CONCISE COMMUNICATION

Cytolethal Distending Toxin in Avian and Human Isolates of Helicobacter pullorum

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Helicobacter pullorum has been isolated from the feces and livers of poultry and is associated with human gastroenteritis. Discrimination of this organism from other enterohepatic Helicobacter species and Campylobacter species has proven difficult. H. pullorum from both avian and human clinical sources has DNA sequence homology and cytotoxic activity that represent a new member of the cytolethal distending toxin (CDT) family of bacterial toxins. CDT is a potential virulence factor in H. pullorum that may serve as a distinguishing phenotype and aid in identification of this organism in veterinary and human clinical samples.

Enterohepatic Helicobacter species (EHSs) are emerging as important pathogens in the genus Helicobacter [1]. Similar to gastric Helicobacter species such as H. pylori, EHSs are responsible for persistent infections associated with chronic inflammation and neoplasia [1]. However, EHSs, as the name implies, colonize the biliary tree and distal gastrointestinal tract. H. pullorum is an EHS originally isolated from 3 different sources: the ceca of asymptomatic poultry, the livers and intestinal contents of hens with lesions suggestive of vibriotic hepatitis, and the feces of human patients with gastroenteritis [2, 3]. It has been isolated in both immunocompetent and immunocompromised patients suffering from acute gastroenteritis [4].

Because H. pullorum can be isolated from poultry and shares culture characteristics with Campylobacter species, it has been classified incorrectly as an atypical Campylobacter when isolated from avian and human sources [3–5]. Thus, it has been suggested that the true prevalence of H. pullorum in poultry and in human and animal disease has been underrepresented [4, 5].

Cytolethal distending toxin (CDT) activity has been reported in a number of EHSs [6, 7]. CDT activity is characterized by the appearance of cellular distension, cytoskeletal abnormalities, G/M cell-cycle arrest, and cytolethality in cultured cell lines treated with bacterial culture supernatants or sonicates of bacteria expressing the toxin [6, 7]. CDT activity is encoded by 3 closely linked genes, cdtA, cdtB, and cdtC, all 3 of which are necessary to transfer CDT activity to a laboratory strain of Escherichia coli [7].

The cdt DNA sequence has been reported to be invariably present in various Campylobacter species [8, 9] isolated from animal and human sources. In contrast to what has been observed in Campylobacter species, not all of the EHSs appear to possess CDT activity and cdt genes. The cdt gene cluster and CDT activity have been identified in H. hepaticus, H. bilis, H. canis, and 2 novel Helicobacter species isolated from mice and woodchucks [6, 7]. H. bilis, first isolated from diseased livers of mice [1], has also been identified in the bile and gallbladder tissues of human patients [10]. H. canis has been isolated from dogs and cats with and without diarrhea, from a diseased canine liver, and from a child with diarrhea [1]. However, H. fennelliae and H. cinaedi, 2 EHSs originally identified in immunocompromised patients with enteritis, chronic bacteremia, or both [11], do not appear to possess the cdt gene cluster, nor do they produce CDT activity [7]. In the current study, we determined whether cdt DNA sequence and CDT activity were present in a series of clinical and avian isolates of H. pullorum. In addition, we investigated whether CDT status could be used as a novel tool in the species identification of this emerging group of gastrointestinal pathogens.

Materials and Methods

Bacteria and cell lines. The bacteria used in this study are listed in table 1. H. pullorum reference strains NCTC12824 (type strain), NCTC12825, and NCTC12827 were obtained from the American Type Culture Collection (ATCC; Manassas, VA). A series of 8 clinical isolates of microaerobic bacteria from patients with gas-
troenteritis that had been submitted to the National Laboratory for Enteric Pathogens in Winnipeg, Manitoba, Canada, by public health laboratories from across Canada were provided by David Woodward (National Laboratory for Enteric Pathogens) [12]. These isolates were provisionally identified as *H. pullorum* [12]. Other strain number Source Reference CDT titerb Cell-cycle arrest

<table>
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<th>Straina</th>
<th>Other strain number</th>
<th>Source</th>
<th>Reference</th>
<th>CDT PCR</th>
<th>CDT titerb</th>
<th>Cell-cycle arrest</th>
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*NOTE.* CDT, cytolethal distending toxin; PCR, polymerase chain reaction; HIV, human immunodeficiency virus.

a Strains were isolated from feces or from cecal contents.

b Amount of sonicate, expressed in micrograms of total protein, required to cause cytopathic effect in 50% of cells in a HeLa cell monolayer.

c Type strain.

Results

Identification of a CDT homologue in *H. pullorum*. The degenerative primers VAT2, WM11, and DHF1, which are based on the amino acid sequence of the *cdtB* subunit [6, 9], produced PCR amplicons of the expected sizes (500 bp for the VAT2/WM11 primer pair and 700 bp for the VAT2/DHF1 primer pair) when used to amplify genomic DNA from *H. pullorum* strain NCTC 12827, which was originally isolated from a patient infected with human immunodeficiency virus who had gastroenteritis [2, 12]. The sequence of the amplicon produced by the VAT2/DHF1 primer pair was determined and was most similar to *cdtB* from *H. hepaticus*, with 61% identity (data not shown). The deduced amino acid sequence was most similar to the *H. hepaticus* CdtB protein, with 73% identity and 87% similarity [7].

