**Campylobacter concisus**: an evaluation of certain phenotypic and genotypic characteristics

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**ABSTRACT**

The clinical relevance of *Campylobacter concisus* in gastrointestinal disease has not been determined definitively. This study investigated the phenotypic and genotypic characteristics of 39 *C. concisus* isolates from Danish patients with diarrhoea, three isolates from healthy individuals and the type strain. A cytolethal distending toxin (CDT)-like effect on Vero cells was observed in 35 (90%) isolates from patients with diarrhoea, in all three isolates from healthy individuals and in the type strain. Analysis of SDS-PAGE protein profiles and PCR amplification of 23S rDNA assigned the isolates into two distinct, but discordant groups. Automated ribotyping (RiboPrinting) identified 34 distinct patterns among the 43 isolates, but cluster analysis did not separate isolates from patients with diarrhoea from isolates from healthy patients. Random amplified polymorphic DNA (RAPD) analysis with three primers identified 37 unique profiles, but requires further evaluation. The isolates obtained from healthy carriers were distinguished by cluster analysis from the isolates obtained from patients with diarrhoea. All the isolates were susceptible to 11 antimicrobial agents tested. Overall, there was considerable variability between the *C. concisus* isolates, but there were no clear phenotypic or genotypic differences between isolates from patients with diarrhoea and isolates from healthy carriers. Further evidence is needed to support the possible role of *C. concisus* as a human enteric pathogen.

**Keywords** Antimicrobial susceptibility, *Campylobacter concisus*, protein profile, RAPD, toxin, typing

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**INTRODUCTION**

*Campylobacter concisus*, a fastidious and slow-growing Gram-negative bacillus found in the human oral cavity, was first described in 1981 [1]. The species has been isolated in higher proportions from relatively shallow and healthy oral sites, suggesting that *C. concisus* is an oral commensal rather than a clinically significant oral pathogen [2]. In addition, *C. concisus* can be isolated from the faeces of patients with diarrhoea, as well as from healthy individuals. Therefore, the possible role of *C. concisus* as a gastrointestinal pathogen has not yet been established firmly [3–7].

In contrast to the well-known human pathogens *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter lari* and *Campylobacter upsaliensis*, *C. concisus* has no known animal hosts, suggesting that *C. concisus* is highly adapted to the human gastrointestinal tract. Inter-personal spread may be an important route of transmission, but is regarded as a relatively uncommon phenomenon for thermophilic *Campylobacter* spp. In a previous study, differences were observed in the age distribution of *C. concisus* patients, compared to the age distribution of patients with *C. jejuni/C. coli* infections [5]. Most isolates were from the very young or elderly, indicating that *C. concisus* is an opportunistic pathogen for patients with immature or compromised immune systems. *C. concisus* is a genotypically heterogeneous species, and this may explain the conflicting reports of its pathogenic potential.
Multiple genomospecies within the species have been described [6–11]. Several potential virulence factors have been proposed for *Campylobacter* spp., including flagella-mediated motility, adhesion to intestinal mucosa, invasion of the epithelial cells of the intestine, and the ability to translocate and to produce toxin. However, the specific virulence mechanisms of *Campylobacter* spp. have not yet been elucidated adequately [12]. The cytolethal distending toxin (CDT) is the most characterised of the *Campylobacter* toxins, and is encoded by the three adjacent genes *cdtA, cdtB* and *cdtC* in *C. jejuni*. The function of CDT in *Campylobacter* pathogenesis is unknown, but it has been shown that *C. jejuni* CDT causes sensitive cells to be blocked in the G2 phase of their cell cycle.

The present study was conducted to determine the phenotypic and genotypic heterogeneity, and the pathogenic potential, of a collection of *C. concisus* isolates.

