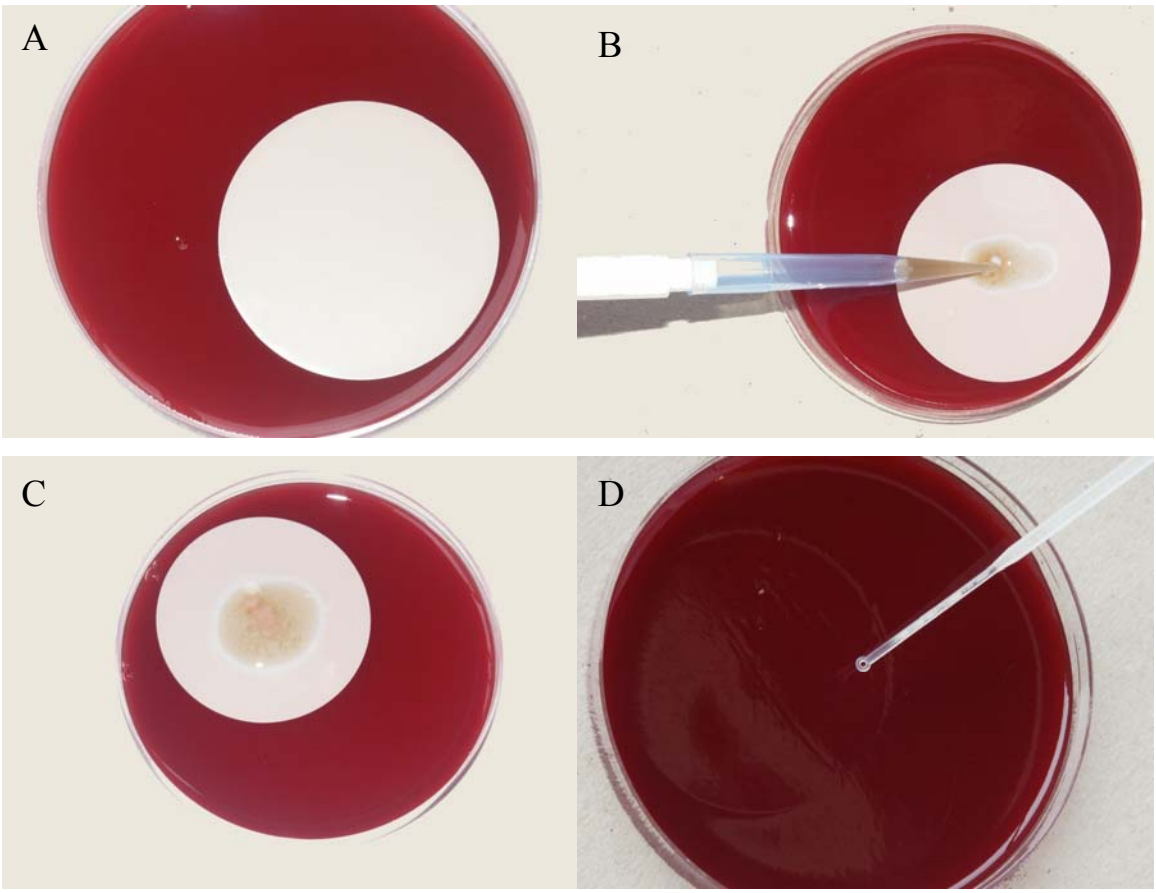

Figure 1 Isolation of EHS using a cellulose acetate filter with a pore size of 0.45 µm.
A. A sterile cellulose acetate membrane filter is applied onto the surface of a blood plate. B. Approximately 250 µl of homogenized tissue is put in the middle of the filter. C. The agar blood plate is incubated at 37°C during half an hour. D. The filter is removed and the filtrate is streaked on the blood plate with a loop

3. Clinical signs and lesions associated with EHS infections in animals

For most of the EHS, the actual clinical significance is not very well known. Much debate on whether EHS are components of the normal microbiota or these bacteria actually have the ability to cause intestinal and hepatobiliary illness has been pursued (Fox et al., 1997; Zenner et al., 1999; Solnick and Schauer, 2001). So far, only *H. hepaticus*, first isolated from laboratory mice, has been clearly recognized as a pathogen and has been associated with a variety of hepatic lesions depending on the mouse strain (Solnick and Schauer, 2001; Ljungh

and Wadstrom, 2002). It can cause chronic active hepatitis and typhlocolitis in immunocompetent mice (Ward et al., 1994; Fox et al., 1996a,b; Whary et al., 1998; Rogers et al., 2004) and can also lead to liver carcinoma in mice of susceptible strains (Ward et al., 1994; Fox et al., 1994; 1996a; Hailey et al., 1998; Whary et al., 1998). In addition, natural and experimental infection with *H. hepaticus* in certain immunodeficient mice can induce inflammatory bowel disease (IBD) (Ward et al., 1996a; Cahill et al., 1997; Kullberg et al., 1998; Chin et al., 2000).

H. bilis has been associated with multifocal hepatitis in mice without clinical signs (Fox et al., 1995). *H. bilis* infection however, also has been linked to typhlocolitis and diarrhoea in immunodeficient rodents (Franklin et al., 1998; Haines et al., 1998). SCID mice naturally infected with *H. bilis* and/or *H. rodentium* may reveal acute diarrhoea (Shomer et al., 1998). *H. cholecystus* is considered as a cause of cholangiofibrosis and centrilobular pancreatitis in Syrian hamsters (Franklin et al., 1996).

Recently, Maurer et al. (2005) suggested that *H. hepaticus, H. bilis, H. rodentium* and *H. cinaedi* play an important role in the pathophysiology of cholesterol gallstone development in mice and possibly in humans. A certain percentage of C57L/J mice infected with one or two of these EHS and fed a lithogenic diet, developed cholesterol gallstones.

Apart from rodents, EHS also have been associated with intestinal and hepatobiliary disease in other mammals and birds (Fox et al., 1997; Zenner et al., 1999; Solnick and Schauer, 2001), such as *H. canis* and *H. pullorum*. *H. canis* has been isolated from a puppy suffering from multifocal necrotizing hepatitis (Fox et al., 1996c) and a cat with episodic diarrhoea (Foley et al., 1999). *H. pullorum* has been linked with vibrionic hepatitis in laying hens by some authors (Stanley et al., 1994; Burnens et al., 1996). This association, however, merely is based on the fact that the bacterial species originally was isolated from laying hens suffering from vibrionic hepatitis (Burnens et al., 1996). Vibrionic hepatitis is primarily characterized by swelling and necrosis of the liver and may cause economic loss by rising poultry flock cull rates (Berry and Whitenack, 1991). The significance of *H. pullorum* in hepatobiliary and intestinal disease in poultry still has to be determined.

4. Zoonotic potential of EHS

During the last two decades, *Helicobacter* colonization of the gastrointestinal tract of humans has been the subject of intensive research (Solnick and Schauer, 2001; Fox, 2002; Ljungh and Wadstrom, 2002).

The recovery of different helicobacters from both immunocompromised and immunocompetent human patients suffering from enteric and hepatobiliary disease has raised the question about the origin and impact of these infections. Hitherto, the causal role of EHS in human hepatoenteric disease often is mostly presumptive (Fox, 1997; Fox et al., 1998; Avenaud et al., 2000; Nilsson et al., 1999; 2000b; 2001; Solnick and Schauer, 2001; Fox, 2002; Rocha et al., 2005).

The rodent EHS *H. hepaticus*, *H. bilis* and *H. cinaedi* are generally considered as zoonotic. *H. hepaticus* may play a role in liver carcinogenesis, IBD, irritable bowel syndrome (IBS) and chronic pancreatitis in humans (Nilsson et al., 2000b; Ge et al., 2001a; Apostolov et al., 2005; Nilsson et al., 2006; Zhang et al., 2006).

H. bilis is the only murine EHS that has actually been isolated from human gallbladder (Andersen, 2001) and has been associated with the development of chronic cholecystitis, biliary duct and gallbladder cancer (Fox et al., 1998; Solnick and Schauer, 2001; Matsukura et al., 2002; Murata et al., 2004; Kobayashi et al., 2005).

H. cinaedi was first isolated from homosexual men, both asymptomatic individuals and men suffering from proctitis, proctocolitis and enteritis. The name 'cinaedi' means homosexual in Latin (Fennell et al., 1984). Next, many reports regarding individuals infected with *H. cinaedi* have been documented. The agent is mainly found in immunocompromised persons often causing a non-lethal disease with a large possibility for recurrence (Uckay et al., 2006). *H. cinaedi* infection has been associated with septicaemia and meningitis in a neonate (Orlicek et al., 1993), bacteraemia in an afebrile patient with X-linked agammaglobulinemia (Simons et al., 2004), acute diarrhoea (Tee et al., 1987), bacteraemia in immunosuppressed persons due to AIDS or cancer (Cimolai et al., 1987; Ng et al., 1987; Sacks et al., 1991; Mammen et al., 1995; Sullivan et al., 1997; Uckay et al., 2006) and multifocal cellulitis and monoarticular arthritis (Burman et al., 1995; Sullivan et al., 1997). Van der Ven et al. (1996) reported a case of a HIV-seropositive man who was suffering from a *H. cinaedi* bacteraemia with

involvement of the soft tissue in the right lower leg causing a localized pain in this area. It was illustrated that endovascular infection was present and may thus be a feature of *H. cinaedi* bacteraemia. Despite the association of this species with extragastric infections, Peňa et al. (2002) detected *H. cinaedi* DNA in antral gastric biopsies obtained from patients. One patient was diagnosed with erosive gastritis. Another patient had a history of colitis. Very recently, *H. cinaedi* DNA was detected in patients with pancreatic exocrine cancer (Nilsson et al., 2006).

Other EHS present in mammals which may be transmitted to human beings are *H. ganmani* and *H. canis. H. ganmani* has been reported in pediatric patients with liver disorders (Tolia et al., 2004), while *H. canis* has been detected in a boy with gastroenteritis and an immunocompetent patient suffering from bacteremia and multifocal cellulitis (Burnens et al., 1993; Solnick and Schauer, 2001; Leemann et al., 2006).

H. pullorum, an EHS occurring in poultry is also believed to be a zoonotic pathogen (Stanley et al., 1994; Steinbrueckner et al., 1997; Atabay et al., 1998; Fox et al., 1998; Ljungh and Wadstrom, 2002; On et al., 2002). Various human cases of gastroenteritis revealed as diarrhoea and hepatobiliary disease as reflected by liver swelling, an increase of liver enzymes and gallbladder cancer associated with *H. pullorum* infection have been reported (Burnens et al., 1994; Stanley et al., 1994; Fox, 1997; Steinbrueckner et al., 1997; Fox et al., 1998; Ponzetto et al., 2000; On et al., 2002). It has also been suggested that *H. pullorum* plays a role in Crohn's disease (Andersson et al., 2002; Bohr et al., 2002). Finally, a case-report about an *H. pullorum*-like organism associated septicaemia has been published (Tee et al., 2001).

H. canadensis, which was originally classified as *H. pullorum,* was cultured for the first time from Canadian patients with diarrhoea, but has afterwards been shown to colonize also wild geese (Fox et al., 2000; Waldenstrom et al., 2003). Very recently, atypical *H. canadensis* strains have been detected in swine (Inglis et al., 2006). *H. canadensis* has geese as reservoir and is acquired by humans as a zoonosis (Waldenstrom et al., 2003).

Altogether, data from studies on biliary and hepatic diseases, as well as pancreatic disorders, suggest that bile-tolerant *Helicobacter* species may induce a chronic infection with possible malignant transformation. Whether they truly participate in the genesis of biliary disease requires, however, additional investigation. At least, there is evidence that both gastric

and intestinal *Helicobacter*s occur in human bile (Queiroz et al., 2003; Kobayashi et al., 2005).

5. Bacterium-host interaction

5.1. Overview

The pathogenesis of most EHS infections is poorly understood both in terms of production of lesions in intestines and liver and in terms of the relationship of the organism to the host tissues at a molecular level. To date, mainly interactions of *H. hepaticus* and *H. bilis* with their hosts have been examined.

Spread of EHS infection by faecal-oral contact between animals is hypothesized (Fox et al., 1996b; Whary et al., 2000). Livingston et al. (1997) demonstrated that *H. hepaticus*-free animals can develop antibodies against this EHS within four weeks after contact with dirty cage bedding from *H. hepaticus*-infected mice. Vertical transmission of *H. hepaticus* has been suggested by Li et al. (1998), but may depend on the mouse strain involved. Nonetheless, it has been recommended to foster pups within 24 h of birth to remain free of *H. hepaticus* (Singletary et al., 2003). Results of a study performed by Scavizzi and Raspa (2006) on the contrary, showed that *H. typhlonius* was present in sex organs of mice without vertical transmission.

The genome of *H. hepaticus* is the only one which has been sequenced completely of all EHS (Suerbaum et al., 2003). Proteins of *H. hepaticus* have orthologs from both *H. pylori* and *C. jejuni*, but *H. hepaticus* is deficient in orthologs from most known *H. pylori* virulence factors, including adhesins, VacA cytotoxin, and nearly all *cag* pathogenicity island (PAI) proteins. However, *H. hepaticus* has orthologs of the *C. jejuni* adhesin PEB1 and the cytolethal distending toxin (CDT), a 71-kb genomic island (HHGI1). *H. hepaticus* also has several genomic islands with a different G+C content than the other sequences of the genome. Interestingly, HHGI1, possessing three basic elements of a type IV secretion system and other virulence protein homologs, constitutes a putative PAI. Within *H. hepaticus*, a large divergence of genome content, including the genomic island HHGI1, is present (Suerbaum et al., 2003). A recent study using male A/JCr mice, demonstrated the role of this PAI in the development of hepatitis in these animals (Boutin et al., 2005).

Almost one decade ago, auto-immunity was demonstrated to contribute to hepatocellular damage in *H. hepaticus* infection. *H. hepaticus*-infected mice indeed may build up antibodies against heat shock protein 70 expressed both by the bacterial agent and injured liver cells (Ward et al., 1996b; Whary et al., 1998).

Ge et al. (2001b) found outer membrane preparation (OMP) proteins in four *H. bilis* strains derived from different host species which were similar to each other but revealed a protein profile different from *H. pylori*, suggesting *H. bilis* has a conserved, unique OMP profile. The divergence in the OMP structure of these two helicobacters was also illustrated by the absence of cross antigenicity between the *H. bilis* OMP and a number of *H. pylori* OMP proteins except for their flagellins. Another finding in this study was the presence of five heat-modifiable proteins (HMP) in the *H. bilis* OMP. Whether these proteins act as porins *in vivo* still needs to be elucidated (Ge et al., 2001b).

H. hepaticus and to a lesser extent *H. bilis*, *H. mastomyrinus, H. aurati, H. trogontum, H. muridarum* and *H. typhlonius* all express urease activity. *H. hepaticus* has urease structural genes which are homologous to those of the gastric *Helicobacter* species (Shen et al., 1998; Beckwith et al., 2001). It contains approximately half of the urease activity of *H. pylori* (Sachs et al., 2003). It is not clear why this urease activity would be essential in the lower bowel and liver, both non-acidic environments. Potential roles for this enzyme in EHS embrace better endurance during passage through the stomach and generating ammonia as a source of nitrogen for protein biosynthesis. Urease activity may also be a factor in pathology, given that ammonia harms host cells and urease itself provokes phagocyte chemotaxis, stimulates immune cells, and induces cytokine production (Beckwith et al., 2001).

Additionally, in *H. hepaticus*, a toxin activity has been identified that causes vacuole formation in a murine liver cell line resulting in a granular appearance of the affected cells. The toxin was called granulating cytotoxin (GCT) referring to the induced morphological cell changes (Taylor et al., 1995). Despite the innovative and interesting character of this finding, no further research involving this toxin was performed for almost a decade. Only recently, Young et al. (2004) alleged that cytopathic effect induced by GCT could be CDT-mediated.

In 2000, Young et al. (2000a) identified three genes encoding CDT and CDT activity in *H. hepaticus*. Genetic and phenotypic evidence of CDT also has been found in *H. bilis, H. pullorum, H. cinaedi and H. canis* (Chien et al., 2000; Young et al., 2000a,b; Kostia et al.,

2003; Taylor et al., 2003). Since CDT is believed to be a virulence factor within EHS, it will be discussed more in detail.

5.2. CDT, a possible virulence factor of EHS (adapted from Microbiological Research 2006, 161, p 109-120).

Background

CDT was first documented as a toxin occurring in *Escherichia* (*E.*) *coli* strains by Johnson and Lior in 1987. They discerned that inoculation of the supernatant of several *E. coli* strains onto cultured Chinese hamster ovary (CHO) cells resulted in progressive cell distention and eventually cell death (Johnson and Lior, 1987a). They further discovered that isolates of *C. jejuni* and *E. coli* Shigella spp. (the former *Shigella* species) were able to induce similar cell changes (Johnson and Lior, 1987b; Johnson and Lior, 1988a). The name of the toxin refers to the morphological cell changes caused by the toxin (Johnson and Lior, 1987a; Johnson and Lior 1988b). To date, various Gram-negative bacterial species including *H. pullorum*, have been shown to produce CDT (Johnson and Lior, 1987a,b; Johnson and Lior, 1988a,b; Pickett et al., 1996; Cope et al., 1997; Okuda et al., 1997; Sugai et al., 1998; Chien et al., 2000; Young et al., 2000a,b; Mooney et al., 2001, Taylor et al, 2003). In *Salmonella enterica* species, a CdtB homologous protein has been demonstrated (De Rycke and Oswald, 2001; Haghjoo and Galan, 2004).

Genetics of CDT

CDT is encoded by three adjacent or slightly overlapping open reading frames (ORFs) assigned *cdtA*, *cdtB* and *cdtC*, assumed to be arranged in an operon. The expression of all three genes is required for CDT activity (Pickett et al., 1994; Scott and Kaper, 1994; Cope et al., 1997). No information has been published on the actual regulation of expression. The expression of the encoded genes however, is variable (Okuda et al., 1995; Pickett et al., 1996; Chien et al., 2000; Bang et al., 2001). It is worthwhile noting that the nucleotide and amino acid sequences do not have any major homology to known genes or proteins and that all three genes have a consensus leader sequence (Pickett et al., 1994; Scott and Kaper, 1994; Cope et al., 1997). The *cdtB* gene appears to be the most conserved gene amongst all *cdt* genes in

terms of differences between bacterial species (Pickett et al., 1996; Mayer et al., 1999; Young et al., 2000a,b; Frisan et al., 2002). Nevertheless, the degree of similarity of this gene and the amino acid sequence of the derived protein may vary between different species.

Structure and action of CDT

Cytolethal distending toxins may be classified in the second group of exotoxins designating true or apparent dimeric toxins. These molecules are composed of the B subunit(s) accounting for the attachment of the toxin to the target cell and the A subunit which encloses the toxic action of the holotoxin (Lara-Tejero and Galan, 2000; Dreyfus, 2003; Salyers and Whitt, 2003). Still, no conclusive evidence about the actual structure of the CDT holotoxin has been provided so far (Aragon et al., 1997; Pickett and Whitehouse, 1999). Several authors (Pickett et al., 1994; Scott and Kaper, 1994; Shenker et al., 2000; Akifusa et al., 2001; Shenker et al., 2004) illustrated that CDT is composed of three polypeptides, while Pickett and Whitehouse (1999) question that the mere assembly of these polypeptides equals the holotoxin.

CDT blocks cell proliferation by activating DNA damage induced cell cycle checkpoint responses (Li et al., 2002). CdtB appears to be both functionally and structurally homologous to the mammalian deoxyribonuclease I (DNAse I) (Elwell and Dreyfus, 2000; Lara-Tejero and Galan, 2000; Avenaud et al., 2004) and the biologically active subunit of the holotoxin. Nonetheless, Dlakic (2000; 2001) suggested that cdtB might also exhibit phosphatase activity. Transient expression of cdtB in eukaryotic cells also resulted in prominent changes in the chromatin of transfected cells (Lara-Tejero and Galan, 2000; Nishikubo et al., 2003). This peptide has the unique ability to directly harm the cellular DNA resulting in cell cycle arrest.

Cells are irreversibly stopped in the G_1, S or G_2 phase and do not enter into mitosis (Elledge, 1996; De Rycke and Oswald, 2001). The phase, during which the block is induced, is dependent on the cell type (Johnson and Lior, 1988a; Cortes-Bratti et al., 2001b; Frisan et al., 2002). Frisan et al. (2002) noted that CDT produced by *Haemophilus ducreyi* arrested epithelial cell lines (HeLa, Hep-2) and normal keratinocytes in the G_2 phase, while primary human fibroblasts were blocked in the G_1 phase as well (Frisan et al., 2002). B lymphocytes undergo apoptosis (Cortes-Bratti et al., 2001b; Frisan et al., 2002). T lymphocytes also may be targeted by CDT and are even suggested to be five times more sensitive to the toxin than

HeLa cells (Gelfanova et al., 1999; Shenker et al., 1999; Mooney et al., 2001; Ohara et al., 2004). 3T3 fibroblasts and mouse Y-1 adrenal cells on the contrary seem to be resistant to CDT (Johnson and Lior, 1988a; Cope et al., 1997; Cortes-Bratti et al., 1999). It was hypothesized that these cells lack the receptor on the cell surface required for binding CDT (Cortes-Bratti et al., 2001a).

CDT induces DNA double strand breaks (DSBs) in eukaryotic cells (Elwell and Dreyfus, 2000; Lara-Tejero and Galan, 2000; Shenker et al., 2000; Frisan et al., 2002). DNA damage is detected by the proteins kinases 'Ataxia telangiectasia mutated' (ATM) and 'ATM and Rad3 related' (ATR) (Alby et al., 2001; Cortes-Bratti et al., 2001b; Li et al., 2002) Mainly ATM is believed to be activated in response to DNA DSBs (Cortes-Bratti et al., 2001a; Shiloh, 2001; D'Amours and Jackson, 2002). Another group of researchers proved that a protein different from ATM, hitherto unidentified and a member of the phospho-inositol (PI) 3 kinase family, is triggered by DSBs. Whether this kinase is ATR remains to be investigated (Alby et al., 2001).

Possible sensors of DNA damage are on the one hand histone H2AX and on the other hand the Mre11 complex, a multisubunit nuclease, comprised of Mre 11, Rad 50 and Nbs 1 proteins. Several reports demonstrate that irradiation (IR) induces extensive phosphorylation of the histone H2AX forming plain foci at the site of damaged DNA (Nelms et al., 1998; Rogakou et al., 1998; Paull et al., 2000). This phosphorylation depends on ATM when cells are treated with IR (D'Amours and Jackson, 2002). In addition, upon IR and also following CDT intoxication, the Mre 11 complex, usually homogeneously distributed within the nucleus in undamaged cells, co-localizes with the phosphorylated H2AX and reorganizes to form large nuclear foci (Nelms et al., 1998; Li et al., 2002; Hassane et al., 2003). These events are characteristic for the normal cellular response after DNA DSBs (D'Amours and Jackson, 2002) and occur both in proliferating and non-proliferating cells (Li et al., 2002).

Once ATM or a related kinase is activated in response to CDT-induced DNA damage, a cascade of events happens resulting in cell cycle arrest. Figure 2 represents schematically the mode of action of CDT.

Concerning the G_2 checkpoint response, the protein kinases chk1 and chk2 are activated via phosphorylation in response to DNA lesions, and both kinases are able to inhibit the phosphatase cdc25C via phosphorylation of serine 216 *in vitro* (Sanchez et al., 1997;

Matsuoka et al., 1998) in the S phase (Alby et al., 2001). In the unperturbed cell cycle, cdc2 is kept inactive via phosphorylation on threonine 14 and tyrosine 15 by the wee1 and myt1 kinases. The cdc25C phosphatase dephosphorylates these residues and activates the cyclin dependent kinase cdc2/cyclinB complex. This is a step needed for entry into mitosis. CDT intoxicated cells however, remain in the G_2 phase since the lack of the dephosphorylation step of the cdc2/cyclinB complex results in an inactive hyperphosphorylated state of cdc2 (Cortes-Bratti et al., 2001b).

As mentioned above, other cell types are blocked in the S and/or G_1 phase as well, such as fibroblasts (Cortes-Bratti et al., 2001a,b; Frisan et al., 2002). In this situation, p53 tumor suppressor serves as a checkpoint for the DNA damage. P53 is a transcription factor that directly activates other genes such as the cyclin dependent kinase inhibitor p21. The oncoprotein MDM2 targets p53 for ubiquitin-dependent degradation and thereby regulates partially the levels of p53. In disconcerted cells, such as CDT poisoned fibroblasts or keratinocytes, p53 is phosphorylated on serine 20 by the chk2 protein kinase resulting in a dissociation of its negative regulator MDM2 which is disturbed in its inhibition of p53-dependent transactivation (Shieh et al., 1997; Cortes-Bratti et al., 2001b; Shiloh, 2001). This event leads to an increased expression of the p53-regulated cyclin-dependent kinase inhibitor p21 and upregulation of p27. Subsequently, the cdk2/cyclinE complex is inactivated by p21 and cells become arrested at the G_1 stage Cortes-Bratti et al., 2001a,b; Frisan et al., 2002).

Another phenomenon observed in CDT intoxicated cells is an assembly of actin stress fibers (Aragon et al., 1997; Cortes-Bratti et al., 1999; Frisan et al., 2003). Nearly all cell types are able to produce stress fibers which are comprised of polymerized actin subunits. They are responsible for the attachment of cells to a substrate and for the cellular shape. They also might be involved in the mobility of cells. Frisan et al. (2003) demonstrated a rearrangement of actin cytoskeleton upon CDT intoxication which appears to be RhoA GTPase mediated and which is part of the ATM-dependent response to DNA damage. The small GTPases of the RhoA protein family are involved in the formation of stress fibers, focal adhesions (Hall, 1998) and cell proliferation (Olson et al., 1995). The activation of this signalling pathway is not toxin dependent, but occurs in response to any genotoxic stress. Frisan and coworkers (2003) suggest a RhoA GTPase dependent linkage between DNA damage, which may be induced by CDT, and alterations in the actin cytoskeleton, possibly needed to prolong cell

survival. Indeed, only a short exposure, to be specific 2 min (Aragon et al., 1997) or 15 min (Cortes-Bratti et al., 1999), to CDT is necessary to induce slowly developing irreversible cellular changes resulting ultimately in cell death (Aragon et al., 1997).

In contrast to the cdtB subunit, the individual role of cdtA and cdtC is markedly less clearly elucidated (Cortes-Bratti et al., 2001a; De Rycke and Oswald, 2001; Avenaud et al. 2004). Several reports present conflicting results regarding their contribution (Cope et al., 1997; Purvén et al., 1997; Shenker et al., 1999; Frisk et al., 2001; Lara-Tejero and Galan, 2001; Mao and DiRienzo, 2002), but in general, it is accepted that cdtB requires cdtA and/or cdtC to get internalized in the target cell preceding cytotoxicity (Cortes-Bratti et al., 2001; Deng et al., 2001; Frisan et al., 2002; Avenaud et al., 2004; AbuOun et al., 2005) which probably occurs by endocytosis via clathrin-coated pits (Cortes-Bratti et al., 2000).