Given the presence of *cdtB* gene sequence homology in *H. pullorum*, we determined whether the organism produced characteristic CDT cytotoxic activity. Sterile sonicates of *H. pullorum* produced cellular distension and cell-cycle arrest at the
G2/M phase of the cell cycle (figure 1) in culture cells. This cytopathic effect and cell-cycle arrest was identical to that which had been described for the EHS *H. hepaticus* [7], although it appears that *H. hepaticus* sonicates possess more CDT activity per microgram of protein (table 1). As previously reported for *H. hepaticus* [7], maximal cytopathic effect was seen at 72 h, and by 96 h cells began to detach from the monolayer.

**CDT expression in clinical strains of *H. pullorum***. A series of 8 clinical isolates, isolated from Canadian patients with gastroenteritis and identified as *H. pullorum* on the basis of phenotypic and genotypic characterization, were obtained [12]. These clinical isolates and 3 reference strains of *H. pullorum* [3] were tested for the presence of the *cdtB* gene by PCR, as well as for production of a CDT cytopathic effect and cell-cycle arrest on 293 cells (table 1). All the reference strains and 6 of 8 clinical isolates were positive for the presence of *cdtB* by PCR. All strains that were positive by PCR also produced a CDT cytopathic effect and cell-cycle arrest, whereas the 2 strains that were negative for CDT by PCR failed to produce a cytopathic effect or cell-cycle arrest.

To further characterize the 2 clinical strains that were negative for CDT sequence and activity, the 16S rRNA sequence of these 2 isolates was determined. Although the *H. pullorum* species-specific PCR primers [3] produced the expected 422-bp amplicon on these 2 strains, the complete 16S rRNA analysis revealed that these strains actually represent a novel *Helicobacter* species closely related to, but distinct from, *H. pullorum* [13].

**Discussion**

A number of EHSs, including *H. fennelliae, H. cinaedi, H. westmeadii, H. pullorum, H. canis*, and *H. rappini*, have been associated with acute infections in human patients [1–3, 11]. In normal hosts, they have been implicated in the development of gastroenteritis, but they also cause invasive disease, including bacteremia and septic arthritis, in immunocompromised patients [11]. Recently, evidence of infection with EHSs, including *H. pullorum*, has been demonstrated in the bile and gallbladder.

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**Figure 1.** Cytopathic effect and cell-cycle arrest induced by *Helicobacter pullorum* cytolethal distending toxin on cultured mammalian cells. Compared with control cells, cells treated with sonicates from *H. pullorum* exhibited marked cytoplasmic distension along with nuclear enlargement and (in ~10%–15% of cells) multinucleation. Analysis of DNA content by flow cytometry revealed an accumulation of cells with 4N and 8N DNA content in monolayers treated with *H. pullorum* sonicates. N, haploid DNA content. Bar, 20 μm.
tissue of Chilean patients with a high incidence of chronic cholecystitis and gallbladder cancer [10].

The accurate identification of the EHSs and the closely related Campylobacter and Arcobacter species has been problematic [14]. The identification of H. pullorum with phenotypic markers has proven to be difficult, and it can be mistaken for Campylobacter species, particularly C. coli and C. lari [5]. Genotypic methods for species identification have been proposed as an adjunct to phenotypic characterization of H. pullorum and other microaerobic spiral bacteria [3, 14, 15]. The initial description of H. pullorum introduced a primer pair that was able to distinguish H. pullorum from the known Helicobacter, Campylobacter, and Arcobacter species that had been characterized at that time [3]. Our results indicate that genotypic methods used to identify these organisms can also be difficult to interpret. Two clinical isolates that were identified as H. pullorum on the basis of the aforementioned species-specific PCR assay were determined to belong to a novel Helicobacter species when the entire 16S rRNA sequence was determined [13]. There were other indications that these 2 strains were distinct from H. pullorum. When the 8 clinical isolates were characterized for genetic diversity by amplified fragment length polymorphism and pulsed field gel electrophoretic analysis, these 2 CDT-negative strains were similar to each other but distinct from other isolates of H. pullorum [12]. In addition, these strains were positive for indoxyl acetate hydrolysis, which is not true for reference strains of H. pullorum.

Ultimately, a combination of genotypic and phenotypic methods may be most useful in the identification of H. pullorum and related organisms. We describe here a novel determinant that could prove useful in the classification of microaerobic spiral bacteria. H. pullorum possesses DNA sequence homology and cytotoxic activity that belong to the CDT family of bacterial toxins [16]. The presence of cdtB sequence homology and production of CDT distinguished H. pullorum from a closely related novel Helicobacter species. Thus EHSs can be divided into those species that possess CDT and those that do not. This is in contrast to published data in which all Campylobacter species studied thus far appear to have cdt gene sequences, although levels of toxin production may vary [8, 9]. PCR–restriction fragment length polymorphism analysis of the cdt gene cluster has been proposed as a method for the speciation of C. jejuni and C. coli [8].

The role of CDT in clinical disease produced by EHSs or Campylobacter species is not known. However, because CDT is not present in the EHSs H. fennelliae and H. cinaedi, which have a predilection for immunocompromised patients, CDT may have a role in the escape of immune surveillance. Evidence from studies of the CDT produced by other bacteria supports the theory that elements of the immune system could be targets of CDT in vivo [16].

References