**MATERIALS AND METHODS**

**Bacterial strains**

Thirty-nine *C. concisus* isolates from Danish patients with diarrhoea, and three from healthy individuals, were studied. All the *C. concisus* isolates were collected during a previous study of the prevalence of campylobacters and related organisms in faeces [5]. Five of the *C. concisus*-positive clinical cases were coinfect with an established bacterial enteric pathogen (three with *Salmonella enterica* subsp. *enterica* serovar Enteritidis, one with *Shigella sonnei*, and one with *Yersinia enterocolitica*). No patients or healthy individuals were related epidemiologically. The *C. concisus* type strain CCUG 13144 was included in all assays, and the identity of the clinical isolates was reconfirmed by including the type strains of *Campylobacter mucosalis*, *C. upsaliensis* and *C. concisus* on all protein profiling gels. Growth conditions were as described below.

**Phenotypic analysis**

*CDT-like activity by Vero cell assay*

Bacterial cell-free supernatants and Vero cells were prepared as described previously [13,14] with modifications [15]. *Campylobacter* cell-free filtrate (50 μL) was added in duplicate at the desired dilutions. A cell-free filtrate from a Vero toxin-producing *Escherichia coli* (VTEC strain H19, serotype O26:H11) was used as a positive control, with Veal Infusion Broth as a negative control. The controls were added in duplicate to each plate in every assay. The CDT-like effect was monitored daily for 3–6 days by phase-contrast microscopy. The CDT-like titre of the supernatants was determined by performing two-fold serial dilutions in a 96-well microtitre plate. The titre of a given assay was expressed as the reciprocal of the highest dilution that caused at least 30% of the cells in a well to be rounded or distended. Toxin production by each *C. concisus* isolate was tested in at least three independent assays with culture supernatants prepared at different times.

**Antimicrobial susceptibility testing**

MICs were determined by the agar dilution method on Mueller-Hinton-II agar supplemented with bovine blood 5% v/v [16]. Plates were incubated for 48 h at 37°C in a microaerobic atmosphere (O2 6%, CO2 7%, H2 7%, N2 80%, v/v). The MIC was defined as the lowest concentration that produced no visible growth. The dilution ranges (mg/L) and antimicrobial agents (Sigma Chemical Company, St Louis, MO, USA) tested were as follows: erythromycin and tetracycline, 0.25–32; nalidixic acid and streptomycin, 1–128; ciprofloxacin, 0.03–16; gentamicin, 0.5–32; colistin and chloramphenicol, 0.5–64; sulphamethizole, 8–512; neomycin, 1–64; and ampicillin 1–32.

**Whole-cell protein profiling**

This was performed as described previously [6], with the modification that *C. concisus* was harvested from blood agar 5% v/v plates containing yeast extract 1% w/v (SSI Diagnostic, Hillerød, Denmark).

**Molecular analysis**

**PCR amplification of 23S rDNA**

This was performed by the method of Bastyns et al. [17] with modifications [11]. The method was modified by using the two reverse primers (CON1 and CON2) independently, rather than as a mixture, and was used to group the isolates. Sequences of 23S rDNA were amplified using the following primer pairs: mixture A, forward primer MUC1 (5'-ATGAGTAGCGAT-AATTGCGG-3'); mixture B, forward primer MUC1 and reverse primer CON2 (5'-GACAGTATCAAGGATTACC-3'). The sequences of the two reverse primers, CON1 and CON2, are significantly different, but yield a similar-sized PCR fragment (306 bp). Isolates were grouped according to their PCR product with either of the reverse primer sets A or B.

**Automated ribotyping**

This was performed using a RiboPrinter (DuPont Qualicon, Wilmington, DE, USA), as described previously [18], with the modification that *Pvu* II was used instead of *Hae* III. The riboprint profiles were aligned according to the position of a molecular size standard on the RiboPrinter and were then exported to a BioNumerics database (Applied Maths, Sint-Martens-Latem, Belgium), and bands were assigned to the profiles. These were compared with the Dice coefficient (position tolerance 1.0%, optimisation 0.5%), and a clustering dendrogram was produced by the unweighted pair-group method with arithmetic averages (UPGMA). Any discernible difference (i.e., a change of band positions and/or the loss or gain of one or more bands) between patterns was considered significant when assessing inter-strain relationships.