Role of CDT in pathogenesis

To date, a number of authors have studied the mode of action and effects of CDT of various Gram-negative bacteria on epithelial and blood cells in animal models. The possible actions of this toxin are firstly inhibition of epithelial cell proliferation and apoptosis allowing bacterial invasion, secondly cell cycle arrest of immune cells ensuing local immunosuppression and finally inhibition of fibrotic response. Pertaining to the clinical outcome, CDT seems to be an adaptable toxin that operates in many bacteria resulting in different virulence characteristics (Albert et al., 1996; Okuda et al., 1997; Purvén et al., 1997; Stevens et al., 1999; Purdy et al., 2000; Young et al., 2001; Wising et al., 2002; Young et al., 2004).

Only few studies about the role of CDT in the pathogenesis of EHS infections have been reported thus far. Ge et al. (2005) illustrated that *H. hepaticus* CDT is crucial in the persistent colonization of this bacterial agent in the gut of outbred Swiss Webster Mice, especially in males. They observed additionally a correlation between *H. hepaticus* colonization and down-regulation of interleukin (IL)-10, which is pivotal in blocking IBD. Also Young et al. (2004) demonstrated a role for CDT in generating IBD in IL-10-/- mice infected with *H. hepaticus*. The latter research group constructed a CDT-negative *H. hepaticus* mutant using a transposon shuttle mutagenesis system and challenged C57/BL6 interleukin 10 (-/-) mice with this mutant strain. They noticed that, although the isogenic *H.*

hepaticus CDT mutant maintained the capacity to colonize C57BL/6 IL-10$^{-/-}$ mice, animals inoculated with the mutant developed markedly less severe disease than mice inoculated with a wild-type *H. hepaticus* strain (Young et al., 2004). Very recently, Pratt et al. (2006) determined the role of CDT in the modulation of the host response to *H. hepaticus* using C57BL/6 IL-10$^{-/-}$ mice which were challenged with wild-type *H. hepaticus* and a CDT-deficient isogenic mutant. Only the wild-type strain and not the CDT-deficient mutant was detected till the end of the 8 month-during experiment. Animals infected with the wild-type strain developed severe typhlocolitis and additionally revealed elevated levels of immunoglobulins, while this was not the case for animals infected with the CDT-deficient mutant strain. These results suggest that CDT has an important immunomodulatory function allowing persistence of *H. hepaticus* in IL-10$^{-/-}$ mice. In addition, CDT may alter the host immune response resulting in the development of colitis (Pratt et al., 2006).

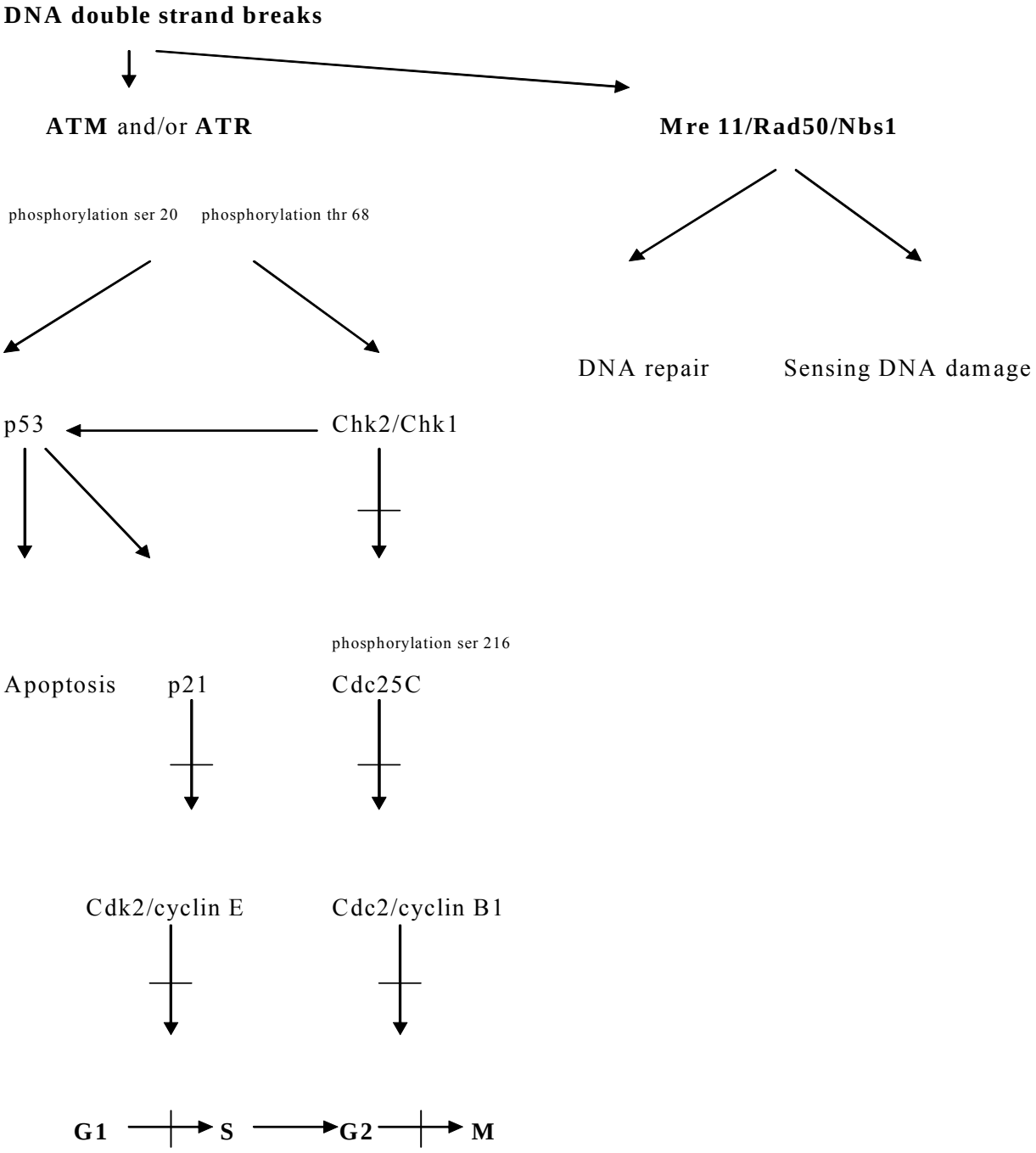

Figure 2 Scheme of mode of action of CDT

6. Treatment of EHS infections in animals and human beings

Hitherto, limited information about an efficient treatment of EHS infections in both animals and humans is available. For birds, no data are presented in literature. Studies about this subject have mainly been performed in *H. hepaticus*- and *H. bilis*-infected laboratory rodents. Since EHS infections not only may cause gastrointestinal disease in these animals, but also may interfere with *in vivo* experiments leading to misinterpretation of data, it is of course important to eliminate EHS from laboratory rodent colonies (Rogers and Fox, 2004; Jacobsen et al., 2005).

The best option to get *Helicobacter*-free rodent colonies may be rederivation by means of embryo transfer. Embryo transfer has been used to free mouse strains from *H. hepaticus* (Van Keuren en Saunders, 2004; Watson et al., 2005). Another possibility to get rid of *H. hepaticus* infection could be caesarean section (Bergin et al., 2005). Since vertical transmission of this bacterium has been implied (Li et al., 1998), the latter may not be appropriate. In contrast, the presence of *H. typhlonius* in sex organs of mice without vertical transmission to their offspring was documented very recently (Scavizzi and Raspa, 2006).
Antibiotic treatment of EHS infected mice and rats might be an alternative (Russel et al., 1995; Foltz et al., 1996; Shomer et al., 1998; Bergin et al., 2005; Jury et al., 2005; Kerton and Warden, 2006). Russell et al. (1995) claimed that orally administered amoxicillin during two weeks eliminates or prevents *H. hepaticus* infection in weanlings, but not in older mice with established enteric colonization. A triple therapy of amoxicillin, metronidazole and bismuth administered orally appears to be effective for eradication of *H. hepaticus*, but not *H. bilis* and *H. rodentium* infections (Foltz et al., 1995; 1996; Shomer et al., 1998). Also recently, an amoxicillin-based triple therapy proved to be successful for the eradication of *Helicobacter* infections in several mouse strains when administered in a diet and in combination with cross-fostering on to *Helicobacter*-free foster mothers (Kerton and Warden, 2006).

With respect to antibiotic treatment for EHS infections in humans, tetracycline, chloramphenicol, ceftriaxone, macrolides and aminoglycosides may be efficient. Ciprofloxacin however, appears to be a poor option for the elimination of *H. cinaedi* infections in humans (Fox et al., 1997; Kuijper et al., 2003; Uckey et al., 2006).

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Scientific aims

During the last decade, the number of case reports on the presence of *Helicobacter pullorum* in samples obtained from human patients suffering from liver and intestinal disease has increased tremendously. In addition, a preliminary study showed that *H. pullorum* was present on 60 % of poultry carcasses pointing to *H. pullorum* as a potentially important food-associated human pathogen. Despite these findings, there is a marked lack of information on the actual prevalence of this species in poultry and humans. Furthermore, very few studies concerning the pathogenesis of *H. pullorum* infections in both poultry and human beings have been performed hitherto.

The **general aim** of this thesis was hence to investigate the occurrence of *H. pullorum* in poultry and human beings and to study interaction of *H. pullorum* with its animal host.

The **specific scientific aims** were to:

1. determine the occurrence of *H. pullorum* in Belgian broilers
2. determine the prevalence of *H. pullorum* in humans with and without gastrointestinal disease
3. determine the genetic relatedness between *H. pullorum* isolates and their *in vitro* susceptibility to different antimicrobial agents
4. characterize potential virulence factors of *H. pullorum* isolates
5. study the *in vivo* interactions of *H. pullorum* isolates with broiler chickens

Chapter 1

The agent *Helicobacter pullorum*: prevalence and *in vitro* susceptibility to different antimicrobial agents

1.1 Occurrence of *Helicobacter pullorum* in broiler chickens and comparison of isolates using amplified fragment length polymorphism profiling

1.2 Prevalence of *Helicobacter pullorum* among patients with gastrointestinal disease and clinically healthy persons

1.3 *In vitro* susceptibility of *Helicobacter pullorum* isolates to different antimicrobial agents

Chapter 1.1

Occurrence of *Helicobacter pullorum* in broiler chickens and comparison of isolates using amplified fragment length polymorphism profiling

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ABSTRACT

A total of 110 broilers from 11 flocks were tested for *Helicobacter pullorum* by polymerase chain reaction; positive samples were re-examined with a conventional isolation method. *Helicobacter pullorum* isolates were examined by amplified fragment length polymorphism (AFLP) fingerprinting for interstrain genetic diversity and relatedness. Sixteen isolates from caecal samples from 2 different flocks were obtained. AFLP analysis showed that these isolates and 4 additional isolates from a different flock, clustered with respect to their origin, which indicates that *Helicobacter pullorum* colonization may occur with a single strain that disseminates throughout the flock. Strains isolated from different hosts or geographical sources, displayed a distinctive pattern.

Helicobacter pullorum is present in approximately one third of live chickens in Belgium and may represent a risk to human health.

INTRODUCTION

Helicobacter pullorum was originally isolated from the faeces and injurious livers of broilers and laying hens (Burnens et al., 1994; Stanley et al., 1994). It was defined as a new species in 1994 by Stanley et al. *H. pullorum* represents a Gram-negative, slightly curved rod with monopolar non-sheathed flagella. It is bile-resistant and requires a microaerobic environment supplemented with H_2 in which growth occurs at 37 and 42°C (Stanley et al., 1994; On et al., 1996; Fox, 1997; Steinbrueckner et al., 1997; Atabay et al., 1998).

Enterohepatic *Helicobacter* species, including *H. pullorum*, are increasingly recognized as microbial pathogens both in humans and animals (Burnens et al., 1996; On et al., 1996; Fox, 1997; Fox et al., 1998; On et al., 2002). *H. pullorum* has been linked with hepatitis in laying hens and also diarrhoea, gastroenteritis and liver disease in humans (Burnens et al., 1994; Stanley et al., 1994; Burnens et al., 1996; Fox, 1997; Steinbrueckner et al., 1997; Fox et al., 1998; Ceelen et al., 2005). *H. pullorum* can contaminate poultry carcasses at the abattoir. Therefore, some authors consider this bacterial species to be a food-borne human pathogen (Atabay et al., 1998; Fox et al., 1998; Gibson et al., 1999).

Hitherto, almost no data are available on the actual prevalence of this species in poultry. Research that could generate these data is hampered by the fastidious growth requirements of *H. pullorum* on the one hand and the phenotypic similarity between member species of the genera *Helicobacter* and *Campylobacter* on the other hand (On et al., 1996; Atabay et al., 1998; Gibson et al., 1999). The occurrence of *H. pullorum* in chickens has merely been studied on only two occasions to our knowledge where the organism was detected using isolation (Burnens et al., 1996; Atabay et al., 1998). Furthermore, no valid epidemiological research methodologies have been recommended thus far.

The objective of the present study was to determine the occurrence of *H. pullorum* in broilers using both PCR and isolation. In addition, amplified fragment length polymorphism profiling was carried out to investigate the genetic relatedness between *H. pullorum* isolates.

MATERIALS AND METHODS

Sample origin

One hundred and ten gastrointestinal tracts and livers of broiler chickens, ten per flock (flock n° 1 – n° 11), collected at a poultry abattoir, were included in this study. Each gastrointestinal tract and liver were deposited in a separate waterproof plastic bag. Samples were taken from the liver, caeca, jejunum and colon for PCR and isolation within three hours after collection. All samples were stored at -20°C and -70°C for PCR and isolation, respectively, until further analysis as described below.

Sample processing

PCR and gel electrophoresis

DNA was extracted from approximately 25 mg caecum, colon, jejunum and liver tissue applying a commercial tissue kit (DNeasy® Tissue Kit, Qiagen, Venlo, The Netherlands). A PCR assay amplifying a 447 bp fragment of the 16S rRNA gene of *H. pullorum* was then used for detection purposes (Stanley et al., 1994). From each sample, 2 µl of the template was added to 8 µl of the PCR mixture, containing 0.03 U/µl Taq polymerase Platinum (Invitrogen Life Technologies, Merelbeke, Belgium), 10 x PCR Buffer (Invitrogen Life Technologies), 3 mM $MgCl_2$ (Invitrogen, Life Technologies), 40 µM of each deoxynucleoside triphosphate (Invitrogen Life Technologies), a final primer concentration of 0.5 µM and sterile distilled water. The conditions used for the amplifications were the following: an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 90 seconds and elongation at 72°C for 90 s, and a final elongation at 72°C for 5 min.

Five microliters of the PCR products of each sample were mixed with 3 µl of sample buffer 5X (50 % glycerol, 1 mM cresol red) and were electrophoresed through an agarose gel containing 1.5 % Multi Purpose agarose (Boehringer, Mannheim, Germany) and 50 ng ethidium bromide per ml 1 x TAE buffer (Amresco, Ohio, USA), pH 8. As molecular size marker, the Gene Ruler 100 bp DNA ladder plus (MBI Fermentas, St. Leon-Rot, Germany) was used. Electrophoresis was implemented at a constant voltage of 170 V in 0.5 x TAE buffer during 75 min. The gels were visualised using the Image Master® VDS (Pharmacia Biotech, Puurs, Belgium).

Table 1 *Helicobacter pullorum* isolates studied using AFLP

Strain		Source	Geographic origin
CE III 2	Flock CLO[a]	Caecal droppings, broiler chicken	Belgium
CE III 3		Caecal droppings, broiler chicken	
CE III 4		Caecal droppings, broiler chicken	
CE III 5		Worker's boot	
CE II 1	Flock n° 5	Caecal tissue, broiler chicken	Belgium
CE II 2		Caecal tissue, broiler chicken	
CE II 3		Caecal tissue, broiler chicken	
CE II 4		Caecal tissue, broiler chicken	
CE II 5		Caecal tissue, broiler chicken	
CE II 6		Caecal tissue, broiler chicken	
CE II 7		Caecal tissue, broiler chicken	
CE II 8		Caecal tissue, broiler chicken	
CE I 1	Flock n° 9	Caecal tissue, broiler chicken	Belgium
CE I 2		Caecal tissue, broiler chicken	
CE I 3		Caecal tissue, broiler chicken	
CE I 4		Caecal tissue, broiler chicken	
CE I 5		Caecal tissue, broiler chicken	
CE I 6		Caecal tissue, broiler chicken	
CE I 7		Caecal tissue, broiler chicken	
CE I 8		Caecal tissue, broiler chicken	
CCUG[b] 33837	NA[c]	Healthy broiler chicken	Switzerland
CCUG 33838		Laying hen, hepatitis	Switzerland
CCUG 33839		Stool, gastroenteritis and hepatitis, human	Switzerland
CCUG 33840		Stool, gastroenteritis, human	Switzerland
G 214		Stool, gastroenteritis, human	Belgium (Ceelen et al., 2005)

[a] CLO: Centrum voor Landbouwkundig Onderzoek, [b] CCUG: Culture Collection of the University of Göteborg, [c] NA: Not applicable for identification

Isolation of H. pullorum

Recovery of *H. pullorum* isolates was attempted on all samples positive in the PCR analysis described above. The samples (200 mg) for isolation of *H. pullorum* were put in a 1.5 ml tube with 400 µl of a mixture composed of 7.5 g glucose, 25 ml brain heart infusion (BHI) broth (Oxoid, Basingstoke, England) and 75 ml sterile inactivated horse serum and were homogenized. The various isolates were inoculated on BHI agar, supplemented with 10 % horse blood, amphotericin B 20 µg/ml (Fungizone; Bristol-Myers Squibb, Epernon, France) and Vitox (Oxoid) (blood agar). A modified filter technique of Steele and McDermott was then used. Briefly, a sterile cellulose acetate membrane filter (0.45 µm) was applied with a sterile pair of tweezers directly onto the surface of the agar. When the filter was adsorbed totally on the agar, approximately 300 µl of the mixture was put in the middle of the filter. After at least one hour of incubation at 37°C and 5 % CO_2, the filter was removed with a sterile pair of tweezers and the filtrate was streaked on the agar with a loop. Incubation was done in microaerobic conditions (5 % H_2, 5 % CO_2, 5 % O_2 and 85 % N_2) at 37°C for minimum three days. Very small, greyish-white, haemolytic colonies were selected and purified on a blood agar plate. The colonial morphology and phenotypic characteristics (Gram-negative, slightly curved rod, catalase and oxidase positive, indoxyl acetate negative) of the isolates were used for presumptive identification. Confirmation of the presumed identity was done on the basis of PCR and sequencing of a 447 bp fragment of the 16S ribosomal RNA gene as described below.

Analysis of nucleotide sequences

The PCR product of the retrieved *H. pullorum* isolates was purified with the Qiaquick PCR purification kit (Qiagen) and sequenced using the same primers applied in the PCR assay with the BigDye Terminator cycle sequencing kit (Applied Biosystems, Lennik, Belgium). Sequencing products were run on the ABI prism^{TM} 3100 Genetic Analyzer (Applied Biosystems), using 50 cm capillaries filled with Performance-Optimized-Polymer 6. The electrophoregrams were exported and converted to the Kodon software package (Applied Maths, Sint-Martens-Latem, Belgium). Sequences were compared to published *H. pullorum* 16S rRNA gene sequences obtained from GenBank (accession numbers AY631956, L36143 and L36144) by using BLAST software.

AFLP

Twenty-two poultry and three human isolates were fingerprinted by AFLP (Table1). These included 16 isolates obtained from flocks n° 5 and n° 9 screened in the present study. In addition, four isolates previously isolated from caecal drops of broilers and farmer's boots from another flock, four reference strains (two of chicken and two of human origin), and one human strain isolated from diarrheic stool in our laboratory were likewise included for comparison.

Restriction endonuclease digestion and ligation of adaptors for AFLP

DNA of *H. pullorum* isolates was extracted using a commercial tissue kit (DNeasy® Tissue Kit, Qiagen). An aliquot containing 200 ng DNA, determined by optic density (260/280nm) measurement using the Spectra Fluor (TECAN, Grödig, Salzburg, Austria), was digested for two hours at 37°C with *Bgl*II (10U/µl) and *Csp*6I (10U/µl) (MBI Fermentas) in TAC-buffer as described by Vos et al. Five microliters of DNA digest was used in a ligation reaction containing 130 µg/ml *Bgl* II adaptor-oligonucleotide and 13 µg/ml *Csp*6I adaptor-oligonucleotide (Invitrogen) (14), 10 X T4 DNA ligase buffer, T4 DNA ligase (1U/µl) (Amersham Pharmacia) and TAC-buffer in a final volume of 20 µl. After incubation for two hours at 25°C, the 20 µl ligation reaction was diluted 25 times.

Direct selective PCR amplification of diluted ligation

Five microliters of the diluted ligation reaction were applied in the PCR assay. The primers used in this PCR assay were primers BGL2F-0, 5'-GAG TAC ACT GTC GAT CT-3' (FAM labeled, 5'-end) and CSP6I-A, 5'-GAG CTC TCC AGT ACT ACA-3' (Kokotovic and On, 1999). The used PCR conditions were the following: an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min and elongation at 72°C for 90 s, and a final elongation at 72°C for 10 min.

Capillary electrophoresis

PCR products were run on the ABI prism™ 3100 Genetic Analyzer (Applied Biosystems), using the Fragile X Rox-1000 size standard and 50 cm capillaries filled with Performance-

Optimized-Polymer 6. Electropherograms were analyzed with Genemapper U 3.5 Software (Applied Biosystems).

Numerical analyses of AFLP profiles

The program BioNumerics version 2.5 (Applied Maths) was used to perform numerical analyses of AFLP profiles. Strain relationships were inferred by use of the Pearson product-moment correlation coefficient and Unweighted Pair-Group with Mathematical Average (UPGMA) clustering, and depicted in a dendrogram (On et al., 2003).

RESULTS

PCR

In table 2, the number of *H. pullorum* DNA positive samples originating from the intestinal tract and liver is presented. In four flocks all samples were negative for *H. pullorum*. In the other seven flocks positive samples were found. In the caecum and colon, a PCR reaction for *H. pullorum* gave positive results in 33.6 % and 31.8 % of the samples, respectively. In total, 10.9 % and 4.6 % of all jejunum and liver samples, respectively, were positive for *H. pullorum*.

Isolation of *H. pullorum*

Eight *H. pullorum* isolates from flock n° 5 and eight *H. pullorum* isolates from flock n° 9, all from the caecum, were obtained. The sequences of the amplified 447 bp fragment of the *H. pullorum* 16S rRNA gene of the *H. pullorum* isolates revealed a similarity of 98 to 100 % to those from Genbank (AY631956, L36142 and L36143).

AFLP

AFLP analysis revealed that isolates derived from each of the individual flocks examined clustered with respect to their flock of origin. The remaining chicken isolates and human strains each displayed a unique profile (Figure).

Table 2 Number of positive poultry tissue samples for *Helicobacter pullorum* in PCR

Flock n°	Tissue			
	Caecum	**Colon**	**Jejunum**	**Liver**
	Number of positive samples			
1	2*	2	1	0
2	3	8	4	0
3	4	1	1	0
4	7	4	1	0
5	8	8	0	0
6	0	0	0	0
7	4	4	0	1
8	0	0	0	0
9	9	8	5	4
10	0	0	0	0
11	0	0	0	0
Total	**37**	**35**	**12**	**5**

* Number of positive animals of ten screened per flock

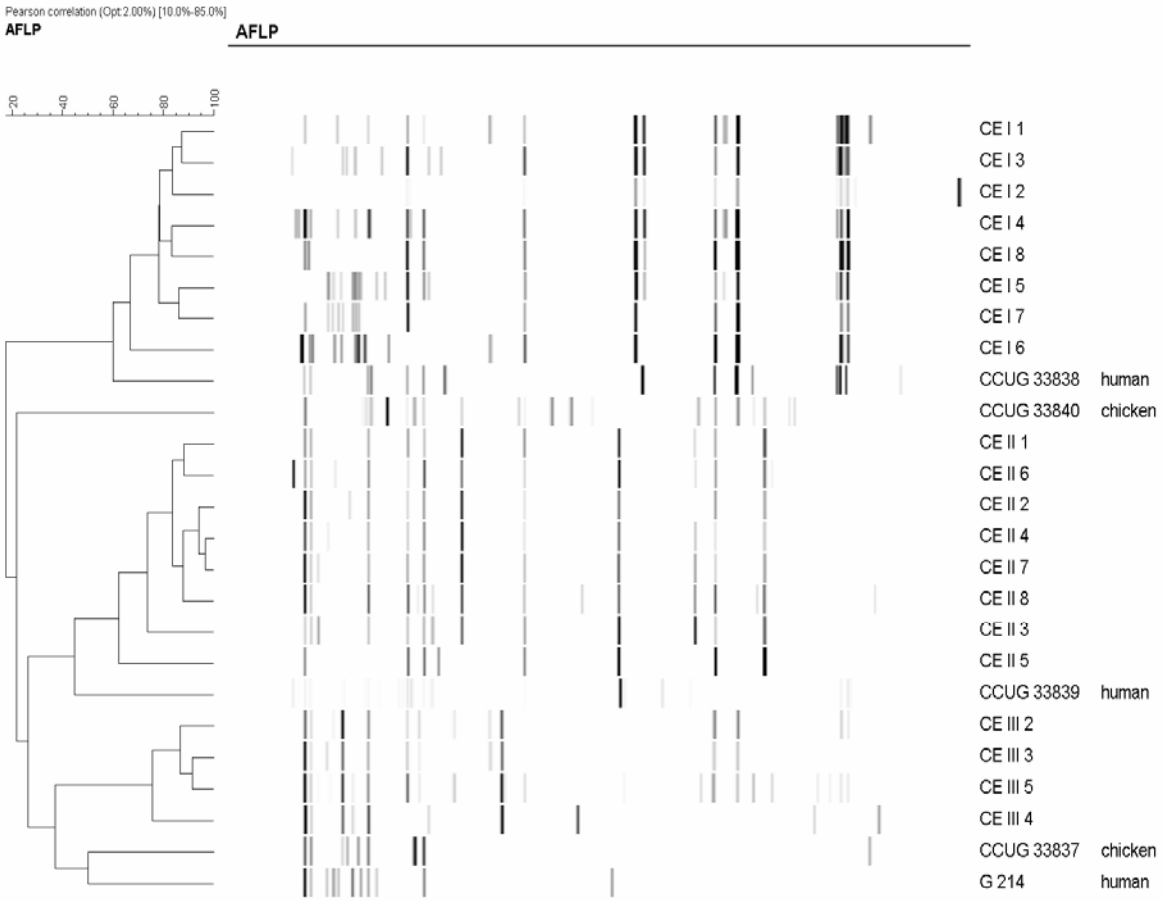

Figure AFLP profiles of *Helicobacter pullorum* isolates and reference strains

DISCUSSION

This report demonstrates that *H. pullorum* is present in 33.6 % of the caecal samples of broiler chickens collected at a poultry slaughterhouse during evisceration using PCR. This microorganism was found in seven flocks out of the 11, while four flocks were negative.

Burnens et al. (1996) found a prevalence rate of 4 % upon sampling caecal contents of broilers. The organism was however detected by isolation, which in view of the fastidious nature of this organism, could explain this markedly lower percentage positive birds. In addition, in our study, the actual caecal tissue was examined for the presence of the organism rather than caecal contents. Microorganisms related to *H. pullorum* have been shown to

adhere closely to the mucosa of the gastrointestinal tract. It has been reported that the phylogenetically related microorganism *C. jejuni* may tightly adhere to the brush borders of the intestine in chickens (Ruiz-Palacios et al., 1981; Sanyal et al., 1984). The same phenomenon has also been documented for *H. pylori* in the stomach (Clyne and Drumm, 1993).

Comparing our study results to those obtained by Atabay et al., the latter research group found a higher occurrence of *H. pullorum* (60 %) on poultry carcasses. This apparent discrepancy could be due to cross-contamination with caecal contents on the surface of broiler carcasses during poultry processing (Atabay et al., 1998; Fox et al., 1998). Furthermore, contamination of the chicken body surface may occur during transport to the abattoir. Faecal excretion of *Campylobacter* may be increased due to stress during transport and consequently contaminate carcasses (Whyte et al., 2001).

H. pullorum DNA was detected in only five liver (4.6 %) and 11 jejunal (10.9 %) samples as opposed to 35 colonic (31.8 %) and 37 caecal (33.6 %) samples. One may hence assume that the lower segments of the intestinal tract are the predominant colonization sites for *H. pullorum* in broiler chickens. *H. pullorum* may gain access to the liver by retrograde transfer from the duodenum. Alternatively, it may translocate from the gut lumen to the portal circulation.

H. pullorum has been associated with vibrionic hepatitis in laying hens, both macroscopically and microscopically (Burnens et al., 1996). In the present study, no gross pathologic lesions were observed in the livers during sampling (data not shown).

Our modest isolation rate of *H. pullorum* from caecal samples may have been the result of examining frozen, compared with fresh samples. However, we successfully recovered 16 isolates from two flocks, allowing (for the first time, to our knowledge) some analysis of the epidemiology of *H. pullorum* in broiler flocks to be undertaken. We used AFLP profiling for this purpose, a highly discriminatory method that has been successfully applied to molecular epidemiological studies of several related species including *H. pylori* (Fox, 1997; Ananieva et al., 2002), *Arcobacter* spp. (Kokotovic and On, 1999) and *Campylobacter* spp.(Siemer et al., 2004; 2005). Isolates derived from each of the individual flocks clustered respectively together, indicating a clonal relationship. In contrast, field and reference strains isolated from different hosts or geographical sources displayed a distinctive

pattern. These data suggest that, as for the related species noted above, AFLP profiling appears to have considerable potential for molecular epidemiological studies of *H. pullorum.* Several authors have suggested that *H. pullorum* has zoonotic potential and is involved in the pathogenesis of diarrhoea and chronic liver diseases in humans (Burnens et al., 1994; Fox et al., 1998; Young et al., 2000; Ceelen et al., 2005). Retail raw poultry meats and other poultry products may constitute vehicles for a *H. pullorum* infection of humans through carcass contamination as previously reported for *Arcobacter* and *Campylobacter* species (Fox et al., 1998; Houf et al., 2000; Antolin et al., 2001; Houf et al., 2001). Concerning health monitoring, PCR may be a helpful method to detect this pathogen not only in intestinal tissue but also in caecal drops from broiler chickens.

In conclusion, this study shows that *H. pullorum* is a rather frequent intestinal coloniser of broiler chickens. PCR and isolation are useful tools for detecting the species in intestinal tissue and in caecal drops. AFLP profiling appears to be a useful tool for molecular epidemiological studies of this species.

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Chapter 1.2

Prevalence of *Helicobacter pullorum* among patients with gastrointestinal disease and clinically healthy persons

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ABSTRACT

The objective of this study was to determine the prevalence of *Helicobacter pullorum* in patients with gastrointestinal disease and in clinically healthy people.

Faecal samples from 531 patients with gastrointestinal disease and 100 clinically healthy individuals were tested for the presence of *Helicobacter pullorum* using PCR analysis based on the 16S rRNA gene. Samples proving positive in PCR were inoculated onto BHI agar supplemented with 10% horse blood and incubated in a microaerobic atmosphere at 37°C for minimum three days for isolation purposes.

Helicobacter pullorum DNA was demonstrated in faecal material from 4.3 % of patients with gastrointestinal disease, but also from 4.0 % of clinically healthy persons. One strain was isolated from one patient with gastrointestinal disease.

It was concluded that *Helicobacter pullorum* is fairly regularly present in faecal samples from humans. Its role in gastrointestinal disease needs further investigation.

INTRODUCTION

In the last two decades, more than 30 species have been described within the genus *Helicobacter* (On et al., 2002). One of these is *Helicobacter pullorum*, a Gram-negative, motile, slightly curved, non spore-forming rod which is oxidase positive, negative for indoxyl acetate esterase, urease production and hippurate hydrolysis. The organism is sensitive to nalidixic acid and mostly catalase positive (On et al., 1996). *H. pullorum* is bile-resistant and requires a microaerobic environment supplemented with H_2 in which growth occurs at 37°C and 42°C (Stanley et al., 1994; On et al., 1996; Fox, 1997; Steinbrueckner et al., 1997). Identification to the species level can be done on the basis of the above mentioned phenotypic traits, although the results of these tests are sometimes difficult to interpret. The correct identity of the species can be confirmed by means of polymerase chain reaction (PCR) (Stanley et al., 1994).

H. pullorum has been detected on several occasions in the caecum and on the carcass of broiler chickens, in the intestine of laying hens and in the faeces of humans. DNA of this bacterial species has been demonstrated in the liver of laying hens and in the biliary tree of humans (Burnens et al., 1994; 1996; Fox, 1997; Fox et al., 1998; Gibson et al., 1999; Steinbrueckner et al., 1997; Young et al., 2000). A number of research groups have associated this organism with vibrionic hepatitis in poultry and with gastroenteritis, diarrhoea, liver and gallbladder disease in human patients (Stanley et al., 1994; Fox, 1997; Fox et al., 1998; Young et al., 2000). It has been suggested that *H. pullorum* also may play a role in Crohn's disease (Andersson et al., 2002; Bohr et al., 2002).

Broilers seem to be the source of infection for humans, due to contamination of the carcass with intestinal contents in the abattoir (Fox, 1997; Atabay et al., 1998). The number of infections with *H. pullorum* in people most probably has been and still is underestimated because of the phenotypic similarities between the genera *Helicobacter* and *Campylobacter* on the one hand and the specific isolation requirements of *H. pullorum* on the other hand (Steinbrueckner et al., 1997; Atabay et al., 1998; Gibson et al., 1999; Young et al., 2000). Consequently, a significant number of patients with diarrhoea may have been misdiagnosed in the past (Steinbrueckner et al., 1997; Atabay et al., 1998; Young et al., 2000).

Despite the increasing number of reports stating *H. pullorum* as a significant food-associated human pathogen, there is a total lack of information on the prevalence of this species in human beings. In fact, no data whatsoever on the actual carrier rates in humans are available.

The aim of the present study was to determine the prevalence of *H. pullorum* in human patients with gastrointestinal disease. For comparatory purposes, clinically healthy persons were likewise included.

MATERIALS AND METHODS

Sample origin

Five hundred and thirty-one faecal samples were obtained from patients with gastrointestinal disease and anonymized at the Department of Gastroenterology, University Hospital of Ghent, Belgium, before being analyzed.

One hundred faecal samples were collected on an anonymous basis from clinically healthy volunteers.

All samples were stored at -20°C and -70°C for PCR and isolation respectively, until further analysis.

Sample processing

PCR and gel electrophoresis

DNA was extracted from faeces (weighing approximately 200 mg) using a commercial stool kit (QIAamp® DNA Stool Mini Kit, Qiagen, Venlo, The Netherlands).

A PCR assay amplifying a 447 bp fragment of the 16S rRNA gene of *H. pullorum* was used (Stanley et al., 1994). Per sample 2 µl of the prepared DNA was added to 8 µl of the PCR mixture, containing 0.03 U/µl taq polymerase platinum (Invitrogen, Life Technologies, Merelbeke, Belgium), 10 x PCR Buffer (Invitrogen, Life Technologies), 3 mM $MgCl_2$ (Invitrogen, Life Technologies), 40 µM of each deoxynucleoside triphosphate (Invitrogen, Life Technologies), a final primer concentration of 0.5 µM and sterile distilled water. The conditions used for the amplifications were the following: an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 90 s and elongation at 72°C for 90 s, and a final elongation at 72°C for 5 min.

Five microliters of the PCR products of each sample were mixed with 3 µl of sample buffer 5X (50 % glycerol, 1 mM cresol red) and electrophoresed through an agarose gel containing 1.5 % Multi Purpose agarose (Boehringer, Mannheim, Germany) in 1 x TAE buffer (Amresco, Ohio, USA), pH 8 and containing 50 ng ethidium bromide per ml. As molecular size marker the Gene Ruler 100 bp DNA ladder plus (MBI Fermentas, St. Leon-Rot, Germany) was used. Electrophoresis was implemented at a constant voltage of 170 V in 0.5 x TAE buffer during 75 minutes. The gels were visualised using the Image Master® VDS (Pharmacia Biotech, Puurs, Belgium).

Isolation of H. pullorum

All samples positive in the PCR were qualified for isolation. The samples (200 mg) for isolation were put in a 1.5 ml tube with 400 µl of a mixture composed of 7.5 g glucose, 25 ml brain heart infusion (BHI) (Oxoid, Basingstoke, England) and 75 ml sterile inactivated horse serum and homogenized.

The various samples were inoculated on BHI agar, supplemented with 10 % horse blood, amphotericin B 20 µg/ml (Fungizone; Bristol-Myers Squibb, Epernon, France) and Vitox (Oxoid). The filter technique of Steele and McDermott (1984) was used, although slightly modified. Briefly, a sterile cellulose acetate membrane filter (0.45 µm) was applied with a sterile set of tweezers directly to the surface of the agar. When the filter was absorbed totally on the agar, approximately 300 µl of the mixture was put in the middle of the filter. After at least one hour of incubation at 37°C and 5 % CO_2, the filter was removed with a sterile set of tweezers and the filtrate was streaked on the agar with a loup. Incubation was done in microaerobic conditions (5 % H_2, 5 % CO_2, 5 % O_2 and 85 % N_2) at 37°C for minimum three days. Very small, greyish-white, haemolytic colonies were selected and purified on a BHI agar blood plate. The colonial morphology and phenotypic characteristics (Gram-negative, slightly curved rod, catalase and oxidase positive, indoxyl acetate esterase negative) of the isolates were used for presumptive identification. Confirmation of the presumed identity was done on the basis of PCR and sequencing of the 16S ribosomal RNA gene as described below.

Analysis of nucleotide sequences

The PCR product of the retrieved *H. pullorum* isolate was purified with the Qiaquick PCR purification kit (Qiagen) and sequenced using the same primers applied in the PCR assay with the BigDye Terminator cycle sequencing kit (Applied Biosystems, Lennik, Belgium). Sequencing products were run on the ABI prism^{tm} 3100 Genetic Analyzer (Applied Biosystems), using 50 cm capillaries filled with Performance-Optimized-Polymer 6. The electropherograms were exported and converted to the Kodon software package (Applied Maths, Sint-Martens Latem, Belgium). Sequences were compared to published *H. pullorum* 16S rRNA sequences obtained from GenBank (accession numbers AY631956, L36143 and L36144) by using BLAST software.

RESULTS

Twenty-three out of the 531 faecal samples (4.3 %) from gastrointestinal patients were found positive for *H. pullorum* in the PCR. In the group of clinically healthy individuals, four out of the 100 samples harboured *H. pullorum* DNA.

H. pullorum was isolated of the faeces from one gastrointestinal patient.

The sequence of the amplified 447 bp fragment of the *H. pullorum* 16S ribosomal RNA gene of the *H. pullorum* isolate revealed a similarity of 99 % to those from Genbank (AY631956, L36143 and L36144).

DISCUSSION

The present study shows that faecal material from 4.3 % of patients with gastrointestinal disease, but also from 4.0 % of clinically healthy persons harbours *H. pullorum* DNA. The finding of equivalent percentages in both groups may question the presumed association of *H. pullorum* with gastrointestinal and liver disease. One could indeed state that *H. pullorum* may reside in the normal gut flora of human beings. Its presence in faecal samples might also be passive, representing acquisition from contaminated food without replication of the organism in the intestinal tract. It can hence not be excluded that demonstration of *H. pullorum* in human faeces might be an accidental finding.

Several authors have, however, related *H. pullorum* to gastroenteritis resulting in diarrhoea, and liver and gallbladder disease in humans (Burnens et al., 1994; Steinbrueckner et al., 1997; Fox et al., 1998). Encountering *H. pullorum* DNA in faeces from gastrointestinal patients as well as from clinically healthy individuals does not necessarily exclude this microorganism from being pathogenic. Indeed, predisposing factors which are hitherto unknown may cause some of the *H. pullorum* strains to make the transition from being a harmless inhabitant or passer-by of the intestinal tract to causing clinical disease. This hypothesis may be complemented with the possible existence of strains with differing virulence, with the highly virulent strains triggering diarrhoeal disease. Differences in virulence between strains have been described for other *Helicobacter* species such as *H. hepaticus* (Suerbaum et al., 2003) and *H. pylori* (Dunn et al., 1997; Israel et al., 2001). In fact, 70 to 90 % of the population in developing countries carries *H. pylori*, while only 25 to 50 % of them develop gastric disease (Dunn et al., 1997). The actual evolvement into gastric disease depends on bacterial factors, host characteristics and/or interaction between host and bacterium (Israel et al., 2001). A well-known phenomenon is that strains possessing the *cagA* gene, a component of the pathogenicity island (*cag*A$^+$), are substantially more virulent than *cag*A$^-$ strains (Kuipers et al., 1995; Lee et al., 1997; Sharma et al., 1998; Israel et al., 2001).

For *H. pullorum*, very few data are available on the actual virulence markers despite the increasing number of clinical reports involving this pathogen. The only study entering upon this research area demonstrates the production of the cytolethal distending toxin (CDT)

by *H. pullorum* which is speculated to play an aetiological role in the development of diarrhoea (Young et al., 2000).

Host factors such as age, genetic background and immune response but also ethnical aspects and regional factors might also play a role in the clinical outcome of a *H. pullorum* infection. These all have been discussed for *H. pylori* on numerous occasions (Taylor et al., 1995; Dunn et al., 1997; Kim et al., 2004; Vilaichone et al., 2004) and to a lesser extent for *H. hepaticus* (Ward et al., 1996; Ihrig et al., 1999; Whary et al., 2001). A report of Whary et al. (2001) who infected different mouse strains with *H. hepaticus* emphasizes the significance of the host response. Various studies about the development of hepatitis, liver cancer and inflammatory bowel disease (IBD) in mice infected with *H. hepaticus* demonstrate that a genetic basis for susceptibility to *Helicobacter*-induced disease is of importance. Indeed, differences between mouse strains regarding the development of liver disease or IBD are commonly noticed (Ihrig et al., 1999; Ward et al., 1994a,b; 1996).

Only one *H. pullorum* strain originating from a patient with gastrointestinal disease was obtained by culture stressing the difficulty to isolate this microorganism from human stool. It is indeed common knowledge that *Helicobacter* species are fastidious bacterial pathogens that are difficult to isolate (Andersen et al., 2001; Al-Soud et al., 2003, Silva et al., 2003).

In conclusion, this is the first elaborate report on the prevalence of *H. pullorum* in both patients with gastrointestinal disease and clinically healthy humans proving that *H. pullorum* is fairly regularly present in the stool of people belonging to both groups. To date, the molecular basis of *H. pullorum* colonization and virulence is poorly understood and further studies to unlock more of the secrets of the lifestyle of this potential pathogen and its encumbrance for public health are hence necessary.

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Chapter 1.3

In vitro susceptibility of *Helicobacter pullorum* isolates to different antimicrobial agents

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ABSTRACT

The *in vitro* activity of 13 antimicrobial agents against 23 *Helicobacter pullorum* isolates from poultry (21) and human (2) origin and one human *Helicobacter canadensis* strain was tested by the agar dilution method. With the *Helicobacter pullorum* isolates, monomodal distributions of Minimum Inhibitory Concentrations were seen with lincomycin, doxycycline, gentamicin, tobramycin, erythromycin, tylosin, metronidazole and enrofloxacin in concentration ranges considered as indicating susceptibility in other bacteria. The normal susceptibility level for nalidixic acid was situated at or slightly above the MIC breakpoints proposed for *Campylobacteriaceae*. Ampicillin, ceftriaxone and sulphamethoxazole-trimethoprim showed poor activity against *Helicobacter pullorum.* For the *Helicobacter canadensis* strain, a similar susceptibility pattern was seen, except for nalidixic acid and enrofloxacin, whose MIC of > 512 µg/ml and 8 µg/ml respectively, indicated resistance of this agent. With spectinomycin, a bimodal distribution of the MICs was noted for the tested strains; eight *Helicobacter pullorum* isolates originating from one flock showed acquired resistance (MIC > 512 µg/ml).

INTRODUCTION

Helicobacter pullorum was first described by Stanley et al. (1994). The species constitutes a Gram-negative, non-spore forming, gently curved, slender rod with monopolar non-sheathed flagella (Stanley et al., 1994; On, 1996; Atabay et al., 1998). *H. pullorum* is associated with vibrionic hepatitis in laying hens and enteritis and diarrhoea in humans (Stanley et al., 1994; Fox et al., 1998; Young et al., 2000). Several authors have suggested that *H. pullorum* is involved in the pathogenesis of chronic liver diseases in humans (Fox et al., 1998; Gibson et al., 1999; Ananieva et al., 2002). Broilers appear to constitute the source of infection for humans, due to carcass contamination with intestinal contents during the slaughter process (Atabay et al., 1998; Fox et al., 1998). A significant portion of cases of human diarrhoea could have been misdiagnosed in the past, because of the specific requisites for isolation of the species on the one hand and biochemical similarities between the *Helicobacter* genus and *Campylobacter* genus on the other hand (Atabay et al., 1998; Gibson et al., 1999).

Despite the increasing number of reports emphasizing the significance of *H. pullorum* in human beings, hardly any data about the antibiotic sensitivity of *H. pullorum* are available in the literature. *H. pullorum* is naturally sensitive to polymyxin B, a phenotypic characteristic distinguishing this species from the other *Helicobacter* spp (Atabay et al., 1998. Resistance to cefalotin and cefoperazone has been reported (Stanley et al., 1994; On, 1996). Different resistance percentages exhibited by *H. pullorum* to nalidixic acid were encountered by several research groups. On (1996) and Atabay et al. (1998) reported 6 % and 28 % *in vitro* resistance respectively, while antimicrobial susceptibility assays showed 55 % resistance to this antimicrobial agent among the tested strains in a study of Melito et al. (2000). Thus far, no susceptibility studies comprising widely used antibiotics with *H. pullorum* strains have been reported.

In the present study, the susceptibility of 23 *H. pullorum* isolates and one *H. canadensis* strain against 13 antimicrobial agents was investigated by the agar dilution method.

MATERIALS AND METHODS

Bacterial strains

A total of 23 isolates of *H. pullorum* were tested: 21 poultry isolates and two human isolates. The poultry strains originated from five different flocks in Belgium and Switzerland. One *H. canadensis* strain (CCUG 47163) was tested likewise (Table 1). The *H. pullorum* strains from the Belgian flocks were isolated from caecal samples using the filter technique of Steele and McDermott. The identity of *H. pullorum* was presumed on the basis of the bacteria being Gram-negative, slightly curved and rod-shaped, oxidase and catalase positive, indoxyl acetate esterase negative and sensitive to polymyxin B. The correct identity of the species was confirmed using a PCR assay developed by Stanley et al. (1994). *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 29213) were included as control strains. The *Helicobacter* strains were inoculated on brain heart infusion (BHI) agar (Oxoid, Basingstoke, England) and incubation occurred for minimally three days in a microaerobic environment (5 % H_2, 5 % CO_2, 5 % O_2 and 85 % N_2) at 37°C.

Antimicrobial agents

The following antibiotics were tested: ampicillin, lincomycin, doxycycline, spectinomycin, gentamicin, tobramycin, ceftriaxone, metronidazole, erythromycin, tylosin, nalidixic acid and sulphamethoxazole-trimethoprim, all purchased from Sigma (St. Louis, MO, USA) and enrofloxacin (Bayer, Brussels, Belgium). Drug solutions were prepared immediately prior to use.

Table 1 Bacterial isolates used in this study

		Strain number	Source
Reference strains of *H. pullorum*		CCUG* 33837	Broiler, Switzerland
		CCUG 33838	Human stool, Switzerland
		CCUG 33839	Human stool, Switzerland
		CCUG 33840	Laying hen, Switzerland
Field isolates of *H. pullorum* isolated from broilers in Belgium	Flock I	CE I 1	Caecal droppings
		CE I 2	Caecal droppings
		CE I 3	Caecal droppings
		CE I 4	Caecal droppings
		B I 1	Farmer's boots
	Flock II	CE II 1	Caecum
		CE II 2	Caecum
		CE II 3	Caecum
		CE II 4	Caecum
		CE II 5	Caecum
		CE II 6	Caecum
		CE II 7	Caecum
		CE II 8	Caecum
	Flock III	CE III 1	Caecum
		CE III 2	Caecum
		CE III 3	Caecum
		CE III 4	Caecum
		CE III 5	Caecum
		CE III 6	Caecum
Type strain of *H. canadensis*		CCUG 47163	Human stool, Canada

* CCUG: Culture Collection of the University of Göteborg

Agar dilution method

The Minimum Inhibitory Concentrations (MICs) of the tested antimicrobial agents were determined by the agar dilution method using BHI agar, containing doubling dilutions of the above stated antimicrobials with final concentrations ranging from 0.03 to 512 µg/ml. Inocula of the *Helicobacter* strains were prepared in phosphate-buffered saline by adjusting bacterial

suspensions directly derived from the culture plate to a density of 3 on the McFarland turbidity scale. The control strains were utilized at a density of 0.5 McFarland standard and consequently 1:10 diluted. The various strains were seeded on the antibiotic-containing plates and on antibiotic-free control plates with a Steers inoculum replicator (MAST, London, UK). Incubation followed in a microaerobic environment for three days at 37°C. The MIC was defined as the lowest concentration that almost entirely inhibited growth. The MIC tests were performed in duplicate.

RESULTS

The results of the MIC tests are summarized in Table 2. A monomodal distribution for the MICs was seen for all antimicrobial agents, except for spectinomycin having a bimodal appearance with a second peak at > 512 µg/ml, *i.e*. the MIC value for all isolates retrieved from flock II. These isolates may hence be designated as having acquired resistance to this antibiotic. Lowered susceptibility or resistance to ampicillin, ceftriaxone and sulphamethoxazole-trimethoprim of all isolates was noted. Susceptibility to lincomycin, doxycycline, gentamicin, tobramycin, erythromycin, tylosin, metronidazole, enrofloxacin and nalidixic acid may be assumed. *H. canadensis* however, was resistant to enrofloxacin and nalidixic acid.

Table 2 MIC distribution of various antimicrobials for *Helicobacter pullorum* and *Helicobacter canadensis* isolates

	Number of *H. pullorum* and *H. canadensis* strains with MIC of (μg/ml)															
Antimicrobial	≤0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512
Ampicillin											8	10*	2	2	1	1
Lincomycin								3	4	14	3*					
Doxycycline			3	7	9	4	1*									
Gentamicin			1	1	12	10*										
Spectinomycin									1	12*	1	1	1			8
Tobramycin				1	4	9	7*	3								
Ceftriaxone [a]										2*	2	5	13			
Metronidazole							3	13	7*	1						
Enrofloxacin	20	3							1*							
Erythromycin					11	12*	1									
Tylosin							3	5	7	6*	3					
Sulphamethoxazole-trimethoprim [b]													4	16*	1	2
Nalidixic acid									2	1	6	9	5			1*

[a] MIC values of two strains were not determined

[b] MIC value of one strain was not determined

* Including the *Helicobacter canadensis* strain

DISCUSSION

This study constitutes the first report of *in vitro* susceptibility testing of *H. pullorum* and *H. canadensis* strains against various antimicrobial agents. The selection of these antimicrobials was based on current recommendations of the National Committee of Clinical Laboratory Standards (M31-A2) (2002). This list was completed with other antibiotics that are fairly frequently used in human patients with gastrointestinal disease and poultry. Nalidixic acid was included in the tests to clarify the alleged susceptibility of *H. pullorum* against this antibiotic.

According to the NCCLS, the agar dilution is the method of choice for testing *H. pylori*, *Campylobacter jejuni* and related species (M31-A2) (2002) (Solnick, 2003). Since *Campylobacter* and *Helicobacter* spp. necessitate comparable incubation circumstances and time, the agar dilution method was used in the present study to determine the minimum inhibitory concentrations for *H. pullorum* and *H. canadensis*.

Hitherto, for *Helicobacter* spp., no internationally accepted criteria for susceptibility testing are available. Part of the explanation probably lies herein that the specific growth requirements and the fastidious nature of *Helicobacter* make it difficult for establishing standardized MIC determination procedures. The quality control limits given for nonfastidious bacteria in aerobic environment are not conforming (Hakanen et al., 2002). For *Campylobacter* and *Arcobacter* spp., breakpoint values for a number of antimicrobials used in the present study (ampicillin, doxycycline, gentamicin, tobramycin, ceftriaxone, sulphamethoxazole-trimethoprim, nalidixic acid) have been suggested by several authors (Van Looveren et al., 2001; ter Kuile et al., 2002; Fera et al., 2003) Based on these breakpoints, one may assume that the *H. pullorum* strains are sensitive to doxycycline, gentamicin, tobramycin, enrofloxacin and erythromycin and resistant to ampicillin, ceftriaxone and sulphamethoxazole-trimethoprim. The high MIC values for ampicillin are noteworthy since other tested *Helicobacter* species are mostly sensitive to this antimicrobial agent (Flores et al., 1985; Fox, 1997; Kiehlbauch et al., 1995; Hachem et al., 1996; Loo et al., 1997. For nalidixic acid, it is difficult to draw clear-cut conclusions about susceptibility versus resistance of the tested *H. pullorum* strains, based on the breakpoints (16-32 µg/ml) for *Campylobacter* spp. and *Arcobacter* spp. given by the authors mentioned above. Also

according to Stanley et al. (1994), a *H. pullorum* strain may be denoted as being resistant to this antimicrobial when the MIC is higher than 32 µg/ml. In the present study, the MIC values of nalidixic acid for 20 tested *H. pullorum* strains are situated at or above 32 µg/ml. This represents the normal susceptibility level of this species to nalidixic acid and does not imply these strains display acquired resistance to nalidixic acid.