**Random amplified polymorphic DNA (RAPD) analysis**

In brief, RAPD analysis was performed with Ready-To-Go RAPD Analysis Beads (Pharmacia Biotech, Freiburg, Germany), containing pre-mixed, pre-dispersed AmpliTaq enzyme mixture A, forward primer MUC1 (5'-ATGAGTAGCGAT-AATTGCGG-3'); mixture B, forward primer MUC1 and reverse primer CON2 (5'-GACAGTATCAAGGATTACC-3'). The sequences of the two reverse primers, CON1 and CON2, are significantly different, but yield a similar-sized PCR fragment (306 bp). Isolates were grouped according to their PCR product with either of the reverse primer sets A or B.

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DNA polymerase, as well as all necessary buffer ingredients and nucleotides. Template DNA extraction procedures and cycling parameters were as described previously [18]. The type strain CCUG 13144 was included in each batch of assays as a positive amplification control, and all the isolates were tested in two independent experiments on separate days to ensure the reproducibility of the results. Fluorescently labelled primers 1281, 1254 and HLWL85 were used in three independent amplifications, and the resultant PCR products were detected on an ABI PRISM 310 (Applied Biosystems, Allerod, Denmark). Profiles were analysed with BioNumerics software using Pearson’s product-moment similarity coefficient and UPGMA clustering to determine profile relatedness. The isolates were grouped according to combined profiles based on each of the three primers, with a cut-off level of 70% similarity.

RESULTS
CDT-like activity and antimicrobial susceptibility
A CDT-like effect on Vero cells was detected in 35 (90%) of 39 isolates from patients with diarrhoea, in all three (100%) isolates from healthy individuals, as well as in the C. concisus type strain. Repeat tests with a given supernatant were fully reproducible, and in at least three independent assays, toxin production by each isolate did not vary by more than one dilution step. Six (14%) isolates, including one from a healthy individual and the type strain, produced high (1:64) CDT-like titres, whereas 11 (25.6%) isolates, including one from a healthy individual, produced a low (1:8) CDT-like titre. The remaining 26 (60.4%) isolates expressed intermediate CDT-like titres of 1:16 and 1:32. All 43 isolates in this study were susceptible to all 11 antimicrobial agents tested (data not shown).

SDS-PAGE of proteins
The 43 isolates could be divided into two variants: group 1, which contained the C. concisus type strain CCUG 13144 (of oral origin) and five isolates from patients with diarrhoea; and group 2, which differed from group 1 by having additional bands at 200 kDa and 31 kDa. All three isolates from healthy individuals belonged to group 2. Considerable heterogeneity was found in the lower regions of the gels.

PCR amplification of 23S rDNA
By using primer combinations of either MUC1-CON1 or MUC-CON2, the isolates could be assigned into two distinct genotypes, with 14 (33.3%) of the 42 clinical isolates in genotype 1, and 28 (66.7%) isolates in genotype 2. Of the three isolates from healthy individuals, two were assigned to genotype 1, and one to genotype 2.

Automated ribotyping
RiboPrinting differentiated the 43 isolates into 34 distinct patterns (RiboGroups). Seven RiboGroups each contained two isolates, and one RiboGroup contained three isolates. One isolate (no. 1091) from a healthy individual was in the same RiboGroup as an isolate from a patient with gastroenteritis (Fig. 1). The type strain, originally isolated from the oral cavity of a patient with periodontal disease, belonged to the same RiboGroup as a gastroenteritis-related isolate. The remaining two isolates from healthy carriers (6118 and 10375) had unique riboprints and showed less relatedness to the major cluster (Fig. 1).

RAPD typing
Analysis of combined RAPD-DNA profiles, based on each of the three primers, identified 37 unique reproducible profiles. Six isolates were not tested with this method. The two tested isolates (6118 and 1091) from healthy carriers clustered separately from the isolates obtained from patients with diarrhoea and from the type strain (Fig. 2).