A bimodal frequency distribution of MICs for spectinomycin was seen. The MIC for all isolates from flock II was higher than 512 µg/ml, while the MICs of the other tested isolates ranged from 8 to 128 µg/ml. The former isolates hence may be designated as having acquired resistance. Spectinomycin is an aminocyclitol antibiotic which binds to the 30 S ribosomal subunit and acts on the protein synthesis during the mRNA-ribosome interaction by preventing elongation of the polypeptide chain at the translocation step (Prescott, 2000). Acquired resistance to spectinomycin may develop as a result of a single step mutation in the chromosomal gene *rps*E, coding for the ribosomal small subunit protein S5 (Bilgin et al., 1990; Wilcox et al., 2001). *Escherichia coli* mutants with alterations at position 1192 in 16S rRNA, namely G/C changing to G/U base pair, have been described as well (Sigmund et al., 19984; Bilgin et al., 1990). Plasmid-mediated resistance to spectinomycin is uncommon (Prescott, 2000), although resistance due to R-plasmid specified adenylylation and phosphorylation of the antibiotic has been described in several bacteria including *Campylobacter* spp (Kawabe et al., 1987; Pinto-alphandary et al., 1990, Prescott, 2000).

The other tested antimicrobial agents showed a monomodal distribution of MICs indicating that MIC values obtained in this study reflect normal susceptibility levels of this species to these antibiotics.

In the present study the MIC of nalidixic acid for the *H. canadensis* strain was found to be higher than 512 µg/ml, that of enrofloxacin was 8 µg/ml which is remarkably higher than the MICs recorded for *H. pullorum* (≤ 0.03-0.06). This confirms the results of Fox et al. (2000) who found four *H. canadensis* isolates to be resistant to nalidixic acid in a disk diffusion test. Resistance to nalidixic acid is considered to be specific for the species *H. canadensis*.

In conclusion, notwithstanding the lack of standardized guidelines on MIC testing for the species *H. pullorum*, this study allows to assess the normal *in vitro* susceptibility of this species for several antimicrobials. Acquired resistance was only detected to spectinomycin.

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Chapter 2

Study of bacteria-host interactions

2.1 *In vitro* testing of virulence markers in *Helicobacter pullorum*

2.1.1 The cytolethal distending toxin among *Helicobacter pullorum* isolates from human and poultry origin

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Chapter 2.1

In vitro testing of virulence markers in *Helicobacter pullorum*

Chapter 2.1.1

The cytolethal distending toxin among *Helicobacter pullorum* isolates from human and poultry origin

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ABSTRACT

Helicobacter pullorum has been associated with diarrhoea, gastroenteritis and liver disease in humans and with hepatitis in poultry. The purpose of the present study was to examine whether cytolethal distending toxin was present among ten poultry and three human *Helicobacter pullorum* isolates and whether a different level of cytolethal distending toxin activity was noted. A PCR assay was performed to detect the *cdtB* gene. In addition, epithelial Hep-2 cells inoculated with sonicate from all strains were observed microscopically and DNA analysis of these cells was done by flow cytometry. All *Helicobacter pullorum* isolates harboured the *cdtB* gene, but functional cytolethal distending toxin activity was only demonstrated in the human *Helicobacter pullorum* strain CCUG 33839. A significant number of cells treated with sonicate from this strain were enlarged. The nuclei were distended proportionally. Giant cells and multinucleated cells were observed as well. In addition, stress fibers accumulated. DNA analysis by flow cytometry revealed 31 % of these cells at the S/G_2 stage of the cell cycle. The tested poultry and human *Helicobacter pullorum* isolates all possess the *cdtB* gene, but under the circumstances adopted in this study only the human strain CCUG 33839 seems to show biological activity typical for CDT *in vitro*.

INTRODUCTION

Helicobacter pullorum is an enterohepatic pathogen with the manifest ability to colonize the distal intestinal tract and liver of poultry and human beings. This species has been associated with diarrhoea in gastrointestinal patients and hepatitis in chickens (Stanley et al., 1994; Steinbrueckner et al., 1997; Atabay et al., 1998; Fox et al., 1998). Despite the increasing number of reported clinical cases involving this pathogen, hitherto, very few data are available on the pathogenesis of the infection. To our knowledge, the only two studies entering upon this research area demonstrate the chemical and biological characterization of lipopolysaccharides in *H. pullorum* (Hynes et al., 2004) and the production of the cytolethal distending toxin (CDT) by mainly human isolates (Young et al., 2000a). CDT is a toxin which originally was described by Johnson and Lior in *Escherichia coli* strains (Johnson and Lior, 1987a). Since then, CDT activity has been apprenticed in other Gram-negative bacteria as well, including *Campylobacter* spp., some *Shigella* spp., *Salmonella enterica* serovar Typhi, a number of enterohepatic *Helicobacter* spp., *Haemophilus ducreyi* and *Actinobacillus actinomycetemcomitans* (Johnson and Lior, 1987b; Johnson and Lior, 1988; Pickett et al., 1996; Okuda et al., 1997 ; Sugai et al., 1998; Cortes-Bratti et al., 1999; Chien et al., 2000; Young et al., 2000a,b; Svensson et al., 2002; Kostia et al., 2003; Taylor et al., 2003; Haghjoo and Galan, 2004). CDT is composed of three polypeptides (subunits cdtA, cdtB and cdtC) encoded by three genes designated as *cdt*A, *cdt*B and *cdt*C which all are required for the production of an active CDT (Frisk et al., 2001; Lara-Tejero and Galan, 2001). The toxin has the unique ability to stop the proliferation of various cells (De Rycke and Oswald, 2001; Frisan et al., 2002). Cells are blocked before entering into mitosis (Whitehouse et al., 1998; Elwell and Dreyfus, 2000; De Rycke and Oswald, 2001). The toxic effect is characterized by cellular enlargement and distention, resulting in cell death (Whitehouse et al., 1998; Cortes-Bratti et al. 1999). Additionally, a CDT-mediated accumulation of actin stress fibers has been described by Aragon et al. (1997).

CdtB is the biologically active subunit of the holotoxin. It is generally accepted that cdtB requires cdtA and/or cdtC to get internalized in the target cell preceding cytotoxicity (Elwell et al., 2001; Cortes-Bratti et al., 2001; Deng et al., 2001; Frisan et al., 2002; Avenaud et al., 2004; AbuOun et al., 2005) which probably occurs by endocytosis via clathrin-coated

pits (Cortes-Bratti et al., 2000). The true individual role of the cdtA and cdtC units however, has not been elucidated yet (Cortes-Bratti et al., 2001; De Rycke and Oswald, 2001; Lee et al., 2003; Avenaud et al., 2004).

In the present study, a PCR test was used to investigate to what extent the *cdtB* gene was present among a collection of *H. pullorum* isolates from poultry and human origin. Secondly, it was meant to determine whether a different CDT-like activity in between these isolates was noticeable. The latter information was gathered by inoculating Hep-2 cells with filtered bacterial sonicate and using flow cytometry, haemacolor and phalloidin staining as assessment techniques.

MATERIALS AND METHODS

Bacterial strains

The *H. pullorum* strains from poultry and human origin used in this study are listed in Table 1. *Campylobacter jejuni* NCTC 11168 which has been proven to harbour the *cdt* genes and produce CDT (Purdy et al., 2000), was included as a positive control. *Helicobacter canadensis* CCUG 47163 (Fox et al., 2000) was adopted as a negative control. Bacterial strains were stored at -70°C in a medium consisting of 7.5 g glucose, 25 ml brain heart infusion (BHI) broth (Oxoid, Basingstoke, England) and 75 ml sterile inactivated horse serum. The bacterial strains were inoculated on BHI agar, supplemented with 10 % horse blood and Vitox (Oxoid) and incubated for 72 hours under microaerobic (5 % H_2, 5 % CO_2, 5 % O_2 and 85 % N_2) conditions at 37°C.

Detection of cdtB gene using PCR

DNA from agar-grown cultures of *H. pullorum*, *C. jejuni* and *H. canadensis* was extracted with a commercial kit (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany) according to the recommendations of the manufacturer. DNA from *C. jejuni, C. coli, C. lari*, *H. bilis*, *H. canis* and *H. hepaticus* served as positive control whereas DNA from *H. canadensis*, *H. pylori* and *H. fennelliae* was included as negative control (Pickett et al., 1996; Young et al., 2000a,b; Chien et al., 2000; Fox et al., 2000). A PCR assay amplifying a 714 bp fragment of the *cdtB* gene was used (Chien et al., 2000). For this purpose, degenerate primers VAT2 (forward,

GTNGCNACBTGGAAYCTNCARGG) and DHF1 (reverse, DACNGGRAARTGRTC), which are based on the amino acid sequence of the cdtB subunit, were synthesized (Invitrogen Life Technologies, Merelbeke, Belgium) and applied in the PCR assay. Per sample, 2 µl of the prepared DNA was added to 8 µl of the PCR mixture, containing 0.04 U/µl Taq polymerase platinum (Invitrogen Life Technologies), 10 x PCR Buffer (Invitrogen Life Technologies), 1.5 mM $MgCl_2$ (Invitrogen Life Technologies), 200 µM of each deoxynucleoside triphosphate (Amersham Pharmacia Biotech, Puurs, Belgium), a final primer concentration of 0.8 µM and sterile distilled water. The used conditions for the amplifications were the following: an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 42°C for 2 min and elongation at 72°C for 3 min, and a final elongation at 72°C for 8 min.

Amplified fragments (5 µl aliquots) were separated by electrophoresis in an agarose gel containing 1.5 % Multi Purpose agarose (Boehringer, Mannheim, Germany) in 1 x TAE buffer, pH 8 and were stained with ethidium bromide. The gels were visualised using the Image Master® VDS (Pharmacia, Biotech).

Analysis of nucleotide sequences

The *cdtB* PCR products were purified with the Qiaquick PCR purification kit (Qiagen) and sequenced using the primers VAT2 and DHF1 with the BigDye Terminator cycle sequencing kit (Applied Biosystems, Lennik, Belgium). Sequencing products were run on the ABI prism™ 3100 Genetic Analyzer (Applied Biosystems), using 50 cm capillaries filled with Performance-Optimized-Polymer 6. The electropherograms were exported and converted to the Kodon software package (Applied Maths, Sint-Martens Latem, Belgium) and compared to known *cdtB* sequences in the databases using multiple sequence alignment. The accession numbers for the nucleotide sequences of the *cdtB* gene obtained in this study are AF123536 and AF220065.

***In vitro* Hep-2 cell line assay**

Preparation of bacterial cell sonicates

Bacterial cultures grown for 72 h on BHI agar blood plates were harvested by washing the plates with phosphate-buffered saline (PBS) followed by centrifugation (4000 x *g*, 20 min,

4°C) and resuspending the pellet in 1.5 ml PBS. The bacteria were disrupted by eight 30-s pulses on ice with the Sonicator, ultrasonic processor, XL 2015 (MISONIX, Farmingdale, NY), a process followed by centrifugation for 10 min (4000 x *g*, 4°C). Then, the sonicated lysate was filtered through a 0.45 µm and subsequently 0.20 µm pore size filter (IWAKI, International Medical, Brussels, Belgium). The protein concentration was determined using the Bio-Rad (Hercules, CA, U.S.A.) protein assay method with bovine serum albumin (Sigma, St. Louis, MO, USA) as a standard and stored at -70°C. Before using them in experimental assays, all preparations were thawed and diluted to 750 µg/ml total protein.

Cell line

The cultured Hep-2 cell line was obtained from the European Collection of Cell Cultures (ECACC). Cells were grown in Eagle's Minimum Essential Medium (EMEM) (Gibco, Invitrogen, Merelbeke, Belgium) containing 10 % fetal calf serum, 1 % glutamine and 1 % non essential amino acids.

Morphological changes in cultured cells

Hep-2 cells were seeded onto 13-mm-circular glass slides (VWR, Leuven, Belgium) in a 24-well plate at a concentration of 1 x 10^4cells/ml and were incubated at 37°C in 5 % CO_2 for 3 h. Following, 10 µl of the filtered bacterial sonicate (non-diluted and two fold serial dilutions ranging from 1:2 till 1:16) was added. PBS was likewise included as a negative control. After an incubation time of 72 h at 37°C in 5 % CO_2, cells were washed three times with PBS and then stained with haemacolor stain. Glass slides were mounted with coverslips and observed microscopically in at least three time-independent assays. Attention was especially paid to cellular and nuclear distention.

Flow cytometry

Flow cytometry assay of the DNA content of the cultured cells was performed as described by Young et al. (2000b). Briefly, cells were seeded in 25-square-centimeter tissue culture flasks at a concentration of 1×10^5 cells/ml and subsequently inoculated with 100 µl filtered bacterial sonicate (non-diluted and two fold serial dilutions ranging from 1:2 till 1:16). PBS was likewise included as a negative control. Following incubation for 72 h at 37°C in 5 %

CO_2, the cells were removed by trypsinization and transferred to a falcon tube. After centrifugation, the pellet was resuspended in a solution containing Triton X-100 and kept at 37°C for 20 min. Subsequently, a staining solution comprising propidium iodide was added. Cells were incubated at 4°C for 3 h before DNA content was analyzed on a FACScalibur flow cytometer (Beckton-Dickenson, San Jose, California, USA). Data acquisition was performed on 1 x 10^4 cells for each experiment using Cell Quest software.

Staining of F-actin with phalloidin-Texas Red X

Hep-2 cells were seeded onto 13-mm-coverslips in a 24-well plate at a concentration of 2 x 10^4 cells/ml. After 3 h, cell cultures were inoculated with 20 µl of non-diluted filtered sonicate of *C. jejuni, H. canadensis* and *H. pullorum* CCUG 33839 and incubated for 72 h at 37°C in 5 % CO_2. PBS served as negative control. Thereafter, they were gently washed twice with 0.5 ml PBS+ (PBS containing Ca^{2+} and Mg^{2+}) at 37°C, fixed with 0.5 ml 3 % paraformaldehyde for 10 min at room temperature, gently washed again with 0.5 ml PBS+ and then permeabilized with 0.5 ml 0.1 % Triton X-100 in PBS+ for 2 min at room temperature. Following washing with PBS+, 0.25 ml phalloidin-Texas Red X (Molecular Probes, Eugene, Oregon, USA) (1:100 in PBS+) was added to each well and the 24-well plate was incubated at 37°C for 1 h. To remove unbound phalloidin, coverslips were gently washed twice with 0.5 ml PBS+ before visualization of the actin filaments with a Leica TCS SP2 laser scanning spectral confocal system (Leica Microsystems GmbH, Heidelberg, Germany).

RESULTS

Detection of cdtB gene using PCR

PCR amplification with the primer pair VAT2 and DHF1 resulted in a 714 bp fragment for all *H. pullorum* strains, indicating all tested *H. pullorum* strains possess the *cdtB* gene. *C. jejuni, H. bilis* and *H. canis* likewise harboured the *cdtB* gene. *H. canadensis, H. pylori* and *H. fennelliae* were negative in the PCR.

Analysis of nucleotide sequences

The sequences of the amplified 714 bp fragment of the *cdtB* gene of the *H. pullorum* isolates revealed a similarity of 98 to 99 % to those from Genbank.

Production of CDT

Morphological changes in cultured Hep-2 cells

C. jejuni caused marked morphological alterations given that a majority of Hep-2 cells were detached from the bottom of the wells and were enlarged with a rounded appearance. The nucleus had a proportionally increased size. Several Hep-2 cells revealed two nuclei. Giant cells and multi-nucleated cells were observed as well, although the latter was not a frequently observed phenomenon (Figure 1). These changes were noticeable up until the last dilution of the bacterial sonicate (1:16).

Concerning the *H. pullorum* strains, the non-diluted sonicate from the *H. pullorum* strain CCUG 33839 elicited the same morphological changes as *C. jejuni*, though to a lesser extent (Figure 1). Hep-2 cells inoculated with sonicate derived from the other *H. pullorum* strains showed no difference to the control cells treated with PBS or sonicate from *H. canadensis*.

Table 1 Bacterial strains used in this study and results of flow cytometry assay using Hep-2 cells treated with non-diluted filtered sonicate of 13 *Helicobacter pullorum* isolates, *Campylobacter jejuni* NCTC 11168 and *Helicobacter canadensis* CCUG 47163

Strain	Source	Mean percentage of cells in S/G_2 phase ± SD*
H. canadensis CCUG**47163	Patient suffering from diarrhoea, faeces, U.K.	6.0 ± 0.6
C. jejuni NCTC *** 11168	Patient suffering from diarrhoea, faeces, Canada	70.6 ± 9.5
H. pullorum CCUG 33837	Broiler at slaughter, Switzerland	6.5 ± 3.4
H. pullorum CCUG 33838	Patient suffering from gastroenteritis and hepatitis, faeces, Switzerland	6.2 ± 1.9
H. pullorum CCUG 33839	Human faeces, Switzerland	31.0 ± 6.1
H. pullorum CCUG 33840	Laying hen with hepatitis, Switzerland	7.2 ± 1.6
H. pullorum CE I 1	Caecal drops, flock I	9.7 ± 2.7
H. pullorum CE I 2	Caecal drops, flock I	7.9 ± 2.5
H. pullorum CE I 3	Caecal drops, flock I	6.8 ± 1.1
H. pullorum B I 1	Farmer's boots, flock I	8.2 ± 3.2
H. pullorum CE II 1	Caecum, flock II	7.4 ± 0.9
H. pullorum CE II 2	Caecum, flock II	8.3 ± 1.0
H. pullorum CE III 1	Caecum, flock III	6.1 ± 1.9
H. pullorum CE III 2	Caecum, flock III	6.7 ± 0.3
H. pullorum G 214	Patient suffering from diarrhoea, faeces, Belgium	8.9 ± 0.7
	PBS	3.8 ± 0.8

* SD: standard deviation, **CCUG: Culture Collection of the University of Göteborg, *** NCTC: National Collection of Type Cultures

Table 2 DNA content analyses of Hep-2 cells treated with two-fold serial dilutions of filtered sonicate of *Helicobacter pullorum* CCUG 33839 and *Campylobacter jejuni* NCTC 11168

Strain	Dilution	Percentage of cells in S/G_2 phase
	1:2	81.1
C. jejuni NCTC*	1:4	78.9
11168	1:8	59.7
	1:16	51.9
	1:2	17.6
H. pullorum	1:4	8.7
CCUG** 33839	1:8	3.8
	1:16	3.0

*NCTC: National Collection of Type Cultures, **CCUG: Culture Collection of the University of Göteborg

Flow cytometry

The results are shown in Tables 1 and 2. CDT of the *C. jejuni* strain caused an arrest in the S/G_2 phase in 70.6 % of the inoculated Hep-2 cells. Only 3.8 % and 6.0 % of the Hep-2 cells inoculated with PBS or sonicate from *H. canadensis* respectively, were at the S/G_2 stage (Figure 2). From the 13 tested *H. pullorum* sonicates, only CDT of the human *H. pullorum* strain CCUG 33839 notably haltered cell division, resulting in 31.0 % of Hep-2 cells being in the S/G_2 phase when non-diluted filtered sonicate was added (Figure 2). The number of cultured cells to be found in the S/G_2 phase diminished in parallel with the two-fold dilutions of the added sonicate up to a 1:4 dilution at which an effect was no longer apprenticed (Table 2). The portion of Hep-2 cells situated in the S/G_2 phase following inoculation with sonicate from the other *H. pullorum* strains was merely slightly above that of Hep-2 cells treated with sonicate from *H. canadensis* (Table 1).

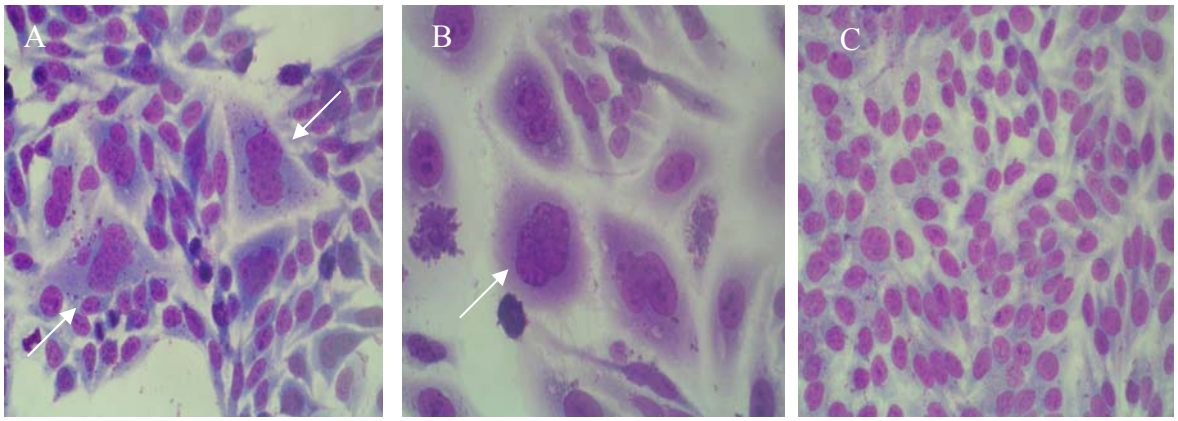

Figure 1 Hep-2 cells exposed to non-diluted filtered cell sonicates of *Helicobacter pullorum* CCUG 33839 (A), *Campylobacter jejuni* NCTC 11168 (positive control) (B) and *Helicobacter canadensis* CCUG 47163 (negative control) (C) for 72 hours (Haemacolor, 400 x). The arrows demonstrate enlarged multinucleated cells

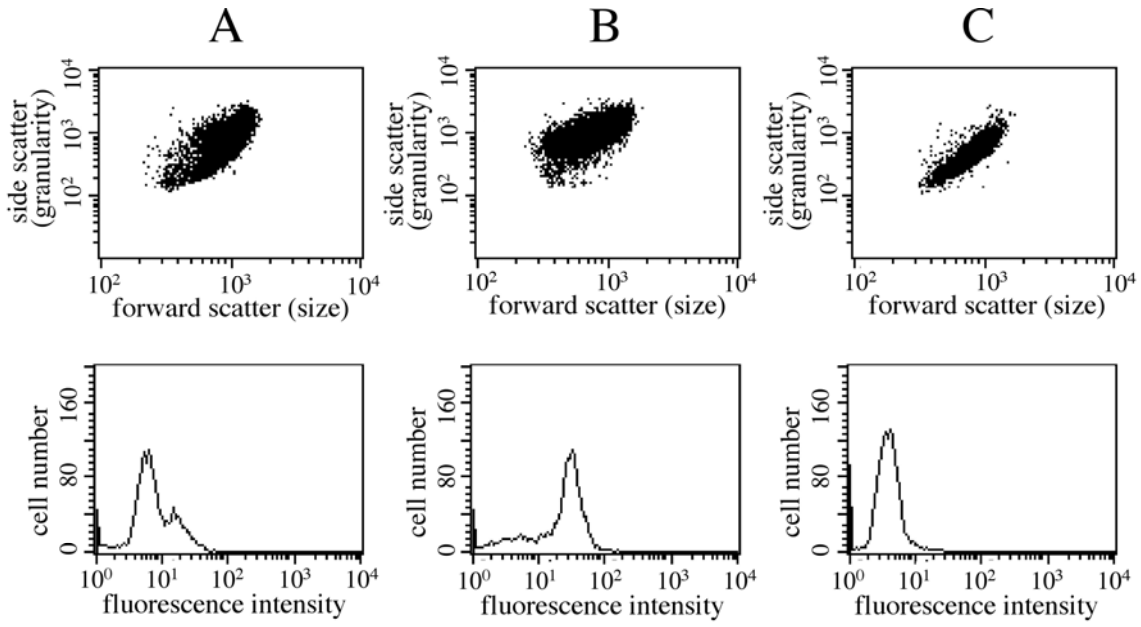

Figure 2 Analysis of DNA content by flow cytometry revealed an accumulation of cells in the S/G_2 phase treated with non-diluted cell sonicate originating from *Helicobacter pullorum* CCUG 33839 (A), *Campylobacter jejuni* NCTC 11168 (B), specifically 70.6 % and 31.0 % respectively. Only 6.0 % of the cells treated with non-diluted filtered sonicate from *Helicobacter canadensis* (C) was situated at the S/G_2 stage

Staining of F-actin with phalloidin-Texas Red X

An accumulation of stress fibers was observed in Hep-2 cells treated with sonicate originating from *C. jejuni*, and to a lesser extent in cells inoculated with sonicate from *H. pullorum* CCUG 33839. Of the Hep-2 cells brought in contact with sonicate from *H. canadensis* or PBS, only a few showed accumulations of actin stress fibers (Figure 3).