DISCUSSION
The definitive identification of non-oral strains of C. concisus, isolated from a number of different sites throughout the human gastrointestinal tract, including faeces, was described in 1989 [8]. However, knowledge is still limited regarding the clinical importance of C. concisus, the occurrence of virulence factors, and genetic diversity. This may be because the isolation techniques used currently in many diagnostic laboratories may not support the growth of C. concisus and other potentially pathogenic non-jejuni/coli Campylobacter spp. These organisms may be fastidious, requiring an H2-enhanced microaerobic atmosphere, special temperature conditions and prolonged incubation, or may be unable to tolerate the antimicrobial agents included commonly in
selective media [19–22]. Furthermore, correct identification may require additional advanced techniques [5,23–26]. Therefore, isolation of this species may be restricted to clinical microbiology laboratories with special interests in C. concisus, as this species, at present, is not an established intestinal pathogen.

The present study detected a CDT-like effect on Vero cells in 35 (90%) of 39 isolates from patients with diarrhoea, and in all three isolates from healthy individuals. The isolates from healthy individuals differed from each other with respect to toxin activity, but this activity was comparable to that observed among gastroenteritis-associated isolates. The type strain, isolated originally from the oral cavity of a patient with periodontal disease, also showed high CDT-like activity, suggesting that this marker is not adequate for identifying potential gastroenteric pathotypes of C. concisus.

In C. jejuni, CDT is the most characterised of the putative Campylobacter toxins. All human isolates of C. jejuni seem to contain the cdt gene locus, although differences in expression of the cdt gene exist [27,28]. It has been shown that C. jejuni CDT causes progressive cellular distension, and ultimately death, in Chinese hamster ovary (CHO), Vero, Hep-2 and HeLa cells [29]. The cdt genes in C. jejuni have been cloned and sequenced [30], and the cdtA, cdtB and cdtC genes encode proteins with predicted molecular sizes of 30.1, 29.0 and 21.1 kDa, respectively. The function of CDT proteins in Campylobacter pathogenesis is unknown, although it has been shown that C. jejuni CDT causes sensitive cells to be blocked in the G2 phase of their cell cycle, indicating that the CDT has a mechanism of action different from that of other bacterial toxins [31,32]. A direct causal role of CDT in campylobacteriosis remains to be demonstrated, although a significant relationship

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Fig. 1. RiboGroup patterns of 43 isolates ordered according to their similarity, using the unweighted pair-group method with averages (UPGMA) clustering. The Dice similarity coefficient was used. The three isolates from healthy carriers are underlined. The scale refers to the percentage of similarity.
between C. jejuni in-vitro toxigenicity (to Hep-2 and Vero cells) and the development of post-infectious irritable bowel syndrome has been reported [12,33]. Whether the CDT-like activity observed in the present study is a result of the expression of C. concisus cdt genes remains to be proved following cloning of the C. concisus cdt genes. However, Vero cells treated with cell-free supernatants of the C. concisus isolates were enlarged markedly (c.2–5-fold) in comparison with control cells (results not shown), which is a common phenomenon observed with Vero cells treated with the supernatant of C. jejuni CDT-producing strains, as well as the supernatant from other CDT-producing bacteria.

Virulence factors of C. concisus have only been investigated in a few studies [11,34–36]. Russell et al. [34] found that ten clinical C. concisus isolates adhered to and invaded Hep-2 cells more efficiently than did C. jejuni and C. coli controls, although only one strain each of C. jejuni and C. coli were tested. Musmanno et al. [35] studied two clinical isolates of C. concisus, both of which failed to adhere to or invade cells, although one isolate produced a cytotoxic effect (elongation of cells) on CHO cells, similar to that shown by C. jejuni subsp. doylei, and induced intracytoplasmatic vacuole formation similar to that caused by cytotoxic Helicobacter pylori. Istivan et al. [11] characterised a haemolytic phospholipase A2 activity in 19 clinical C. concisus isolates, all of which produced vacuolating and cytolytic effects on CHO cells in tissue culture and contained the pldA gene, homologous to the pldA gene in C. coli. An animal model, using BALB/cA mice pretreated with cyclophosphamide and intragastrically challenged with C. concisus, showed transient colonisation of the liver and ileum, and signs of gastrointestinal disease, including weight loss and loose stools. However, histological examination did not find evidence of infection in colonised organs, and the findings were not reproducible in two later studies, possibly because of rapid clearance of the organism [37].