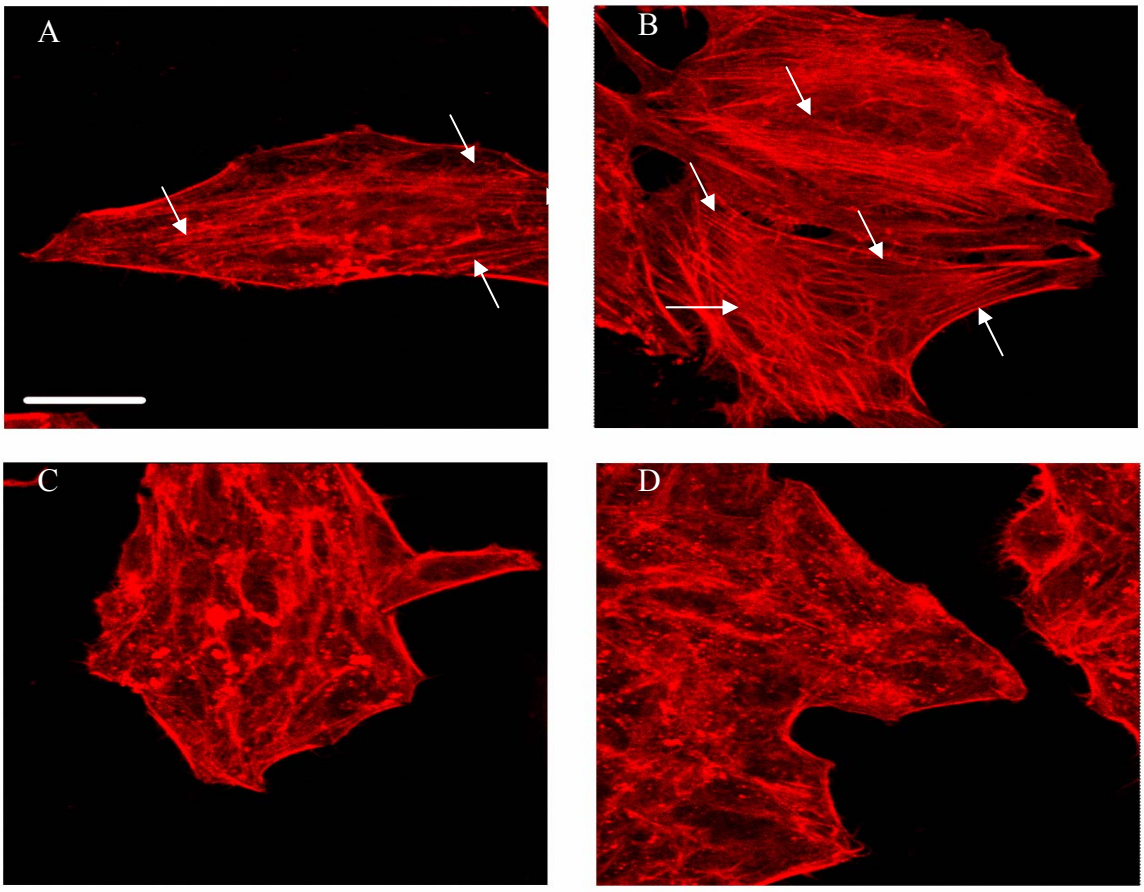

Figure 3 Confocal microscopy changes of actin filaments in Hep-2 cells treated with non-diluted cell sonicate from *Helicobacter pullorum* CCUG 33839 (A), *Campylobacter jejuni* NCTC 11168 (B), *Helicobacter canadensis* CCUG 47163 (C) and PBS (D). The arrows demonstrate an accumulation of F-actin stress fibers (bar = 10 µm)

DISCUSSION

This study is the first to report the testing of human and poultry *H. pullorum* strains for the presence of the *cdtB* gene and the production of CDT using three techniques. Haemacolor staining and flow cytometry were used to detect the antiproliferative effect of CDT by demonstrating cellular and nuclear enlargement and analyzing DNA content, respectively. As an additional phenotypic criterium for assessing CDT production, the appearance of stress fibers was investigated. Frisan et al. (2003) demonstrated a rearrangement of the actin cytoskeleton upon CDT intoxication which appears to be RhoA GTPase mediated and which is part of the 'Ataxia telangiectasia mutated' (ATM)-dependent response to DNA damage. The small GTPases of the RhoA protein family are involved in the formation of stress fibers, focal adhesions (Hall, 1998) and cell proliferation (Olson et al., 1995). Frisan et al. (2003) suggest a RhoA GTPase dependent linkage between DNA damage, which may be induced by CDT, and alterations in the actin cytoskeleton, possibly needed to prolong cell survival (Frisan et al., 2003).

The results of the present study exhibit that all tested *H. pullorum* isolates harbour the *cdtB* gene, but that CDT activity was only substantially present in the human *H. pullorum* strain CCUG 33839. The other *H. pullorum* strains did not show significant CDT activity despite harbouring the cdtB subunit encoding *cdtB* gene as demonstrated by PCR and sequencing. CdtB is considered to be the biologically active subunit of the holotoxin. Variation in posttranslational modification of this subunit between the *H. pullorum* strain CCUG 33839 and the other *H. pullorum* strains might result in differences in CDT activity between these strains.

Since *cdtA* and *cdtC* genes are also required for activity of the holotoxin (Elwell and Dreyfus, 2000; Frisk et al., 2001; Lara-Tejero and Galan, 2001), variation in these genes may result in differences in CDT activity. Abuoun et al. (2005) suggested that a point mutation at codon 167 in the *cdtC* gene could have consequences for the activity of the holotoxin. In a study of Bang et al. (2003), the *cdtB* gene was present in all tested *C. coli* and *C. jejuni* strains, whereas *cdtA* and *cdtC* were only detected in 95 % of the *C. coli* strains and 90 % of the *C. jejuni* strains. Isolates missing one of the latter genes demonstrated very low or no CDT activity. In none the *H. pullorum* strains used in the present studies, *cdtA* and *cdtC* genes were

demonstrated by PCR although several primers described in literature for the amplification of these genes in *H. hepaticus* and *Campylobacter* spp. (Eyigor et al., 1999a; Bang et al., 2003) or developed on the basis of a consensus of the gene sequences of *H. hepaticus* and *C. jejuni* using KODON (Applied Maths) were used (data not shown). This might indicate that these genes are absent in our strains. It may, however, also be due to variation in the degree of similarity of the *cdtA* and *cdtC* genes between and within different bacterial species (Scott and Kaper, 1994; Pickett et al., 1996; Pickett and Whitehouse, 1999; Cortes-Bratti et al., 2001; Frisan et al., 2002; Lee et al., 2003), resulting in non-annealing of the primers.
Polymorphisms in the *cdtABC* flanking regions might be another reason for variation in CDT activity among the tested strains, although they all possessed the *cdtB* gene. Yamano et al. (2003) suggested that the *cdtABC* flanking region in *A. actinomycetemcomitans* is very polymorphic and may explain the variability in CDT titers among several strains. They suggested that the DNA area upstream of *orf1* in *A. actinomycetemcomitans* is important for expression of the entire gene.

In the present study, CDT activity was tested in Hep-2 cells. Although these cells have been used to demonstrate CDT activity in several bacterial species (Johnson and Lior, 1988; Purven and Lagergard, 1992; Scott and Kaper, 1994; Pickett and Whitehouse, 1999), it can not be excluded that they are not very sensitive to CDT produced by *H. pullorum* strains. The degree of similarity between the amino acid sequences of cdtA and cdtC subunits, which most probably are involved in attachment of the toxin to the host-cell surface and in internalization of cdtB, may vary tremendously between and within different bacterial species (Scott and Kaper, 1994; Pickett et al., 1996, 1999; Cortes-Bratti et al., 2001; Frisan et al., 2002; Lee et al., 2003). This may result in variation in sensitivity of host cells for CDT produced by different bacterial species or possibly strains. Eyigor et al. (1999b) hypothesized that low CDT activity of *C. coli* isolates might be due to low sensitivity of the HeLa cells used in their study.

In conclusion, the tested poultry and human *H. pullorum* isolates all possess the *cdtB* gene, but under the circumstances used in this study only the human *H. pullorum* strain CCUG 33839 seems to show biological activity typical for CDT *in vitro*. The existence of other virulence markers including the production of other toxins and the phenomenon of

adhesion and colonization capacity need to be examined to unlock more of the secrets of the lifestyle of this emerging pathogen.

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Chapter 2.1.2

Cell death by mitotic catastrophe in mouse liver cells caused by *Helicobacter pullorum*

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ABSTRACT

Helicobacter pullorum is a member of the enterohepatic *Helicobacter* species group. *Helicobacter pullorum* has the ability to produce cytolethal distending toxin which induces cell cycle arrest in a number of cell types resulting in cellular distention and eventually cell death.

The mouse hepatic cell line H 2.35 was exposed to filtered cell sonicate from 11 poultry and three human *Helicobacter pullorum* isolates. Morphological changes were investigated using light microscopy, transmission electron microscopy and time-lapse video microscopy. Additionally, DNA analysis of treated cells was done by flow cytometry.

Cytotoxicity was seen for all *Helicobacter pullorum* isolates after incubation for 72 hours with different levels of toxic activity. Features characteristic for mitotic catastrophe were noticed involving chromatin condensation, formation of multinuclear distended cells and micronucleation. In addition, intranuclear pseudoinclusions were observed in sonicate treated cells. Finally, cells exposed to sonicate eventually underwent cell death with the morphological features of necrosis. The toxic factor proved to be soluble, trypsin–sensitive and stable at 56°C and at -70°C. The molecular weight was estimated to be over 50 kDa.

These characteristics are equal to those of cytolethal distending toxin indicating that this toxin is involved in the morphological cell changes observed in the mouse liver cell line.

INTRODUCTION

Helicobacter pullorum is a member of the enterohepatic *Helicobacter* species (EHS) group (Fox, 1997). This species may infect the intestinal tract of poultry and human beings. It has also been detected in the liver of laying hens and biliary tree and gallbladder of humans (Stanley et al., 1994; Fox et al., 1998). *H. pullorum* has been associated with vibrionic hepatitis in poultry and with diarrhoea, gastroenteritis and hepatobiliary disease in human beings (Stanley et al., 1994; Steinbrueckner et al., 1997; Atabay et al., 1998; Fox et al., 1998; Ceelen et al., 2005) as reflected by liver swelling, an increase of liver enzymes and gallbladder cancer (Fox et al., 1998). Despite the awareness of the potential hazard of *H. pullorum* for human health, very few studies on the pathogenicity of this species have been reported. Hynes et al. (2004) characterized the lipopolysaccharides in *H. pullorum* chemically and biologically. Young and co-researchers (2000a) and our research group (Ceelen et al., 2006a) showed that several *H. pullorum* strains produce the cytolethal distending toxin (CDT) which induces cell cycle arrest in a number of cell types resulting in cellular distention and eventually cell death. On molecular level, only the *cdtB* gene encoding the active compound of the toxin has been sequenced (Young et al., 2000; Ceelen et al., 2006). Hitherto, no other potential virulence markers have been studied in *H. pullorum*.

Taylor et al.(1995) showed that another EHS, *H. hepaticus*, produces a toxic factor causing a cytopathic effect (CPE) characterized microscopically by granule formation in a mouse liver cell line (CCL 9.1). This toxin was called granulating cytotoxin (GCT) referring to the induced morphological cell changes. Despite the innovative and interesting character of this finding, no further research involving this toxin was performed for almost a decade. Only recently did Young et al. (2004) hypothesize that CPE induced by GCT could in fact be CDT mediated.

In the present study it was light microscopically examined whether and to what extent *H. pullorum* isolates from poultry and human origin cause CPE in mouse liver cells. The CPE was additionally scrutinized using transmission electron microscopy and time-lapse video microscopy. DNA content analysis of treated cells was performed using flow cytometry. Finally, the nature and molecular weight of the toxic factor were partially determined.

METHODS

Cytotoxic assay

Bacterial strains

The 14 *H. pullorum* strains from poultry and human origin used in this study are listed in Table 1. *H. hepaticus* ATCC 51448, *H. canadensis* CCUG 47163 and *Campylobacter jejuni* NCTC 11168 were included as well. Bacterial strains were stored at -70 °C in a medium consisting of 7.5 g glucose, 25 ml brain heart infusion (BHI) broth (Oxoid, Basingstoke, England) and 75 ml sterile inactivated horse serum. The bacterial strains were inoculated on BHI agar supplemented with 10 % horse blood and Vitox (Oxoid) (BHI agar blood plates) and incubated for 72 hours under microaerobic conditions at 37°C. This microaerobic environment was maintained in vented jars (Led Techno, Heusden-Zolder, Belgium) which were evacuated to -20 mm Hg and then equilibrated with a gas mixture consisting of 5 % H_2, 5 % CO_2, 5 % O_2 and 85 % N_2.

Preparation of bacterial cell sonicates

Bacterial cultures grown for 72 h on BHI agar blood plates were harvested by washing the cultures with phosphate-buffered saline (PBS) followed by centrifugation (1500 x *g*, 20 min, 4°C) and resuspending the pellet in 1.5 ml PBS. The bacteria were disrupted by eight 30-s pulses on ice with a sonicator, ultrasonic processor, XL 2015 (MISONIX, Farmingdale, NY), a process followed by centrifugation for 10 min (1500 x *g*, 4°C). Then, the sonicate was filtered through a 0.45 µm and subsequently 0.20 µm pore size filter (IWAKI, International Medical, Brussels, Belgium). The protein concentration was determined using the Bio-Rad (Hercules, CA, U.S.A.) protein assay method with bovine serum albumin (Sigma, St. Louis, MO, USA) dissolved in PBS as a standard. The sonicate was stored at -70°C. All preparations were thawed and diluted to 750 µg total protein/ml before using them in experimental assays.

Cell line

The H 2.35 mouse hepatocyte cell line was obtained from the European Collection of Cell Cultures (ECACC). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen, Merelbeke, Belgium) according to the manufacturer's recommendations.

Table 1 Bacterial strains used in this study

		Strain number	Source
Reference strains of *H. pullorum*		CCUG* 33837	Broiler at slaughter, Switzerland
		CCUG 33838	Patient suffering from gastroenteritis and hepatitis, faeces, Switzerland
		CCUG 33839	Human faeces, Switzerland
		CCUG 33840	Laying hen with hepatitis, Switzerland
Field isolates of *H. pullorum* isolated from broilers in Belgium	Flock I	CE I 1	Caecal droppings
		CE I 2	Caecal droppings
		CE I 3	Caecal droppings
		CE I 4	Caecal droppings
		B I 1	Farmer's boots
	Flock II	CE II 1	Caecum
		CE II 2	Caecum
	Flock III	CE III 1	Caecum
		CE III 2	Caecum
Human strain of *H. pullorum*		G 214	Patient suffering from diarrhoea, faeces, Belgium
C. jejuni		NCTC** 11168	Patient suffering from diarrhoea, faeces, U.K.
Type strain of *H. hepaticus*		ATCC*** 51448	Liver, mouse
Type strain of *H. canadensis*		CCUG 47163	Patient suffering from diarrhoea, faeces, Canada

*CCUG: Culture Collection of the University of Göteborg, **NCTC: National Collection of Type Cultures, ***ATCC: American Type Culture Collection

Cytotoxicity assay

H 2.35 cells were seeded in 24-well plates or in 25-square-centimeter tissue culture flasks at a concentration of 1 x 10^5cells/ml and were incubated at 37°C in 5 % CO_2 for 3 h. Thereafter, 100 µl of bacterial sonicate (hence containing 75 µg of total protein) was added. PBS was likewise included as a negative control.

The 24-well plate cell cultures were used for light microscopical and transmission electron microscopical examination as well as for time lapse video microscopy analysis. The tissue culture flask cell cultures were used for flow cytometry.

Light microscopy

The inoculated and control cells were further incubated at 37°C in 5 % CO_2 and examined for cytotoxic effect by means of light microscopy every 24 h till 96 h post-inoculation in at least three time-independent assays.

Flow cytometry

Following incubation of the inoculated and control cells flasks for 72 h, the cells were removed by trypsinization and transferred to a falcon tube. After centrifugation, the pellet was resuspended in a solution containing Triton X-100 and kept at 37°C for 20 min. Subsequently, a staining solution comprising propidium iodide was added. Cells were incubated at 4°C for three hours before DNA content was analyzed on a FACScalibur flow cytometer (Beckton-Dickinson, San Jose, California, USA). Data acquisition was performed on 1 x 10^4 cells for each experiment using Cell Quest software. DNA content analyses were performed in three time-independent assays.

Transmission electron microscopy

Inoculated and control cells were fixed in glutaraldehyde 2 % buffered with 0.1 M Na-cacodylate (pH 7.4) after 48 h incubation. Following post-fixation in 1 % osmiumtetroxide overnight, cultures were dehydrated in a graded series of ethanol (70 %, 85 %, 95 %, 100 %, 10 min each) and embedded in LX medium. Semithin sections of 2 μm were stained with Toluidine blue and examined using a Leitz Aristoplan light microscope equipped with a Leitz orthomat E photocamera. Ultrathin sections of 60 nm were cut with a diamond knife on a Reichert Jung Ultracut U microtome (Jung, Vienna, Austria), mounted on formvar-coated copper grids and stained with uranyl acetate and lead citrate. Samples were viewed with a Jeol EXII transmission electron microscope at 80 kV. In this assay, only *H. pullorum* CCUG 33839 sonicate was included.

Time-lapse video microscopy

Following incubation of the inoculated and control cell cultures during 48 h, cells were placed on an invert microscope (Fluovert Leitz, Wetzlar, BRD, Germany) in a temperature-controlled space at 37°C for an additional 24 h. The cells were viewed with a 40 x objective lens and images were collected via a camera C-1966-01 (Hamamatsu Phototonics, Mamamatsu-City, Japan) which was connected to a U-matic video recorder VO-5850P (Sony, Tokyo, Japan). One image was recorded every 20 sec via an interval timer (VEL, Leuven, Belgium). Data were registered on a videotape using a video typewriter VTW-210 (FOR.A, Japan) and images were played at a rate of 25 images per second which is analogous to an acceleration of 500 x. Special attention was paid to cellular alterations as well as cell movements. As in the previous assay only *H. pullorum* CCUG 33839 sonicate was included.

Partial characterisation of the toxic factor

Pretreatment of bacterial sonicate with trypsin, heat and cold

When CPE was detected, the CPE inducing sonicate was subjected to the following treatments: (1) 100 µl of sonicate was incubated for 30 min at 37°C with 10 µl 2.5 % trypsin (Sigma). Thereafter, the activity of trypsin was stopped by adding soybean trypsin inhibitor (Type II-S: Sigma; 10 mg of soybean was added to 2.5 % trypsin). Finally, the treated bacterial sonicate and 1 % penicillin-streptomycin were added to the cultured cells as described above. Cells inoculated with non-treated bacterial sonicate served as positive control. Negative controls included PBS, trypsin, soybean trypsin inhibitor and 1 % penicillin-streptomycin; (2) bacterial sonicate was incubated at 56°C, 70°C and 100°C for 30 min, 15 min and 10 min, respectively. Thereafter, the heated bacterial sonicate was added to the cultured cells. Non-heated sonicate and PBS were included as positive and negative controls, respectively; (3) bacterial sonicate was kept at -70°C for six months. Following thawing of the aliquots, the cold treated bacterial sonicate was added to the cultured cells. Non-long-term-frozen sonicate and PBS were included as positive and negative controls, respectively. All pretreatments of bacterial sonicate were carried out in three time-independent assays. Cell cultures inoculated with these sonicates were examined light microscopically, as described above.

Molecular weight estimation

The bacterial sonicates of *H. pullorum* CCUG 33839, I CE 2 and I B 1 were subjected to ultrafiltration using Amicon Ultra-15 centrifugal filter devices (Millipore, Bedford, U.S.A) with a molecular weight cut off (MWCO) of 30 and 50 kDa. Cells were inoculated with 100 µl of the retentate or permeate fractions and examined light microscopically, as described above. Non-filtered sonicate and PBS were likewise included. Molecular weight estimation was performed in at least three time-independent assays.

RESULTS

Cytotoxic assay

Light microscopy

No CPE was detected in the negative control (Figure 1D) and in the cells inoculated with *H. canadensis* sonicate. Sonicates of all *H. pullorum* isolates induced CPE, albeit with varying degrees. Concerning the level of induced CPE, the tested strains can indeed be subdivided into three groups (Table 2). A first group of *H. pullorum* isolates as well as the *H. hepaticus* and *C. jejuni* strains induced a strong cytopathic effect, characterized by enlarged multinucleated cells. Cells decreased in number finally undergoing cell death by necrosis after 72-96 h (Figure 1, 2). *H. hepaticus* sonicate treated cells, however, already became necrotic after 36-48 h. A second category encomprised *H. pullorum* strains which induced a moderate CPE on the cultured mouse cells, which were enlarged and possessed two or more nuclei. Granulated rounded and detached necrotic cells were detected as well, but to a lesser extent than when cells were treated with sonicate of strains classified in the previous group. A third category was typified by minor CPE. The cells were somewhat distended and cell death was seldomly seen. In this third category, not all cells in a well were affected.

Flow cytometry

Analysis of DNA content revealed that sonicate from *H. pullorum* caused an increase of the DNA amount in treated H 2.35 cells. With regard to the latter, strains could be divided into three groups, confirming the division of strains based on light microscopical analysis (Table 2).

Table 2 Results of light microscopy and flow cytometry of H 2.35 cell cultures inoculated with sonicate of different *Helicobacter pullorum* (Hp) isolates, *Helicobacter hepaticus* (Hh) ATCC 51448, *Helicobacter canadensis* (Hc) CCUG 47163 and *Campylobacter jejuni* (Cj) NCTC 11168

Isolate number	**Light microscopy (degree of CPE*)**	**Flow cytometry (median fluorescence intensity) ± SD°**
Hp CCUG 33839	+++	1022 ± 13.28
Hp G 214	+++	939 ± 45.30
Hp I CE 1	+++	956 ± 0.58
Hp I CE 2	+++	956 ± 0.58
Hp I B 1	+++	944 ± 16.74
Hp II CE 1	+++	924 ± 45.35
Hp II CE 2	+++	964 ± 0.58
Hp III CE 2	+++	885 ± 137.38
Hp CCUG 33837	+++	905 ± 84.70
Hh ATCC 51448	+++	NT**
Cj NCTC 11168	+++	805 ± 94.51
Hp I CE 4	++	659 ± 42.44
Hp III CE 1	++	669 ± 28.04
Hp CCUG 33838	++	679 ± 84.82
Hp I CE 3	+	423 ± 20.05
Hp CCUG 33840	+	463 ± 4.24
Hc CCUG 47163	-	375 ± 12.63
PBS	-	290 ± 36.98

*CPE: cytopathic effect (+++: strong, ++: moderate, +: weak, -: absent), **NT: not tested, °SD: standard deviation

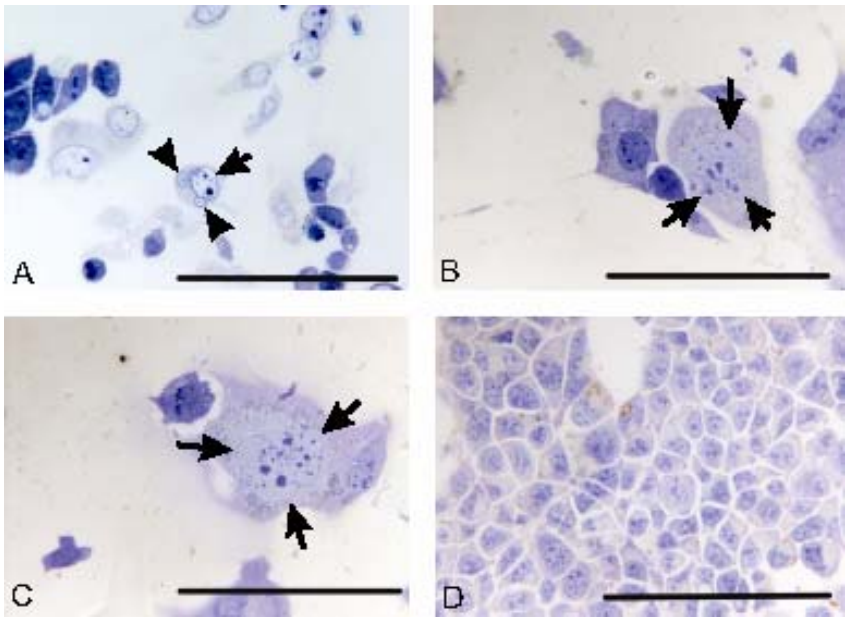

Figure 1 Cytopathic effect on H 2.35 cells 48 h following exposure to filtered *Helicobacter pullorum* sonicate (strain CCUG 33839). Cells are enlarged: a cell dying by necrosis possessing one nucleus and two micronuclei is arrowed (A), a sonicate treated multinucleated (arrows) cell (B), a sonicate treated cell possessing a multilobular nucleus (arrows) (C). Normal confluent control H 2.35 cells after inoculation with PBS showing a homogenous diameter and polygonal outline (D) (H&E) (Bar, 100 μm)

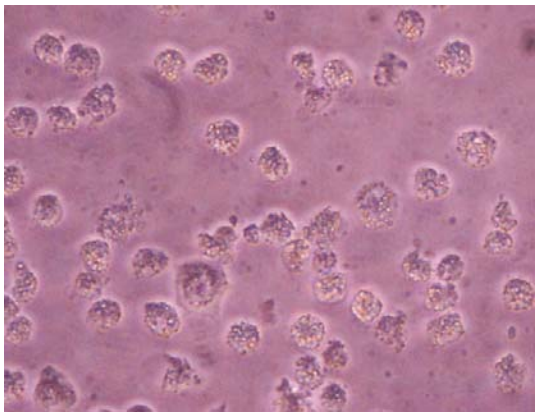

Figure 2 H 2.35 cells 96 h following inoculation with filtered *Helicobacter pullorum* sonicate (strain CCUG 33839) showing cell death by necrosis (phase contrast microscopy) (40 x)

Transmission electron microscopy

In the confluent control cultures, most cells showed a homogenous diameter and had a polygonal outline. In cell cultures exposed to the *H. pullorum* CCUG 33839 sonicate for 48 h, characteristic ultrastructural changes were observed. The most prominent changes were noticed in the nuclear compartment and consisted of multinucleation with either the presence of several nuclei of normal dimension or the formation of micronuclei (Figure 3C). Besides the number of nuclei, a change in chromatin configuration with distinct chromatin condensation was observed. The presence of pseudoinclusions with cytoplasmic content and lipid droplets inside the nucleus could likewise be observed (Figure 3A-B). Cells in the treated cultures displaying the nuclear changes had become very large. Vacuolization of the cytoplasm was observed in the enlarged cells, but was not an obligatory feature. The cell density of the treated cell cultures was much lower as compared to that of untreated cultures, due to cell death with the morphological features of necrosis. Nuclei in mitosis were absent in treated cultures. Apoptotic cells were noticed very seldomly in treated cultures. Their number was not increased in comparison to control cultures.

Time-lapse video microscopy analysis

The confluent control cells had a homogenous diameter and a polygonal outline. Almost no cell death by necrosis in these cells nor striking cell movements were observed. The *H. pullorum* sonicate treated cells were enlarged and displayed cell extensions and occasionally more than one nucleus could be seen. The cells displayed cell surface movements. Ultimately the cells detached and died by necrosis. Time-lapse video microscopy confirmed that vacuolization of the cytoplasm was not an obligatory event in the giant cells.

Partial characterisation of the toxic factor

Molecular weight estimation

Toxic effects were noticed when the 30-kDa and 50-kDa retentate fractions were added to the cell cultures, but not when permeate fractions were added. This indicates that the molecular weight of the toxic substance was more than 50 kDa.

Pretreatment of bacterial sonicate with trypsin, heat and cold

The toxic factor was inactivated by trypsin 0.25 %; cells treated with the trypsinised sonicate were showing the same morphology as cells inoculated with PBS. Toxic activity of bacterial sonicate was resistant to heating at 56°C for 30 min, was inactivated by heating at 70°C and 100°C for 15 and 10 min, respectively and was stable at -70°C for six months.

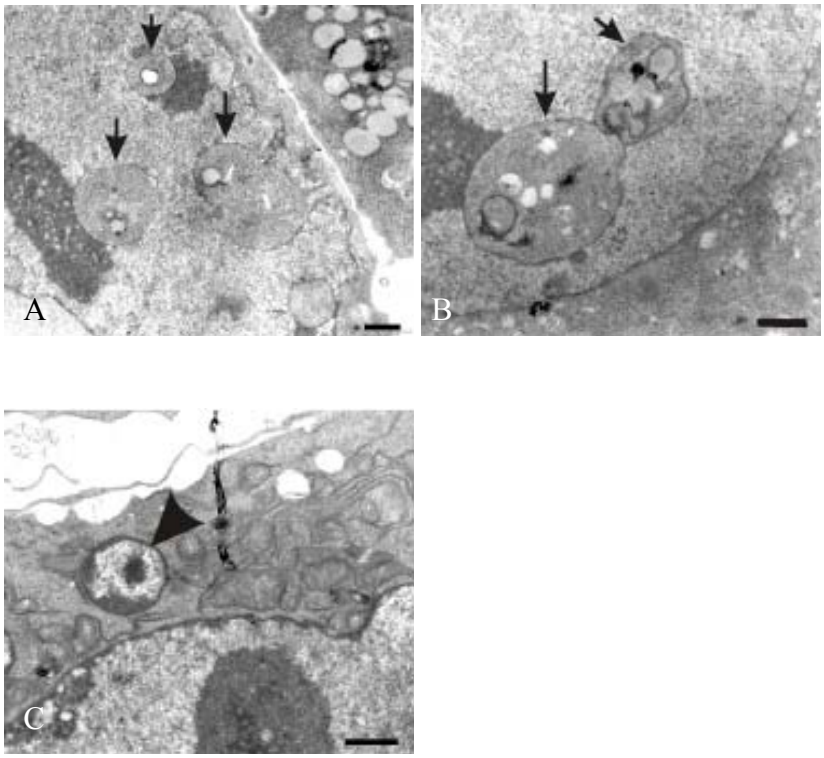

Figure 3 Transmission electron microscopical analysis of H 2.35 cells treated with filtered *Helicobacter pullorum* sonicate (strain CCUG 33839) 48 h post-treatment: intranuclear pseudoinclusions (arrows) with cytoplasmic content and lipid droplets inside (A-B), micronucleus (arrow) (C) (Bar, 100 μm)

DISCUSSION

The present study highlights that *H. pullorum* sonicate treated H 2.35 cells exhibit phenotypic changes characteristic for mitotic catastrophe, a cell death occurring during or shortly after a dysregulated or failed mitosis. The formation of binucleated cells, micronucleation and multinuclear giant cells observed in the present study are characteristic morphological alterations of this type of cell death (Chan et al., 1999; Erenpreisa et al., 2000; Huang et al., 2005; Michalakis et al., 2005; Perletti et al., 2005; Malorni and Fiorentini, 2006). The Nomenclature Committee on Cell death (NCDD) recommends the use of the terms such as "cell death preceded by multinucleation" or "cell death occurring during the metaphase", which are more precise and more informative (Kroemer et al., 2005). No consensus about the morphological changes of chromatin has been achieved hitherto. Some authors mention cells revealing decondensed chromatin (Ianzini and Mackey, 1997; Roninson et al., 2001), while others talk about clumps of condensed chromatin in cells undergoing mitotic catastrophe (Chan et al., 1999; Erenpreisa et al., 2000; Michalakis et al., 2005). The latter phenomenon was noted in the present study. Additionally, nuclear pseudoinclusions were detected in sonicate treated H 2.35 cells. The research group of Fox likewise described the presence of intranuclear pseudoinclusions *in vivo* in A/JCr mice suffering from proliferative hepatitis following a persistent infection with *H. hepaticus* (Fox et al., 1996).

Analogous changes were observed in cells treated with sonicate prepared from *H. hepaticus* and *C. jejuni* strains. In view of this, we speculate that also *H. hepaticus* and *C. jejuni* induce cell death by mitotic catastrophe.

In our experiments, treated cells died by necrosis, a phenomenon that was detected by means of time-lapse video microscopy, TEM analysis and a trypan blue exclusion method (data not shown). Mitotic catastrophe usually results in apoptosis, although mitotic blockade also may culminate in necrosis (Yeung et al., 1999; Michalakis et al., 2005).

Mitotic catastrophe may be considered as a molecular apparatus in mammalian cells that avoids aneuploidization of daughter cells. A DNA damage checkpoint triggers a signalling pathway involving different protein kinases which results in a cell block and cellular damage. The main function of this checkpoint is to block the cell cycle at the G_1 or G_2

stage before entering mitosis or to delay mitotic exit and consequently to prevent cytokinesis (Erenpreisa et al., 2000; Bayart et al., 2004; Huang et al., 2005; Niida et al., 2005). DNA damage involves pyrimidine dimers and double strand breaks and can be due to exposing of cells to γ-irradiation (Castedo et al., 2004; Huang et al., 2005) or to cytolethal distending toxin (CDT) (Cortes-Bratti et al., 2001; Ceelen et al., 2006b). CDT is a bacterial protein that is widely distributed among Gram-negative bacteria including *H. pullorum* (Johnson and Lior, 1987a,b; Johnson and Lior, 1988; Pickett et al., 1996; Cope et al., 1997; Okuda et al., 1997; Sugai et al., 1998; Chien et al., 2000; Young et al., 2000a,b; Mooney et al., 2001). The toxin activates identical signalling pathways in response to DNA damage as described for mitotic catastrophe (Chan et al., 1999; Castedo et al., 2004; Huang et al., 2005; Niida et al., 2005) with in the middle of the network the protein kinases 'Ataxia telangiectasia mutated' (ATM) and 'ATM and Rad3 related' (ATR) (Cortes-Bratti et al., 2001; Li et al., 2002; Ceelen et al., 2006b). Exposing various cell lines to CDT results in enlarged multinucleated cells as observed in several studies (Pickett et al., 1996; Ceelen et al., 2006b). In our study the cytotoxic activity on the mouse hepatocytes was destroyed by treatment with trypsin or heating (70 and 100°C) and the toxin appeared to have a molecular mass of more than 50 kDa. Moreover, cytotoxicity appeared 72-96 h post-inoculation. All these characteristics are equal to those of CDT (Johnson and Lior, 1987a; Johnson and Lior, 1988; Albert et al., 1996; Pickett et al., 1996). *H. hepaticus* and *C. jejuni* strains both possess CDT activity (Purdy et al., 2000; Young et al., 2000b; Young et al., 2004; Ceelen et al., 2006a) and induced similar cell changes as *H. pullorum,* whereas sonicate from the *H. canadensis* strain, which does not show CDT activity (Young et al., 2000a; Ceelen et al., 2006a), did not cause any CPE.

Taking all these observations into consideration, we may hypothesize that CDT is probably responsible for the cellular changes described in the present study. Nonetheless, whether these observations can be allocated to CDT only is not actually proven, but mainly indicative. It can in fact not be excluded that *H. pullorum* produces another not yet identified toxic factor which may cause these cellular changes in itself or may work synergistically with CDT. Taylor et al. (1995) demonstrated that a toxic factor is present in sonicate of *H. hepaticus* that induces granule formation in a mouse liver cell line. This factor was therefore named GCT. More recently however, Young et al. (2004) illustrated that CPE induced by

GCT could be CDT mediated. Results from our study confirm these indications. These research areas need hence undoubtedly to be further explored.

To conclude, this study is the first to report induction of features characteristic for mitotic catastrophe in liver cells exposed to sonicate of human and poultry *H. pullorum* isolates. Different levels of toxic activity were seen for different *H. pullorum* isolates. Microscopical investigation and DNA analysis of sonicate treated cells suggest that CDT may play a role in induction of CPE. Further research is necessary to determine the exact role of CDT in hepatocyte toxicity.

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Chapter 2.2

In vivo interaction of poultry and human *Helicobacter pullorum* isolates with broiler chickens

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ABSTRACT

Four groups of 23 broilers were inoculated at one day of age with one of four different *Helicobacter pullorum* strains from human and poultry origin. A fifth group of eight control animals was inoculated with phosphate-buffered saline. At five time points post-inoculation, up to slaughter age, animals of every group were sacrificed and caecum, colon, jejunum and liver were examined both macroscopically and microscopically. In addition, bacterial titration and PCR analysis for *Helicobacter pullorum* were performed. Faecal material was also collected on a regular basis for PCR analysis for *Helicobacter pullorum*.

Chickens remained clinically healthy throughout the experiment. At necropsy, macroscopic lesions characterized by red streaks on the serosa of the jejunum and foamy content in the caeca with on its serosal side dark brownish streaks, were noticed.

Helicobacter pullorum was detected from the start till the end of the experiment in all infected groups. The predominant site of colonization was the caecum and the bacterium was excreted in the faeces till at least 42 days of age. This implies that broiler chickens may act as a reservoir for *Helicobacter pullorum* hence constituting a possible source of infection for human beings.

INTRODUCTION

Helicobacter pullorum belongs to the group of urease-negative enterohepatic *Helicobacter* species (EHS). It was isolated for the first time from the caecum of asymptomatic broilers and the liver and intestine of laying hens suffering from vibrionic hepatitis (Stanley et al., 1994; Burnens et al., 1996). *H. pullorum* was detected on 60 % of poultry carcasses (Atabay et al., 1998) and in 33.6 % of caecal content of broilers at slaughter (Ceelen et al., 2006). Hitherto, there is still much debate on the actual pathogenic potential of *H. pullorum* in the chicken.

This agent has also been found in the faeces of humans with gastrointestinal disease and clinically healthy people (Ceelen et al., 2005). Fox et al. (1998) additionally detected *H. pullorum* DNA in the biliary tree and gallbladder of Chilean women suffering from chronic cholecystitis. In human beings, *H. pullorum* has mainly been associated with diarrhoea, gastroenteritis and hepatobiliary disease (Stanley et al., 1994; Atabay et al., 1998, Steinbrueckner et al., 1997; Fox et al., 1998; Ceelen et al., 2005) as reflected by liver swelling, an increase of liver enzymes and gallbladder cancer (Fox et al., 1998).

In view of the above, this bacterial species represents an emerging pathogen that may cause food-borne illness due to carcass contamination of broilers at slaughter (Atabay et al., 1998; Fox et al., 1998; Ceelen et al., 2006).

Despite the fairly frequent occurrence of *H. pullorum* in chickens and its possible association with hepatoenteric disease, up until now, no experimental study involving *H. pullorum* has yet been performed to discover more about the interactions of *H. pullorum* with its natural host. Consequently, the present study was set up to investigate the colonization capacity, preferred colonization site and the level of faecal excretion of *H. pullorum* in poultry. Attention was also paid to the possible induction of inflammation and lesions in the intestines and liver of the inoculated animals.

MATERIALS AND METHODS

Chickens

Fertilized eggs from broiler breeder hens (Claeys, Kruishoutem, Belgium) were used in this study. The eggs were hatched at our laboratory and 100 chickens were housed on litter with free access to food and water. The animals received 12 h of light per day.

H. pullorum strains

The poultry *H. pullorum* strains CE II 1 and CCUG 33840 and human *H. pullorum* strains LMG 16318 and G 214 (Table 1), stored at -70°C, were used in this study. They were grown on brain heart infusion (BHI, Oxoid, Drongen, Belgium) agar, supplemented with 10 % horse blood, amphotericin B 20 µg/ml (Fungizone; Bristol-Myers Squibb, Epernon, France) and Vitox (Oxoid) (BHI blood agar). Incubation was done under microaerobic conditions (5 % H_2, 5 % CO_2, 5 % O_2 and 85 % N_2) at 37°C for 72 hours. *H. pullorum* cultures were harvested by washing the plates with phosphate-buffered saline (PBS) followed by centrifugation (1500 *g*, 10 min, 4°C). The pellet was subsequently resuspended in PBS to an optical density (OD) of 0.7 at 600 nm which corresponds to approximately 7.5 $\log_{10}$ CFU/ml.

Experimental design

The broilers were randomly divided at the day of hatching into four groups (A-D) of 23 birds and one group (E) of eight animals, and transferred to separate units of the experimental facility. All animals of groups A-D were inoculated with one of the *H. pullorum* strains at one day of age (Table 1). Two-hundred µl of bacterial inoculum containing 6.8 $\log_{10}$ CFU was given to each chicken via the gizzard. Group E was maintained as a control group and received 200 µl PBS. All chickens were clinically examined on a daily basis.

At day 3, 10, 17, 24 and 41 post-inoculation (PI), pooled faecal samples were collected from every group for PCR analysis as described below.

At day 1, 8, 15 and 22 PI, four animals in groups A-D and one animal in group E were euthanized. The remaining animals were sacrificed at day 42 PI.

Necropsy was subsequently performed on all euthanized chickens and attention was paid to possible macroscopic lesions. Samples were taken from the liver, jejunum, caecum and colon

for detection of *H. pullorum* using PCR, isolation, immunohistochemistry and for histological examination of lesions as described below.
The experiment was performed with the approval of the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University.

Table 1 Groups of broilers inoculated with four *Helicobacter pullorum* strains and one control group inoculated with PBS

Group of broilers	*H. pullorum* strain	Source of *H. pullorum* strain
A	CE II 1	Caecal tissue, broiler chicken, Belgium
B	CCUG 33840	Laying hen with hepatitis, Switzerland
C	LMG 16318	Human faeces, Switzerland
D	G 214	Human faeces, gastroenteritis, human, Belgium
E		PBS

PCR analysis and gel electrophoresis

DNA was extracted from approximately 25 mg liver, jejunum, caecum and colon tissue applying a commercial tissue kit (DNeasy® Tissue Kit, Qiagen, Venlo, The Netherlands).
DNA was extracted from faeces (weighing approximately 200 mg) using a commercial stool kit (QIAamp® DNA Stool Mini Kit, Qiagen).
A PCR assay amplifying a 447 bp fragment of the 16S rRNA gene of *H. pullorum* was then used followed by agarose gel electrophoresis as previously described (Stanley et al., 1994; Burnens et al., 1996).

Isolation of *H. pullorum*

A 20 % weight/volume suspension of the samples (tissue and contents) in PBS was prepared. The number of CFU per gram of sample was determined by plating 100 µl of 10-fold serial dilutions in PBS on BHI blood agar plates which were incubated microaerobically for five days at 37°C. In doing this, a previously described technique was used adopting a sterile cellulose acetate membrane filter (0.45 µm) to diminish bacterial contamination (Steele and

McDermott, 1984; Ceelen et al., 2006). On day 42 PI, no serial dilutions were made, and only 100 µl of the undiluted tissue suspension was inoculated.

Amplified fragment length polymorphism (AFLP)

The *H. pullorum* strains used for inoculation and *H. pullorum* isolates retrieved from the various caecal tissue samples PI were fingerprinted by AFLP (Ceelen et al., 2006).

Histological and immunohistochemical analysis

Following fixation in 10 % formaldehyde for 48 h, the tissues were embedded in paraffin, sectioned at 5 µm, mounted on glass slides and stained with haematoxylin and eosin using standard procedures. When bacteria showed close association with epithelial (intestine) or liver cells, immunohistochemistry was performed additionally. For the latter, polyclonal anti-*H. pylori* antibodies (DakoCytomation, Heverlee, Belgium) were adopted (Ananieva et al., 2002).

Five-µm sections of paraffin-embedded tissues were placed on super Frost slides coated with (3-aminopropyl)triethoxy-silane (minimum 98 %) (Sigma-Aldrich, Bornem, Belgium). Sections were deparaffinized, rehydrated, and pretreated by the antigen retrieval microwave technique. After washing, slides were incubated with endogenous peroxidase and were washed afterwards using PBS. Slides then underwent sequential application of 30 % goat serum, the primary rabbit anti-*H. pylori* B 0471 Dako 1/320 antibody (DakoCytomation), biotinylated goat anti-rabbit antibody 1/500 (DakoCytomation), StreptABComplex/horseradish peroxidase (DakoCytomation), and finally 3,3'-diamino benzidine tetrahydrochloride (Sigma-Aldrich) with each time a wash step. Sections were counterstained by use of an aqueous-based hematoxylin staining and mounted with a permanent mounting medium (Prosan, Merelbeke, Belgium).

Statistics

Possible difference in the number of bacteria in the caecum and colon at the different time points PI were investigated using analysis of variance. Therefore, the number of bacteria (log transformed) was included as dependent variable and group as fixed variable. Post-hoc pairwise comparisons between the groups were performed using Scheffe's tests. In case the

assumptions of normality and homogeneity of variance were not fulfilled, non-parametric Kruskal-Wallis analysis of variance was used. Differences were considered statistically significant when P-values were lower than 0.05. Bacterial numbers on the other sampled sites were not analysed statistically because of the very low colonisation levels. Statistical analyses were performed using SPSS version 14.00.

RESULTS

Clinical signs

No obvious clinical signs were noticed. Two animals in group D died at the age of one week. No lesions were noticed during necropsy of these animals nor could any poultry-specific bacterial pathogens be isolated from caecum, jejunum, liver or spleen.

PCR analysis and gel electrophoresis

In group E, all faecal and tissue samples were negative for *H. pullorum.*

In group A-D, all faecal samples were positive in PCR for *H. pullorum* till day 42 PI.

The results of PCR analysis of liver, jejunum, caecum and colon are given in Table 2. At day 1 PI, no *H. pullorum* DNA was detected in liver and jejunum samples except for group C. At the next time points, irrespective of the group, *H. pullorum* DNA was occasionally present in liver and jejunum. The majority of the caecum and colon samples were positive for *H. pullorum* from day 1 until day 42 PI among all *H. pullorum*-inoculated groups.

Isolation of *H. pullorum*

H. pullorum was not isolated from any of the animals from the control group.

Results of the bacteriological titration of the caecum and colon of the groups A-D at various times until day 22 PI are shown in Figure 1.

Significant differences for the *H. pullorum* titres in the caecum and colon were seen at day 1 and 22 PI, but not at the other time points. At day 1 PI, caecal titres in group A and C differed significantly from each other, but not from the other groups; colon titres were borderline significantly different in the overall analysis of variance (P=0.034), but none of the pairwise comparisons between the groups were statistically significant using the Scheffé's test

(P=0.078 for group C and D). At day 22 PI, only the caecal titres in group B and D were significantly different, but not the other comparison in pairs. Colon titres were different in the overall analysis ($P < 0.001$). The p-values of the significant comparisons in pairs between groups A-D, B-C and B-D were 0.008, 0.012 and 0.001, respectively.

At day 42 PI, *H. pullorum* was isolated from all the caecum samples except for one sample in group B, from one colon sample in groups A and B and from five and three colon samples in groups C and D, respectively.

At day 1, 8 and 15 PI, *H. pullorum* was isolated from one jejunum sample in group C (4.9;5.1;1.7 $\log_{10}$ CFU/g tissue, respectively) and at day 22 PI, two samples were positive (2.7;3 $\log_{10}$ CFU/g tissue) in this group. At day 22 PI, a titre of 4.4 $\log_{10}$ CFU/g tissue was demonstrated in one jejunum in group A and at day 42 PI a titre of 2.7 $\log_{10}$ CFU/g tissue was found in one jejunum in group B. *H. pullorum* was not isolated from the remaining jejunum samples.

From none of the *H. pullorum*-inoculated animals, *H. pullorum* could be isolated from the liver tissue.

AFLP

AFLP analysis revealed that the four strains used for inoculation each displayed a unique profile. All retrieved isolates from the caeca of one and the same group clustered with the strain used for inoculation.

Table 2 PCR results of *Helicobacter pullorum* isolated from different organs from broilers inoculated with four strains

Organ	Group A					Group B				
	1 day [a]	8 days	15 days	22 days	42 days	1 day	8 days	15 days	22 days	42 days
Liver	-/-/-/- [b]	-/+/-/-	-/-/+/+	-/-/+/-	-/-/-/-/-/+/+	-/-/-/-	+/-/+/-	-/-/+/+	+/-/+/-	-/-/-/-/-/-/-
Jejunum	-/-/-/-	-/-/-/+	-/+/-/-	+/-/-/+	-/-/-/-/-/-/-	-/-/-/-	-/+/+/+	-/-/+/-	-/-/-/-	-/+/-/-/-/+/-
Caecum	+/+/+/+	+/+/+/+	+/+/-/+	+/+/-/-	+/+/+/-+/+/-	-/+/+/+	+/+/+/-	+/-/-/+	+/+/-/+	-/+/-/+/-/+/-
Colon	+/+/+/-	+/+/-/-	-/-/-/-	+/-/+/+	+/-/-/+/+/+/+	+/+/+/+	+/+/-/-	+/+/+/+	-/-/+/+	+/+/+/+/+/+/+
	Group C					Group D				
Liver	-/-/-/-	-/+/-/-	-/-/-/-	+/-/-/-	-/-/-/-/-/-/-	-/-/-/-	-/-/-/+	-/-/-/-	-/-/+/-	-/-/+/+/+
Jejunum	-/-/+/+	-/-/-/-	+/-/-/+	+/-/-/-	-/-/+/-/-/-/-	-/-/-/-	-/-/-/-	-/-/-/-	+/-/-/-	-/+/+/+/-
Caecum	+/+/+/+	+/-/+/+	+/+/+/-	+/+/+/-	-/+/+/+/-/-/-	+/+/+/+	+/+/+/+	+/+/-/+	-/-/+/-	+/+/-+/-
Colon	+/+/+/+	-/+/+/-	+/-/-/-	+/+/+/+	+/+/+/-/-/-/-	+/+/+/+	+/-/-/+	-/-/+/-	+/+/+/+	+/-/+/+/+

[a] Number of days post-inoculation, [b] In all groups, four animals were examined at day 1, 8, 15 and 22 days post-inoculation. At day 42 post-inoculation, seven animals were tested except for group D in which only five animals were included

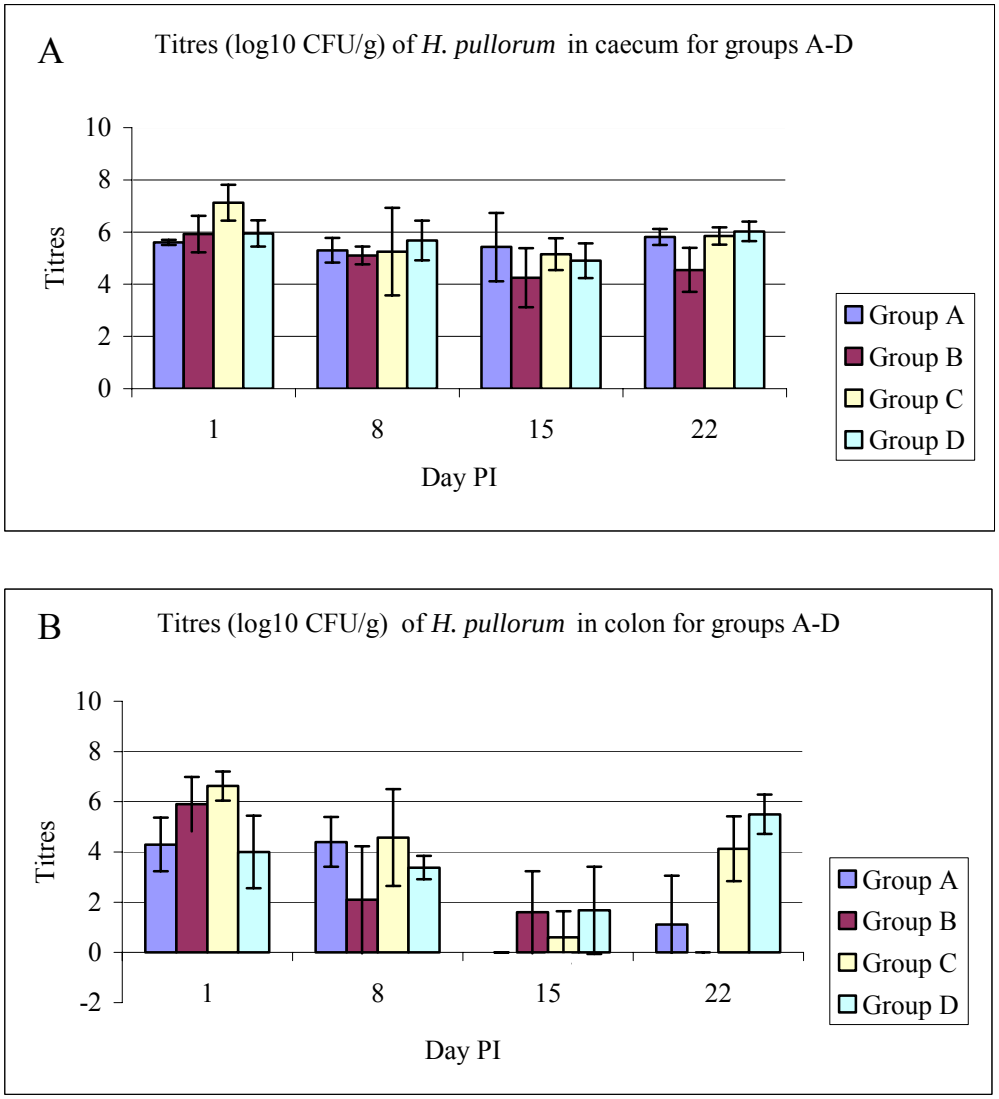

Figure 1 Titres (log_{10} CFU/g) of *Helicobacter pullorum* in groups A-D at day 1, 8, 15 and 22 post-inoculation. Caecum (A), colon (B)

Necropsy findings

Marked macroscopic lesions were not observed in group E.

No lesions were apparent in the liver nor colon in groups A-D at any time point.

At day 1 PI, the vast majority of animals of groups A-D revealed yellowish caeca with a foamy content which was most overt in group C (Figure 2). No macroscopic lesions were seen in the jejunum of the animals of group A-D at this point of time.

At day 8 PI, caeca were swollen, especially in group D. Foamy caecal contents were seen in most animals of groups A-D. The contents were yellowish to brownish in colour. No marked lesions were observed in the jejunum of these animals.

At day 15 PI, half of the caeca and jejuni had foamy contents distributed evenly over groups A-D.

At day 22 PI, most of the caeca in group A-D were swollen and presented dark brown-blackish longitudinal streaks on the serosal surface (Figure 3). Some of these also displayed foamy contents. One to two animals of each group (A-D) additionally had a jejunum presenting transverse red streaks on the serosal surface with occasionally and irrespective of the group, a foamy content. The jejunum of one animal in group D had a swollen appearance (Figure 4).

At day 42 PI, two-third of the caeca revealed dark brownish longitudinal streaks and were enlarged. One chicken in group B revealed a very pale, inflated caecum with yellowish malodorous content. Approximately half of the inoculated chickens showed jejuni with a focal swollen aspect and foamy contents. Again, no marked differences between groups were noted at this time point.

Histological and immunohistochemical analysis

In the control group (group E), in general, no marked microscopic changes were noticed in the intestines. In two caecal samples of this group however, mild focal aggregates of lymphoplasmahistiocytic cells and some heterophils were noticed in the lamina propria at day 42 PI. The caecal epithelium remained undamaged. No lesions were present in the liver of the uninfected animals at day 1, 8, 15 and 22 PI. A very mild portal infiltration of round cells was noticed in half of the livers sampled at day 42 PI.

At day 1 PI, histological abnormalities were observed in the caeca of animals from groups A-D. A moderate infiltration of heterophils was found in the lamina propria in all animals of these groups. In the other samples, no microscopic changes were observed.

At day 8 PI, scattered heterophils were found in the caecal lamina propria of two chickens (group D). No signs of inflammation were visible in the caeca of other animals, nor in the colon, jejunum and liver of any animal at this point of time.

Fifteen days following inoculation, one animal in group A revealed a mild heterophilic infiltration in both the caecum and colon. In one animal of group C, a very mild monomorphonuclear infiltrate was present in the caecum. Inflammation was not noticed in the caecum and colon of the other broilers, nor in the jejunum and liver samples.