All 43 isolates in the present study were fully susceptible to all 11 antimicrobial agents tested,
including erythromycin and nalidixic acid. Little published information is available regarding the antimicrobial susceptibility of this species. Greig et al. [38] found that eight isolates from South Africa were all sensitive to ciprofloxacin, tetracycline, ampicillin and gentamicin, although seven were resistant to erythromycin, while Van Etterijck et al. [3] found that 51% of isolates from Belgium were resistant to nalidixic acid. The drug of choice for treatment of Campylobacter infections is normally erythromycin or a fluoroquinolone [22], although increasing numbers of resistant C. coli and C. jejuni isolates are now being reported worldwide [39]. However, it seems that resistance has not emerged in C. concisus, and the present study suggests that erythromycin (or a newer macrolide) should be considered if treatment with antimicrobial agents is needed for C. concisus infections.

C. concisus is considered to comprise at least two genomospecies [8,9]. In the present study, SDS-PAGE divided the 43 isolates into two broad variants, containing 14% and 86% of the isolates, respectively. The protein bands of lower molecular size were highly heterogeneous, indicating that this size range may be used to further subdivide the groups in future studies and increase the discriminatory index of this typing method. The prevalence of the two groups in the present study was comparable to that found in a previous study [6] of C. concisus isolates from Denmark, in which the distribution of isolates in groups 1 and 2 was 15% (15/98) and 85% (82/98), respectively, and patients infected with group 2 displayed more signs of infection than patients infected with group 1, although this latter observation was not statistically significant. Another Danish study [40] evaluated 44 isolates from patients with diarrhoea for their reactions with plant lectins. Although the lectin typing system was both stable and reproducible, no correlation between lectin reaction pattern and patient category was observed.

The present study used PCR amplification of the 23S rDNA region to assign the isolates into two definitive genotypes, genotype 1 (MUC1-CON1) and genotype 2 (MUC1-CON2), comprising 33.3% and 66.7% of the isolates, respectively. In contrast, the relative frequencies of the same genotypes among 21 Australian clinical isolates and reference strains were 71.4% and 28.6%, respectively [11] (T. S. Istivan, personal communication). DNA:DNA hybridisation experiments [8] with a number of reference strains, including CCUG 13144 (type strain) in the present study, and CCUG 20034 in the study by Istivan et al. [11], revealed that these two strains belonged to different DNA subgroups, in agreement with the combined Australian and Danish results, which also separated these two strains into two different groups following repeated testing, thereby indicating that a true difference in the geographical distribution of these genotypes is the most plausible explanation. Alignment of groups obtained by 23S rDNA and SDS-PAGE analysis in the present study showed remarkable disagreement in separation of strains, probably because of the different typing targets analysed.

Ribotyping and RAPD typing have been used to elucidate the complex epidemiology of thermophilic Campylobacter spp. infections for outbreak investigations and surveillance purposes, and to study disease associations [18,41–43]. Ribotyping identified 34 RiboGroups among the 43 isolates in the present study. Additional RAPD-DNA analysis with three primers identified unique profiles for all 37 isolates tested; six isolates, including one from a healthy carrier, were not tested by this method because of a sudden loss of reproducibility, which is an inherent problem of the technique and stresses the importance of including a control strain in each experiment. Only two isolates from healthy carriers were tested, and the ability of RAPD analysis to discriminate between the two major populations needs further evaluation with more isolates from healthy carriers. However, the substantial heterogeneity observed among the C. concisus isolates in this study supports previous findings from Belgium and South African laboratories, which have applied highly discriminatory genotyping techniques to study clinical isolates of C. concisus [3,10]. Taken together, the molecular epidemiological studies of C. concisus indicate that the species consists of at least two genomospecies with extensive genetic diversity. Studies of C. jejuni have revealed diversity at the phenotypic level for almost every characteristic that has been implicated in pathogenicity. However, this phenotypic variation does not always coincide with observed or predicted differences in virulence between clinical and non-clinical isolates [12]. Clearly, further work is still needed to determine whether C. concisus is a significant human enteric pathogen.
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