At day 22 PI, the mucosa of the majority of the caeca was infiltrated with a small amount of monomorphonuclear cells, a finding which was evenly distributed over the groups.

At day 42 PI, a mild to moderate inflammation was present in the caeca of three-quarters of the infected animals with no marked differences between the groups. In most samples a slight to modest increase of round cells and occasionally heterophils was noticed. Focal blunting and fusion of villi were visible. Sporadically, karyorrhectic cells were seen in the surface epithelium. In one animal (group A), a slight loss of enterocytes and a mildly flattened surface epithelium were noticed. In another animal (group B), ulceration of the surface epithelium was noticed, the number of crypts was markedly decreased and a diffuse infiltration of a mixture of inflammatory cells was present in the lamina propria.

Most of the liver samples did not show significant microscopic lesions. A minority of the samples revealed small monomorphonuclear aggregates ad random irrespective of the group. None of the livers showed microscopic lesions characteristic for vibrionic hepatitis. The colonic samples showed no pathological alterations.

At day 1 PI, small rod-shaped, slightly curved bacteria positively stained with polyclonal anti-*H. pylori* antibodies were present mainly in the lumen of the caecum in all inoculated groups. Additionally, a small number was associated with the caecal epithelium. These bacteria were found in the lumen of the colon as well, albeit to a minor extent. At the following points of time, these rod-shaped bacteria remained to be noted in the caecum. Most of these showed close association with the epithelium and a significant portion was found in the caecal crypts. The bacteria were also seen in association with the colonic epithelium, but not consistently (Figure 5). These rod-shaped bacteria were not found in the jejunum nor liver samples of groups A-D and in any samples of group E.

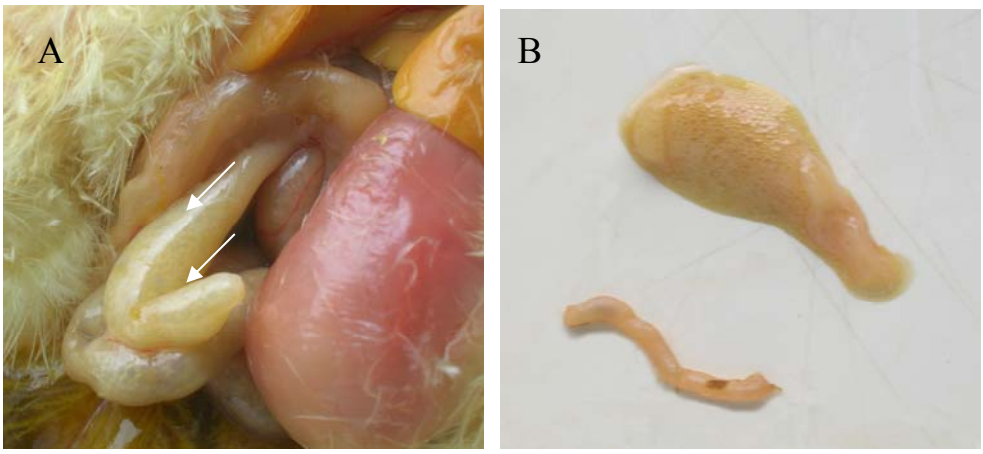

Figure 2 Yellowish distended caeca within foamy content (arrows) (day 1 post-inoculation, group C) (A), distended foamy caecum (above; group C) compared with normal caecum (below; group E) at day 1 post-inoculation (B)

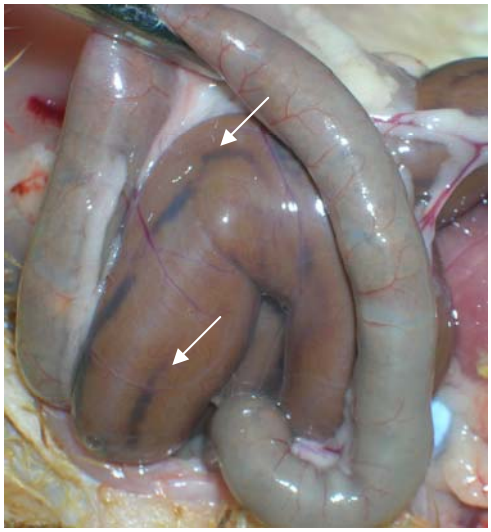

Figure 3 Dark brown-blackish longitudinal streaks (arrows) on the serosal side of the caeca (day 22 post-inoculation, group C)

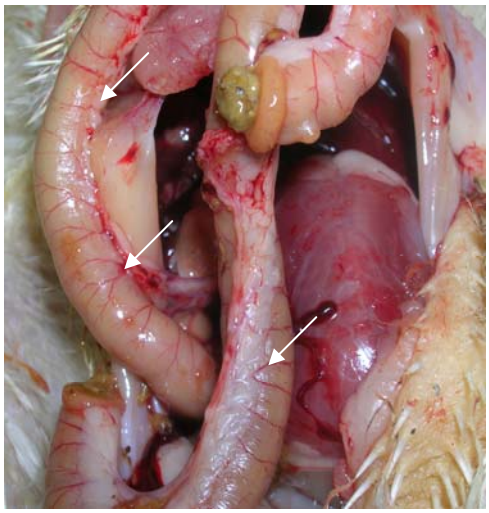

Figure 4 Jejunum revealing transversal red streaks (arrows) on the outside (day 22 post-inoculation, group D)

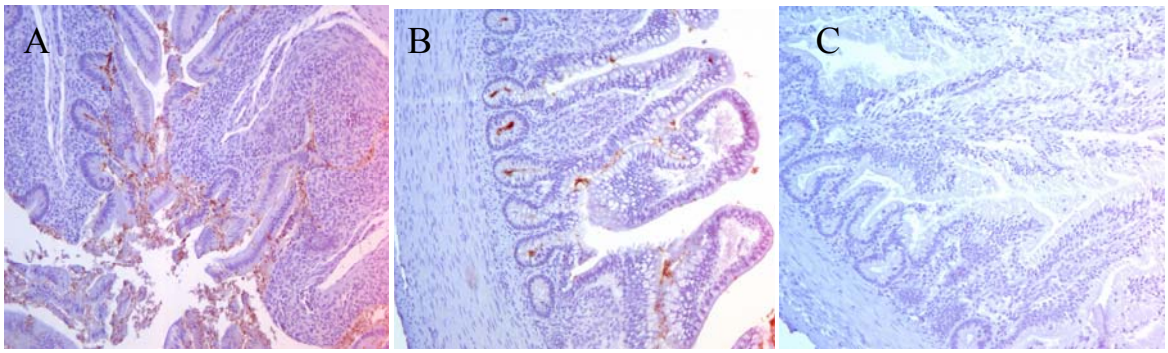

Figure 5 Immunohistochemistry using anti-*Helicobacter pylori* antibodies reacting with *Helicobacter pullorum.* Small rod-shaped, slightly curved bacteria are present in the lumen and crypts of the caecum and colon, but not in the jejunum. Bacteria are also associated with the epithelium of the caecum and colon. Caecum (day 15 post-inoculation, group B) (A), colon (day 8 post-inoculation, group C) (B), jejunum (day 22 post-inoculation, group C) (C). 40 x

DISCUSSION

In the experiment presented here, the intestines and liver from experimentally *H. pullorum*-infected chickens were macroscopically and microscopically examined at five time points PI. Noteworthy is the presence of distended caeca with foamy contents 1 and 8 days PI in most of the *H. pullorum*-infected chickens. Chickens exposed to a toxigenic *Campylobacter jejuni* strain may also reveal a distended intestinal tract with foamy contents, a feature hitherto considered a criterion corresponding to *C. jejuni*-induced disease (Clark and Bueschkens, 1988). The findings in this study question the statement as to *C. jejuni* being solely responsible for these marked necropsy findings.

At the end of the experiment, large caeca with dark brownish streaks on the serosal side were visible in a substantial part of the *H. pullorum*-inoculated animals. They were not detected in the control group. The animals were tested weekly for the presence of *Campylobacter* spp. by means of PCR analysis (Shen et al., 2001) and *Salmonella* spp. by enrichment in tetrathionate broth followed by subculture on Brilliant Green agar. All samples remained negative till the end of the experiment (data not shown) indicating that these common chicken pathogens (Welkos, 1984; Shane et al., 1992; Desmidt et al., 1998) were not the source of the lesions noticed mainly in the caeca.

H. pullorum infection in laying hens has in the past been associated with vibrionic hepatitis (Stanley et al., 1994; Burnens et al., 1996), a disease primarily characterized by swelling and necrosis of the liver and possibly causing economic loss by increased poultry flock cull rates (Berry and Whitenack, 1991). In the current experiment, no macroscopic nor microscopic lesions were present in the liver samples. This could be explained by the relatively young age of the broiler chickens and thus the lack of hepatic lesions when euthanizing the animals. Similar phenomena have been noticed in *H. hepaticus*-infected mice where hepatic lesions only start to develop at an older age depending on the mouse strain (Li et al., 1998; Whary et al., 1998).

At the onset of this study, the caecum in chickens was considered the preferred colonization site by this agent, although no clear evidence on this had been given in literature. The present study actually showed that the bacterial agent was indeed mainly present in the

caeca, and specifically interacted with the epithelial cells starting from the age of approximately one week. The bacterium was also observed frequently in the caecal crypts.
In general, a concordance between the three detection techniques for *H. pullorum* was noticed indicating isolation, PCR and immunohistochemistry are valuable identification methods. Nonetheless, in a minority of animals, samples tested positive in PCR while no *H. pullorum* was yielded using isolation. The higher sensitivity of PCR could account for this. Sporadically however, the opposite was seen which could be explained by the fact that only a small sample of tissue was used for PCR analysis, whereas both contents and a larger section of tissue were adopted for the isolation method. Immunohistochemistry showed the localisation of the bacteria in their niche, but generally is considered a less sensitive and less specific detection method than isolation and PCR (Livingston et al., 1997; Rogers et al., 2004).

At the age of 42 days, *H. pullorum* was detected in high numbers in the caecal samples proving that the agent is able to colonize broilers in high quantities at least till the age of slaughter. Moreover, the bacterium was excreted in the faeces up until slaughter age, which may lead to carcass contamination during the slaughter process. Infected chickens apparently do not develop obvious clinical signs such as diarrhoea and may therefore act as carriers of *H. pullorum* infection. These findings are of particular importance because of the fact that *H. pullorum* is considered as a food-borne pathogen responsible for causing several gastro-intestinal and liver diseases in humans (Stanley et al., 1994, Steinbrueckner et al., 1997; Fox et al., 1998; Andersson et al., 2002; Bohr et al., 2002; Ceelen et al., 2005). The microorganism has indeed been detected in a significant portion of living meat chickens and laying hens and on poultry carcasses on several occasions (Burnens et al., 1996; Atabay et al., 1998; Ceelen et al., 2006; Miller et al., 2006).

In conclusion, the results of this experiment demonstrate that *H. pullorum* is able to colonize broiler chickens and additionally is excreted in their faeces at least until the age of 42 days. The preferred colonization site is the caecum wherein the bacterium shows close association with the surface epithelium. Infected chickens apparently reveal no clinical signs, although macroscopic pathological changes on the caeca may be present. Microscopically, in general only mild lesions are noticed. Consequently, one may conclude that broilers may act as carriers of *H. pullorum* hence constituting a possible source of infection for human beings.

This study is only a first step in the investigation of the interaction of *H. pullorum* with its chicken host and stipulates further research.

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Burnens et al. (1996) demonstrated the presence of *Helicobacter pullorum* in the caecal content of four percent of broilers by isolation. However, due to the fastidious character of *H. pullorum* and its requirement for special growth conditions, isolation is in all probability not the most appropriate technique for demonstrating the prevalence of this EHS. Therefore, in the present thesis, we resorted to a PCR technique based on the 16S rRNA gene (Stanley et al., 1994). Using this assay, *H. pullorum* was detected in 33.6 % of the caeca of 110 Belgian broilers. One could question whether the number of animals included in this experiment is sufficient and allows to conclude that one-third of the Belgian chicken population may be positive. We like to argue that the animals were originating from 11 flocks spread over the territory, and not from a selected region. It is nonetheless reasonable to state that more animals in different countries including Belgium need to be screened for the presence of *H. pullorum* to get a trustworthy impression about the true prevalence of this bacterial agent amongst the world chicken population.

Atabay et al. (1998) isolated *H. pullorum* from 9 of 15 poultry carcasses. Although the number of carcasses they sampled is low, this high occurrence of *H. pullorum* even using isolation may, at first sight, seem surprising. It has, however, been suggested that the surface of chicken carcasses may be cross-contaminated with caecal contents during poultry processing (Atabay et al., 1998; Fox et al., 1998) similary to *Campylobacter* spp. and *Salmonella* spp. (Oosterom et al., 1983; Hald et al., 2001; Olsen et al., 2003; Goksoy et al., 2004). Additionally, contamination of the chicken body surface may arise during transport to the abattoir as has also been described for *Campylobacter* spp. (Whyte et al., 2001). Hence, the discrepancy between our study results and those obtained by Atabay and his research group may be more apparent than factual.

We illustrated that *H. pullorum* is fairly regularly present both in clinically healthy humans and patients suffering from gastrointestinal disease. Most faecal samples obtained from the latter group of people had an abnormal appearance, but unfortunately, no additional clinical data were available. There may be different reasons for detecting *H. pullorum* DNA in the stool of clinically healthy human beings.

First of all, it is possible that *H. pullorum* belongs to the normal human intestinal microbiota. Secondly, it may be speculated that a certain number of the clinically healthy persons found positive for *H. pullorum* included in our study, could have been carriers of this EHS. Steinbrueckner et al. (1997) reported a case of human enteritis strongly associated with *H. pullorum.* It concerned a female patient with several episodes of diarrhoea from which only *H. pullorum* was recovered, and no other diarrheic bacterial nor parasitic pathogens. *H. pullorum* remained detectable when disease signs were no longer present and until three months after the first presentation; a carrier status hence was suggested (Steinbrueckner et al., 1997).

Thirdly, some *H. pullorum* strains may be of very low virulence, resulting in absence of clinically detectable signs of infection. Differences in virulence between strains have been described for other *Helicobacter* species such as *H. hepaticus* (Suerbaum et al., 2003) and *H. pylori* (Dunn et al., 1997; Israel et al., 2001). In fact, 70 to 90 % of the population in developing countries carries *H. pylori*, while only 25 to 50 % of them develop gastric disease (Dunn et al., 1997; Falkow, 2006). The actual evolvement into gastric disease depends on bacterial virulence factors, host characteristics and/or interaction between host and bacterium (Israel et al., 2001). For *H. pullorum*, only little information is available on the actual virulence factors despite the growing number of clinical reports related to this pathogen. Only few studies dealing with this research area have been published in particular demonstrating the production of cytolethal distending toxin (CDT) by *H. pullorum* which is hypothesized to play an aetiological role in the development of diarrhoea (Ceelen et al., 2006a). The latter feature was our focus point in chapters 2.1.1 and 2.1.2 and will be discussed below.

A fourth possible reason for the presence of *H. pullorum* DNA in the stool of clinically healthy people is the difference in host factors such as age, genetic background and immune response. Also ethnicity and regional factors might play a role in the clinical outcome of *H. pullorum* infection. These all have been discussed for *H. pylori* on numerous occasions (Taylor and Parsonnet, 1995; Dunn et al., 1997; Kim et al., 2004; Vilaichone et al., 2004) and to a lesser extent for *H. hepaticus* (Ward et al., 1996; Ihrig et al., 1999; Whary et al., 2001).

In the present studies, faecal material was tested for the presence of *H. pullorum* DNA using a PCR method based on the 16S rRNA gene following DNA extraction by means of a commercial stool kit (QIAamp® DNA Stool Mini Kit, Qiagen, Venlo, The Netherlands).

Although PCR theoretically allows us to obtain accurate results, this method has some disadvantages, particularly due to the preceding methods of sampling and extraction of DNA from human stool. The sensitivity of the PCR method was tested before application by spiking human faecal samples with known concentrations of the human *H. pullorum* strain LMG 16318. A *H. pullorum* concentration of 2000 colony forming units per ml faecal suspension was the lower detection limit (data not shown). Lower DNA concentrations may hence not have been detected in the faeces. We additionally do not know whether *H. pullorum* is excreted in faeces continuously or intermittently. Should the latter be the case for *H. pullorum* as has been described for *Salmonella enterica* serovar Typhimurium (Falkow, 2006), then its occurrence can be underestimated due to sampling at only one time point. Finally, according to the DNA extraction process as outlined by the manufacturer, we started the procedure with an amount of 200 mg of faeces. Since this amount may be considered as being fairly small, one may hypothesize this issue as a third possible reason for an underestimation of the prevalence of *H. pullorum* in the faeces of people.

Although it has not yet been clearly proven that *H. pullorum* has zoonotic potential, the increasing number of reports about *H. pullorum*-associated diarrhoea, gastroenteritis and biliary disease in humans illustrates the possible significance of this microbe as a human pathogen (Burnens et al., 1994; Stanley et al., 1994; Burnens et al., 1996; Fox, 1997; Steinbrueckner et al., 1997; Fox et al., 1998). The actual contribution of *H. pullorum* in pathological processes has probably been underestimated due to the specific requirements for culture of the species, the use of unsuitable culture media and the frequent incorrect classification of *H. pullorum* as *C. coli* and *C. lari* with which various major phenotypic characteristics are shared. As an example, *H. pullorum* is susceptible to polymyxin B, an antibiotic present in most *Campylobacter* isolation media (Corry et al., 1995; On et al., 1996; Atabay et al., 1998; Young et al., 2000).

H. pullorum is considered as a potential food-borne human pathogen by a number of authors (Burnens et al., 1994; Stanley et al., 1994; Atabay et al., 1998; Fox et al., 1998). Results from an experimental study as described in this thesis illustrate that the bacterium can colonize the intestine of chickens and may be excreted in the faeces up until slaughter age. This implies that these animals indeed may act as a reservoir for *H. pullorum* and constitute one of the

sources of infection for human beings. Atabay et al. (1998) were able to isolate the species from chicken carcasses. We additionally demonstrated in our laboratory that a human *H. pullorum* strain was able to survive for an extended period outside the chicken gut at low temperature, although under conditions of high moisture (data not shown). This may to some extent support the assumption of *H. pullorum* being a food-borne pathogen due to contamination of the carcasses at the slaughterhouse. One may suppose that a part of the human exposure to *H. pullorum* may originate from cross-contamination in kitchens during food handling. An upgrading of the hygiene in domestic kitchens hence is important, even if one is not yet sure about the actual clinical relevance of *H. pullorum* in human beings. This likewise markedly may reduce the number of *Campylobacter* spp. and *Salmonella* spp. infections as demonstrated previously. It is indeed essential to wash hands on a regular basis and to use separate surfaces or alternatively to correctly rinse surfaces while raw and cooked foods are being prepared (Humphrey, 2000; Gorman et al., 2002; Barker et al., 2003; Kusumaningrum et al., 2004).

H. pullorum may be translocated to people due to contact with live chickens and their faeces as well. *H. pullorum* was detected by means of PCR and isolation in faeces of two privately owned chickens out of the 53 sampled animals in a small experiment in our laboratory (data not shown). We thus may assume that faecal material also may be an infection source of this pathogen for chicken farmers and hobbyists.

Recently, we reported for the first time the occurrence of *H. pullorum* in a parakeet, more specifically a *Psephotus haematogaster*, during an episode of diarrhoea (Ceelen et al., 2006b). These animals may possibly act as another vehicle of *H. pullorum* infection for human beings, although further research involving more aviary birds species is needed before any firm conclusions can be made in this area.

One of the objectives of this dissertation was to determine the *in vitro* susceptibility of various *H. pullorum* isolates to a series of antimicrobial agents. When we started our experiments, it was only known that *H. pullorum* is naturally sensitive to polymyxin B and resistant to cefalotin and cefoperazone (Stanley et al., 1994; On, 1996; Atabay et al., 1998). In addition, resistance percentages of 6 to 55 % to nalidixic acid were reported by several research groups (On, 1996; Atabay et al., 1998; Melito et al., 2000).

In our study, Minimum Inhibitory Concentration (MIC) determinations were carried out on a relatively limited number of *H. pullorum* isolates. Amplified fragment length polymorphism (AFLP) analysis revealed that isolates obtained from the same flock, clustered together, indicating clonal origin. Therefore, probably, a smaller number of *H. pullorum* strains was actually used for MIC determinations than initially thought. This however, does not alter the fact that this report is the first to give some indications on the normal *in vitro* susceptibility of this species for several antimicrobials. It also demonstrates that development of acquired resistance may occur as illustrated here for spectinomycin. Still, it is obvious that more *H. pullorum* isolates need to be tested in the future. It should be emphasized that the fastidious nature of this species in terms of *in vitro* isolation limits the number of strains that can be included in a similar study.

All isolates obtained from herd II showed acquired resistance to spectinomycin. Although AFLP analysis revealed clonal origin of these isolates, an eight-fold difference in MIC was seen for instance between strains CE II 1 and CE II 2 for gentamicin (1 and 0.12, respectively) and between strains CE II 3 and CE II 4 for tylosin (16 and 2, respectively). This variation in MIC values between these clonal isolates may be due to the specific growth requirements and the fastidious nature of *Helicobacter* making it difficult to institute standardized MIC determination procedures. Up until now, for *Helicobacter* spp., no internationally accepted criteria for susceptibility testing are available. The quality control limits given for nonfastidious bacteria in aerobic atmosphere are not in compliance. Nevertheless, the agar dilution method used in the present study hitherto, is the most suitable way to determine the MIC values for *H. pullorum* and *H. canadensis*, since, according to the International Council for Laboratory Standards (ICLS), this method is the choice for testing *H. pylori*, *C. jejuni* and related species (M31-A2) (2002). Indeed, *Campylobacter* spp. and EHS require similar incubation circumstances and time (Solnick, 2003).

High MIC values of ampicillin were noticed for all *H. pullorum* isolates indicating intrinsic resistance against this antibiotic. Other *Helicobacter* spp., on the contrary, are generally considered as naturally sensitive to ampicillin and amoxicillin, both belonging to the group of the aminobenzylpenicillins displaying a similar antimicrobial activity (Prescott, 2000). Amoxicillin is often used for the treatment of *Helicobacter* infections in humans and animals, usually in combination with metronidazole and omeprazole/bismuth. The finding of

MIC values ≥ 32 µg/ml of ampicillin for the tested *H. pullorum* isolates is hence interesting because *H. pullorum*-infected persons may not benefit from a treatment with ampicillin or amoxicillin.

We mentioned throughout this thesis the fastidious growth requirements for *H. pullorum* rendering its isolation very sensitive for overgrowth by contaminating organisms. In view of this, the high MIC values of ampicillin may also be useful for preparing a selective medium to improve the cultivation of this EHS.

The mechanism of resistance against ampicillin in *H. pullorum* in not known. Resistance to β-lactam antibiotics in *Campylobacter* spp. may be due to restricted ability of these antibiotics to bind penicillin binding proteins (PBPs) or the capacity of the bacterial strains to produce β-lactamase enzymes (Prescott, 2000; Engberg et al., 2006). Low affinity PBPs due to a modification in one or more of the peptides has also been described in *H. pylori* (Dore et al., 1998; van Zwet et al., 1998; Dore et al., 1999; Mégraud, 2001; Wang et al., 2001). We tested β-lactamase activity in our *H. pullorum* strains using diagnostic ROSCO tablets (ROSCO, Taastrup, Denmark) (data not shown). The test gave negative results, indicating that this resistance mechanism is not responsible for the high MIC values in *H. pullorum*. However, although this diagnostic test is recommended by ICLS for demonstrating β-lactamase activity, we do not know for certain whether this test is accurate to detect β-lactamase activity in *Helicobacter* spp. Indeed, regarding *C. jejuni*, the β-lactamase provides resistance to the overall neutrally charged amoxicillin, ampicillin, and ticarcillin but not to the negatively charged penicillin G which constitutes the antibiotic component in the diagnostic ROSCO test (Engberg et al., 2006).

Since the discovery of *H. pullorum* (Stanley et al., 1994), only very few virulence studies with this microbe have been performed. Young et al. (2000) mentioned the presence of CDT activity in human and poultry *H. pullorum* isolates. Hynes et al. (2004) reported about chemical and biological characterization of lipopolysaccharides in *H. pullorum*.

With a view to expand our knowledge about the pathogenicity of this EHS, we examined both poultry and human *H. pullorum* strains for CDT production. Using Hep-2 cells, we only noticed obvious activity characteristic for CDT in one human strain. Previously, Young et al. (2000) assessed the presence of CDT activity in a collection of nine

human isolates, and two chicken isolates. The two latter, plus one human isolate were tested in both the experiment of Young et al. (2000) and ours. While we concluded that the majority of *H. pullorum* isolates did not clearly show CDT activity, Young et al. asserted that their tested isolates did produce CDT. Nonetheless, the results of Young and his group (2000) demonstrated that the cytotoxic titres produced by *H. pullorum* were 12 to 81 times lower than observed in the *H. hepaticus* strain included as a positive control. One may wonder whether the CDT activity reported has any significance *in vivo*. In both *in vitro* assays, epithelial cultured cell lines were adopted to look for CDT activity, more precisely HeLa cells in the study of Young et al. (2000) and a Hep-2 cell line constituting a HeLa derivate in our experiment. Both cell lines were used occasionally in the past to demonstrate CDT activity in various Gram-negative bacteria (Johnson and Lior, 1988; Purvén and Lagergård, 1992; Scott and Kaper, 1994; Pickett and Whitehouse, 1999). However, we cannot rule out that Hep-2 cells are less susceptible to CDT generated by *H. pullorum* strains, which may explain the apparent discrepancy between our results and those of Young et al. (2000). The degree of similarity between the amino acid sequences of cdtA and cdtC subunits, which most probably are involved in attachment of the toxin to the host-cell surface and in internalization of cdtB, may differ enormously between and within different bacterial species (Scott and Kaper, 1994; Pickett et al., 1996; Cortes-Bratti et al., 2001; Frisan et al., 2002; Lee et al., 2003). This may give rise to variation in sensitivity of host cells for CDT generated by different bacterial species or possibly strains. Eyigor et al. (1999) theorized that apparently low CDT production by *C. coli* isolates might be due to low sensitivity of the HeLa cells used in their investigation.

In view of this, we performed a toxicity assay in a mouse liver cell line with the same collection of *H. pullorum* isolates using the same amount of total proteins from sonicate as in the previous experiment. In this assay on the contrary, we did notice cell changes characteristic for CDT for all isolates. The cell changes included an increase of the cell size with proportional nuclear distention. Binucleated cells and micronucleation were observed as well. These findings imply that hepatocytes may be more sensitive to CDT produced by *H. pullorum* than other epithelial cells. Nonetheless, whether these observations can be allocated to only CDT is not actually proven, but mainly indicative. We indeed cannot exclude that *H. pullorum* produces another yet unidentified toxic factor which may cause these cellular changes in itself or may work synergistically with CDT.

CDT is composed of three subunits designated as cdtA, cdtB and cdtC, encoded by the genes *cdtA*, *cdtB* and *cdtC*, as described in the literature review on EHS of this thesis. The variation in these genes among Gram-negative bacteria is well known. The sequence of the *cdtB* gene encoding the cdtB subunit is recognized as the most conserved, but still can vary to a certain extent between different bacterial species. We were able to amplify this gene partially in all tested *H. pullorum* strains using the degenerative primers developed by Chien et al. (2000). The presence of the *cdtB* gene is however no proof for CDT activity. The cdtB compound is indeed mainly responsible for CDT activity, but the cdtA and cdtC subunits apparently are required as well (Pickett et al., 1994; Scott and Kaper, 1994; Aragon et al., 1997; Okuda et al., 1997; Mayer et al., 1999; Pickett and Whitehouse, 1999; Shenker et al., 2000; Saiki et al., 2001; Shenker et al., 2004). In an attempt to detect and identify the total sequences of the *cdtA* and *cdtC* genes, we only succeeded in sequencing the *cdtA* gene partially using the forward primer GNW (Bang et al., 2003) and a self-designed reverse primer. We were not able to amplify the *cdtC* gene. The large variability of these genes among different bacterial agents can be demonstrated by the fact that the partial *cdtA* gene sequence found in *H. pullorum* was not comparable to other *cdtA* gene sequences from the GenBank database. However, the sequence of the deducted amino acids corresponded to that of the cdtA peptide in several thermophilic *Campylobacter* spp. and *H. hepaticus* from the GenBank database following multiple sequence alignment.

At the start of this research, the pathogenic significance of *H. pullorum* in poultry was not clear. We were able to develop a chicken model which is suitable for studying the pathogenesis of *H. pullorum* infection. The animals remained clinically healthy till the age of slaughter. This study was not set up to detect reductions in feed conversion or weight loss. Therefore, we cannot exclude that *H. pullorum* infection may have effects on performance. We did not notice lesions characteristic for vibrionic hepatitis which have earlier been described in *H. pullorum*-infected laying chickens (Burnens et al., 1996). This could be explained by the relatively young age of the broiler chickens at euthanasia. Similar phenomena have been noticed in *H. hepaticus*-infected mice where hepatic lesions only start to develop at an advanced age depending on the mouse strain (Li et al., 1998; Whary et al., 1998).

No laying hens were included in the study and a potential negative outcome of *H. pullorum*-infection in these animals on the egg laying has not been investigated. Further studies may focus on developing a similar *in vivo* model with layers to test the economic impact of *H. pullorum* infection in these animals as well.

Future research needs to focus on the role of *H. pullorum* CDT *in vivo*. This implies the knowledge of the total sequence of the *cdt* gene complex and the purification of the CDT toxin. In addition, the construction of a CDT-negative *H. pullorum* mutant undoubtedly may allow to study the exact role of CDT in pathogenicity of this bacterial agent. A *H. pullorum* mutant with a disrupted CDT coding region may therefore be created similar to that described for *H. hepaticus* (Young et al., 2004).

Furthermore, exploration of new virulence factors is required to entirely elucidate the pathogenicity of *H. pullorum* and its capacity to cause disorders related to liver, intestine and/or pancreas in human beings. In this respect, both *in vitro* assays and animal models need to be developed. *In vitro* research needs to be undertaken to examine the potential adhesion and invasion capacity of this bacterium. *In vitro* assays also may allow to discover toxic activity in *H. pullorum* other than CDT activity linked to the presence of certain virulence genes.

H. pullorum or its DNA has been detected in gallbladder tissue from Chilean women (Fox et al., 1998), liver tissue from hepatocellular carcinoma patients (Ponzetto et al., 2000) and liver and bile from laying hens suffering from vibrionic hepatitis (Burnens et al., 1996). In our studies, *H. pullorum* sonicate was clearly more toxic for a murine hepatocyte cell line than for another epithelial cell culture. This might also indicate a tropism of the bacterium or its metabolites for the liver and perhaps gallbladder. Therefore, further *in vitro* studies using liver cell lines from different host species and the development of *in vivo* models to study liver and gallbladder pathology may be useful. The A/JCr mouse line is known to easily develop hepatic and biliary pathology. This mouse model is moreover a universally accepted model for studying *Helicobacter*-induced pathology in humans and therefore, may provide supplementary information about the interaction of *H. pullorum* with its hosts (Fox et al., 1996; Fox and Lee, 1997).

Finally, the developed chicken model can be further extended by inoculating laying hens in a similar way, keeping broilers and layers for a longer time period with the aim to investigate possible liver injuries and by including more *H. pullorum* strains, both wild type strains and CDT-negative *H. pullorum* mutants.

In conclusion, we demonstrated that *H. pullorum* is present in Belgian broiler chickens. The bacterial agent colonizes the caeca and may remain present in chickens till the age of slaughter. One may deem that these animals are a potential source of infection for human beings due to contamination of the bird's carcasses in the abattoir. We also presented a case report of a *H. pullorum*-infected parakeet potentially constituting another reservoir and source of infection for people. Still, the absence of clear-cut evidence of zoonotic behaviour of *H. pullorum* needs to be underlined. Furthermore, persons both clinically healthy and suffering from gastrointestinal disease may harbour this bacterium fairly frequently. The *H. pullorum* isolates retrieved in these experiments showed decreased susceptibility or were resistant to ampicillin, ceftriaxone and sulphamethoxazole-trimethoprim with consequent implications for the treatment of *H. pullorum*-infected individuals. The contribution of this EHS to human intestinal, hepatic and/or pancreatic disorders is hitherto, however, not very clear. Finally, only little CDT activity of *H. pullorum* was generally noticed in Hep-2 cells. Liver cells on the contrary, revealed damage quite characteristic for CDT. The true role of this toxin *in vivo* nevertheless, remains questionable.

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Summary

Starting approximately two decades ago, research about *Helicobacter* organisms has progressively gained importance. One of the enterohepatic *Helicobacter* species (EHS) was isolated from chickens and humans and assigned *Helicobacter pullorum.* Although the scientific community tagged this species as a potential emerging pathogen, little information about its actual significance was available at the beginning of this thesis.

This thesis starts with an introduction about the relevant literature on the EHS highlighting the need for additional research.

The purpose of the thesis was to investigate the occurrence of *H. pullorum* in poultry and human beings, to study the interaction of *H. pullorum* with its animal host and to analyze some clinical relevant properties of this microorganism.

The experimental research is divided into two main parts, chapters 1 and 2. Chapter 1 includes the prevalence of *H. pullorum* in humans and chickens and its *in vitro* susceptibility to different antimicrobial agents. In chapter 2, bacteria-host interactions are studied both *in vitro* and *in vivo*.

In the first study of chapter 1, the occurrence of *H. pullorum* in broiler chickens was determined.

The caeca, colon, jejunum and liver of 110 animals obtained from 11 different flocks, were tested for the presence of *H. pullorum* using a PCR method based on the 16S rRNA gene; positive samples were re-examined with a conventional isolation method. Therefore, samples were inoculated onto brain heart infusion (BHI) agar supplemented with 10 % horse blood and subsequently incubated in a microaerobic atmosphere at 37°C for minimum three days.

The retrieved *H. pullorum* isolates were examined by amplified fragment length polymorphism (AFLP) fingerprinting for investigating genetic diversity and relatedness between strains.

In the caecum and colon, the PCR reaction for *H. pullorum* gave positive results in 33.6 % and 31.8 % of the samples, respectively. In total, 10.9 % and 4.6 % of all jejunum and liver samples, respectively, were positive for *H. pullorum* DNA.

Sixteen isolates from caecal samples of chickens from two different flocks were obtained. AFLP analysis showed that these isolates and four additional isolates previously obtained from another flock, clustered with respect to their origin. This indicates that *H. pullorum* colonization may occur with a single strain that disseminates throughout the flock. Isolates obtained from different host species or geographical sources, displayed a distinctive pattern.

Because literature data illustrated that this *Helicobacter* species may also be present in human beings, the objective of the second study of chapter 1 was to determine the prevalence of *H. pullorum* both in patients with gastrointestinal disease and clinically healthy people.
In this experiment, faecal material from 531 individuals with gastrointestinal disease and 100 clinically healthy persons was examined for the presence of *H. pullorum* by the same PCR method as used in the first study. Samples proving positive in PCR were selected for isolation purposes in a similar way to the aforementioned study.
H. pullorum DNA was demonstrated in faeces from 4.3 % of patients with gastrointestinal disease, but also from 4.0 % of clinically healthy persons. We were furthermore able to isolate one strain from a patient suffering from diarrhoea.

To complete this first chapter, the *in vitro* activity of 13 antimicrobial agents against 21 poultry and two human *H. pullorum* isolates and one human *H. canadensis* strain was tested by the agar dilution method. With the *H. pullorum* isolates, monomodal distributions of Minimum Inhibitory Concentrations (MICs) were seen with lincomycin, doxycycline, gentamicin, tobramycin, erythromycin, tylosin, metronidazole and enrofloxacin in concentration ranges considered as indicating susceptibility in other bacterial species. The normal susceptibility level for nalidixic acid was situated at or slightly above the MIC breakpoints proposed for *Campylobacteriaceae*. Ampicillin, ceftriaxone and sulphamethoxazole-trimethoprim showed poor activity against *H. pullorum.* With spectinomycin, a bimodal distribution of the MICs was noted for the tested strains; eight *H. pullorum* isolates originating from one flock showed acquired resistance (MIC > 512 µg/ml). For the *H. canadensis* strain, a similar susceptibility pattern was seen, except for nalidixic acid and enrofloxacin, where the MIC was > 512 µg/ml and 8 µg/ml, respectively, indicating resistance.

Chapter 2 is composed of two cardinal parts dealing with pathogen-host interactions examined in *in vitro* assays and an *in vivo* model.

Exploration of the literature urged us to hypothesize that cytolethal distending toxin (CDT) could be present in *H. pullorum*. This toxin is able to cause double strand breaks in the DNA of eukaryotic cells which become enlarged, with proportionally distended nuclei, and finally die due to apoptosis. The effect of *H. pullorum* sonicate was consequently tested on a Hep-2 and a liver cell line in the first two studies of chapter 2.

The presence of the *cdtB* gene among a collection of *H. pullorum* isolates was investigated using PCR in a first experiment described in chapter 2.1.1. We examined herein also the level of the toxic activity and looked for differences between the included isolates. For that reason, epithelial Hep-2 cells inoculated with filtered sonicate from each strain were observed microscopically. Because the presence of enlarged, multinucleated cells is characteristic for CDT activity, DNA analysis using flow cytometry of the treated cells was done as well.

All isolates harboured the *cdtB* gene, but functional CDT activity was only demonstrated in the human *H. pullorum* strain CCUG 33839. A significant number of cells treated with sonicate from this strain were enlarged. The nuclei were distended proportionally. Giant cells and multinucleated cells were observed as well. In addition, stress fibers accumulated. DNA analysis by flow cytometry revealed 31 % of these cells at the S/G_2 stage of the cell cycle. We thus can conclude that the tested poultry and human *H. pullorum* isolates all possess the *cdtB* gene, but that under the circumstances adopted in this study, only the human strain CCUG 33839 showed biological activity typical for CDT *in vitro*.

The description of a cytopathic effect of *H. hepaticus* on liver cells in the middle of the nineties urged us to test filtered cell sonicate from the same *H. pullorum* isolates as adopted in the previous study on the mouse hepatic cell line H 2.35. Chapter 2.1.2 describes this study and discusses the results obtained.

Morphological changes in the cultured liver cells exposed to the cell sonicate were investigated using light microscopy, transmission electron microscopy and time-lapse video microscopy. Additionally, DNA analysis of treated cells was done by flow cytometry.

Cytotoxicity was seen for all *H. pullorum* isolates after incubation for 72 h with different levels of toxic activity. Features characteristic for mitotic catastrophe were noticed involving chromatin condensation, formation of multinuclear distended cells and micronucleation. In

addition, intranuclear pseudoinclusions were observed in sonicate treated cells. Finally, cells exposed to sonicate eventually underwent cell death with the morphological features of necrosis.
The toxic factor proved to be water soluble, trypsin-sensitive and stable at 56°C and at -70°C. The molecular weight was estimated to be over 50 kDa. These characteristics are equal to those described for CDT indicating that this toxin is, at least partially, involved in the morphological changes observed in this cell line.

A final study described in chapter 2.2 constitutes an experiment on the *in vivo* interaction of *H. pullorum* with the chicken host. No experiments in chickens with this bacterial agent had been performed in the past. Four groups of 23 one-day old broilers were inoculated with two strains from poultry and two strains from human origin. A fifth group of eight control animals was inoculated with phosphate-buffered saline. At five time points post-inoculation, up to slaughter age, animals of every group were culled and caecum, colon, jejunum and liver were examined both macroscopically and microscopically. In addition, bacterial titration and PCR analysis for *H. pullorum* were performed on the same samples. Faecal material was also collected on a regular basis for PCR analysis for this bacterial agent.
All animals remained clinically healthy throughout the experiment. At necropsy, there were red streaks on the serosa of the jejunum of animals in the *H. pullorum*-inoculated groups. The caeca of these animals also had dark brownish streaks on the serosa and foamy contents.
The control group remained negative for the presence of *H. pullorum* during the entire experiment. *H. pullorum* was detected from the start till the end of the experiment in all infected groups. The predominant colonization site was the caecum and the bacterium was excreted in the faeces till at least 42 days of age. This implies that broiler chickens may act as a reservoir for *H. pullorum* and may shed the bacteria up to slaughter age, hence constituting a potential source of infection for human beings.

In conclusion, *H. pullorum* appears to be highly prevalent in broilers and may induce mild subclinical lesions. The CDT production may be important for virulence. A limited percentage of people are infected, but the role in human disease remains controversial. The intrinsic antimicrobial susceptibility pattern will need to be taken into account when

envisaging treatment of patients. Future research should focus on the significance of *H. pullorum* in human beings. In addition, long term effects of the infections in layers and/or parent chickens should be examined.

Samenvatting

Sinds ongeveer twee decennia heeft onderzoek over *Helicobacter* organismen enorm aan belang gewonnen. Eén van de enterohepatische *Helicobacter* species (EHS) werd uit kippen en mensen geïsoleerd en *Helicobacter pullorum* genoemd. Hoewel de wetenschappelijke gemeenschap deze species als een mogelijke opkomende pathogeen bestempelde, was er weinig informatie voorhanden over haar daadwerkelijk belang. Op het moment dat het onderzoek beschreven in deze thesis aangevat werd, had men het raden naar de werkelijke prevalentie van *H. pullorum* bij de mens en kip en informatie over virulentiefactoren was heel schaars.

Deze thesis start met een inleiding over de relevante literatuur over de EHS waarin de noodzaak voor verder onderzoek wordt benadrukt.

De doelstelling van deze thesis was het bestuderen van het voorkomen van *H. pullorum* bij pluimvee en mensen, het onderzoeken van de interactie van *H. pullorum* met haar gastheren en het analyseren van enkele klinisch relevante eigenschappen van dit microorganisme.

Het experimentele onderzoek is opgedeeld in twee hoofdstukken. Hoofdstuk 1 omvat de prevalentie en *in vitro* gevoeligheid van *H. pullorum* ten opzichte van verschillende antimicrobiële agentia. In hoofdstuk 2 worden bacterie-gastheer interacties bestudeerd, zowel *in vitro* als *in vivo*.

In de eerste studie van hoofdstuk 1 werd het voorkomen van *H. pullorum* bij braadkippen bepaald.

De caeca, het colon, het jejunum en de lever van 110 dieren afkomstig van 11 tomen, werden getest op de aanwezigheid van *H. pullorum* waarbij gebruik werd gemaakt van een PCR methode gebaseerd op het 16S rRNA gen; positieve stalen werden opnieuw onderzocht door middel van een conventionele isolatie methode. Hierbij werden de stalen geïnoculeerd op brain heart infusion (BHI) agar met 10 % paardenbloed en vervolgens geïncubeerd in een micro-aërofiel milieu bij 37°C gedurende minimum drie dagen.

Genetische diversiteit en verwantschap van de bekomen *H. pullorum* isolaten werden onderzocht door amplified fragment lenght polymorphism (AFLP) 'fingerprinting'.
Wat betreft het caecum en colon, gaf de PCR reactie voor *H. pullorum* positieve resultaten bij respectievelijk 33,6 % en 31,8 % van de stalen. In totaal waren respectievelijk 10,9 % en 4,6 % van alle jejunum en lever stalen positief voor *H. pullorum* DNA.
Zestien isolaten van caecale stalen afkomstig van dieren van twee verschillende tomen werden bekomen. AFLP analyse toonde aan dat deze isolaten en vier voorheen bekomen isolaten van een andere toom, een cluster vormden in relatie tot hun afkomst. Dit duidt erop dat *H. pullorum* kolonisatie kan gebeuren met één enkele stam die zich verspreidt doorheen de toom. Isolaten bekomen uit diverse gastheren of van een verschillende geografische herkomst, vertoonden elk een apart patroon.

Omdat gegevens in de literatuur aantoonden dat deze *Helicobacter* species ook mogelijk voorkomt bij mensen, was de doelstelling van de tweede studie van hoofdstuk 1 het nagaan van de prevalentie van *H. pullorum* in zowel patiënten met gastro-intestinale aandoeningen als klinisch gezonde mensen.
In deze proef werd faecaal materiaal van 531 individuen met gastro-intestinale problemen en van 100 klinisch gezonde personen onderzocht op het voorkomen van *H. pullorum* door middel van dezelfde PCR methode gebruikt in de eerste studie. De PCR positieve stalen werden geselecteerd voor cultivatie die op dezelfde wijze werd uitgevoerd als in de eerste studie.
H. pullorum DNA werd aangetoond in de faeces van 4,3 % van de patiënten met gastro-intestinale aandoeningen, maar eveneens in 4,0 % van de klinisch gezonde personen. We slaagden erin een stam te isoleren uit een patiënt met diarree.

Om dit hoofdstuk te vervolledigen, werd de *in vitro* gevoeligheid van 21 kippen, twee humane *H. pullorum* isolaten en één *H. canadensis* stam tegenover 13 antimicrobiële agentia getest door middel van de agar dilutie methode.
Bij de *H. pullorum* isolaten werd een monomodale verdeling van Minimum Inhibitorische Concentratie (MIC) waarden gezien voor lincomycine, doxycycline, gentamicine, tobramycine, erythromycine, tylosine, metronidazole en enrofloxacine in concentraties welke

beschouwd worden als gevoelig bij andere bacteriële species. De normale gevoeligheid voor nalidixinezuur lag rond of net boven de MIC breekpunten aanbevolen voor *Campylobacteriaceae*. Ampicilline, ceftriaxone en sulphamethoxazole-trimethoprim vertoonden weinig activiteit tegenover *H. pullorum*. Bij spectinomycine werd een bimodale verdeling van MIC waarden opgemerkt voor de geteste stammen; acht *H. pullorum* isolaten afkomstig van éénzelfde toom vertoonden verworven resistentie (MIC > 512 µg/ml). Wat de *H. canadensis* stam betreft, werd een gelijkaardig gevoeligheidspatroon waargenomen, behalve voor nalidixinezuur en enrofloxacine waar de MIC waarden van respectievelijk > 512 µg/ml en 8 µg/ml aantonen dat dit agens resistent is aan deze antimicrobiële middelen.

Hoofdstuk 2 bestaat uit twee grote delen met betrekking tot pathogeen-gastheer interacties die onderzocht werden in zowel *in vitro* proeven als in een *in vivo* model.
Het doornemen van de relevante literatuur deed ons vermoeden dat het "cytolethal distending toxin" (CDT) aanwezig zou kunnen zijn bij *H. pullorum*. Dit toxine is in staat om het DNA ter hoogte van beide strengen in eukaryotische cellen te beschadigen die vervolgens opzwellen met binnenin proportioneel vergrote kernen en uiteindelijk sterven door apoptose. Het effect van *H. pullorum* sonicaat werd daarom getest op een Hep-2 en een levercellijn in de eerste twee studies van hoofdstuk 2.
Het voorkomen van het *cdtB* gen bij een collectie *H. pullorum* isolaten werd onderzocht door middel van een PCR techniek in een eerste experiment dat is beschreven in hoofdstuk 2.1.1. We onderzochten ook de graad van toxische activiteit en keken naar verschillen tussen de ingesloten isolaten. Hiervoor werden epitheliale Hep-2 cellen geïnoculeerd met gefilterd sonicaat van elke stam afzonderlijk, microscopisch bekeken. Omdat de aanwezigheid van vergrote, meerkernige cellen karakteristiek is voor CDT activiteit, werd eveneens DNA analyse van de behandelde cellen door middel van flowcytometrie uitgevoerd.
Alle isolaten hadden het *cdtB* gen, maar functionele CDT activiteit werd enkel aangetoond in de humane *H. pullorum* stam CCUG 33839. Een significant aantal cellen behandeld met sonicaat van deze stam waren opgezwollen. De kernen waren proportioneel vergroot. Reuzencellen en meerkernige cellen werden eveneens opgemerkt. Voorts was er accumulatie van stressvezels zichtbaar. DNA analyse door flowcytometrie toonde aan dat 31 % van deze cellen zich bevond in de S/G_2 fase van de celcyclus.

We kunnen dus concluderen dat alle geteste kippen en humane *H. pullorum* isolaten het *cdtB* gen bevatten, maar dat onder de omstandigheden in deze studie enkel de humane stam CCUG 33839 biologische activiteit typisch voor CDT *in vitro* vertoonde.

In het midden van de jaren negentig werd een cytopathisch effect van *H. hepaticus* op muizencellen beschreven. Dit zette ons aan om gefilterd celsonicaat van dezelfde *H. pullorum* isolaten als gebruikt in de vorige studie, te testen op de muizenlevercellijn H 2.35. Hoofdstuk 2.1.2 beschrijft deze studie en bediscussieert de bekomen resultaten.
Morfologische veranderingen in de levercellijn blootgesteld aan het celsonicaat werden licht-, transmissie elektronen en time-lapse video microscopisch onderzocht. Bijkomend werd DNA analyse van de behandelde cellen uitgevoerd door flowcytometrie.
Cytotoxiciteit werd gezien voor alle *H. pullorum* isolaten na een 72 uur durende incubatie met verschillende gradaties van toxische activiteit. Kenmerken typisch voor mitotische catastrofe werden opgemerkt zoals chromatine condensatie, vorming van meerkernige, vergrote cellen en micronucleatie. Intranucleaire pseudo-inclusies werden ook teruggevonden in met sonicaat behandelde cellen. Uiteindelijk stierven alle cellen die behandeld werden met sonicaat af en vertoonden deze morfologische kenmerken van necrose.
De toxische factor was wateroplosbaar, trypsinegevoelig en stabiel bij 56°C en -70°C. Het moleculaire gewicht bedroeg meer dan 50 kDa. Deze karakteristieken komen overeen met deze van CDT wat een indicatie kan zijn dat dit toxine, minstens gedeeltelijk, betrokken is bij de morfologische veranderingen opgemerkt in deze cellijn.

Een laatste studie, beschreven in hoofdstuk 2.2, omvat een experiment over de *in vivo* interactie van *H. pullorum* met haar kippengastheer. In het verleden werden nog geen experimentele infecties met dit agens bij kippen uitgevoerd. Vier groepen van 23 ééndagskuikens werden geïnoculeerd met twee stammen van kippen en twee stammen van humane oorsprong. Een vijfde groep van acht controledieren werd geïnoculeerd met fosfaat gebufferde zoutoplossing. Dieren werden opgeofferd op vijf tijdstippen post-inoculatie tot op slachtleeftijd en caecum, colon, jejunum en lever werden zowel macro- als microscopisch onderzocht. Bijkomend werden bacteriële titratie en PCR analyse voor *H. pullorum*

uitgevoerd. Eveneens werd faecaal materiaal op regelmatige tijdstippen verzameld voor PCR analyse van dit bacterieel agens.
Alle dieren bleven klinisch gezond doorheen het volledige experiment. Bij autopsie werden rode strepen op de serosale zijde van het jejunum opgemerkt bij dieren geïnoculeerd met *H. pullorum*. De caeca van deze dieren vertoonden eveneens donkerbruine strepen ter hoogte van de serosa en een schuimige inhoud.
De controlegroep bleef negatief op de aanwezigheid van *H. pullorum* gedurende het volledige experiment. *H. pullorum* werd gedetecteerd vanaf de start tot het einde van de proef in alle geïnfecteerde groepen. De voornaamste kolonisatieplaats was het caecum en de bacterie werd in de faeces uitgescheiden tot op een leeftijd van tenminste 42 dagen. Dit duidt erop dat braadkippen kunnen fungeren als reservoir voor *H. pullorum* en mogelijk de kiem uitscheiden tot op slachtleeftijd. Zij vormen dus een potentiële infectiebron voor de mens.

Als besluit kan worden gesteld dat *H. pullorum* in hoge mate voorkomt bij braadkippen en mogelijk milde subklinische letsels induceert. De CDT productie zou belangrijk kunnen zijn voor virulentie. Een beperkt aantal mensen is besmet met *H. pullorum*, maar de klinische betekenis van infecties met dit microorganisme voor de mens blijft omstreden. Dit bacterieel agens is van nature weinig gevoelig aan ampicilline, ceftriaxone en sulphamethoxazole-trimethoprim, wat in acht moet worden genomen tijdens de behandeling van patiënten. Verder onderzoek naar het belang van *H. pullorum* bij de mens is zeker een noodzaak. Eveneens zouden de effecten van een infectie met *H. pullorum* bij leghennen en/of ouderkippen moeten worden onderzocht.

Author's curriculum

Liesbeth Ceelen werd geboren op 8 maart 1979 te Leuven. Na het behalen van het diploma hoger secundair onderwijs, richting Grieks-wiskunde, aan het Paridaens Instituut te Leuven, begon ze in 1997 met de studies Diergeneeskunde aan de Universiteit van Antwerpen (RUCA). In 2000 slaagde ze aan deze universiteit met de grootste onderscheiding en ontving de "Prijs decanaat Faculteit Medische en Farmaceutische Wetenschappen, Universiteit Antwerpen" voor beste studente in de derde kandidatuur Diergeneeskunde. De studies werden verder gezet aan de Universiteit van Gent, waar ze in 2003 afstudeerde als dierenarts, met grote onderscheiding.

Onmiddellijk daarna trad zij in dienst bij de vakgroep Pathologie, Bacteriologie en Pluimveeziekten en ontving ze een IWT beurs voor het uitvoeren van wetenschappelijk onderzoek met als titel "*Helicobacter pullorum*: oorzaak van enterohepatische aandoeningen bij de mens en pluimvee?"

In 2006 behaalde ze aan de Universiteit van Gent het diploma "Master of Laboratory Animal Science" categorie D volgens FELASA (Federation of European Laboratory Animal Science Associations) met grote onderscheiding en voltooide ze eveneens de doctoraatsopleiding in de Diergeneeskundige Wetenschappen.

Liesbeth Ceelen is auteur of mede-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften. Zij was meermaals spreker op internationale congressen.

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