Journal of Medical Microbiology (2005), **54**, 615–619 DOI 10.1099/jmm.0.45988-0

Prevalence of potential virulence markers in Polish *Campylobacter jejuni* and *Campylobacter coli* isolates obtained from hospitalized children and from chicken carcasses

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Received 20 December 2004
Accepted 15 March 2005

The pathogenicity of thermotolerant *Campylobacter* species, common food-borne pathogens, depends on certain factors unevenly distributed among strains of different origin. The prevalence of such markers has never been examined in a population of Polish *Campylobacter* strains of human and poultry origin. Therefore, we analysed the presence of the *cadF*, *cdtA*, *cdtB* and *cdtC* genes and the *iam* sequence in *Campylobacter jejuni* ($n = 115$) and *Campylobacter coli* ($n = 57$) isolates from children with diarrhoea and from chicken carcasses. The *cadF* gene was present in nearly 100 % of *Campylobacter* isolates tested, regardless of their origin or species. In contrast, the *iam* region was found in 83·3 % and 100 % of *C. coli* isolates from children and chickens, respectively, but in only 1·6 % and 54·7 %, respectively, of *C. jejuni* isolates. Similarly, the detection rates of *cdt* genes varied between human and chicken isolates. All three *cdt* genes were found in nearly all *C. jejuni* isolates from both children and chickens, but in only 5·6 % of human *C. coli* isolates as compared to 87·2 % of chicken *C. coli* isolates. This different distribution of genetic markers between human and chicken isolates indicates that some *Campylobacter* infections in children may have additional sources other than contaminated chicken meat.

INTRODUCTION

Campylobacter jejuni and *Campylobacter coli* are the most frequent cause of diarrhoeal disease in humans throughout the world (Allos, 2001). However, the results of a 5-year study in Polish children with diarrhoea showed that the mean frequency of *C. jejuni* and *C. coli* infections did not exceed 9·1 %, and the majority of cases occurred at an age of 0–5 years (Rozynek & Dzierzanowska, 1994).

Searches for possible sources of *Campylobacter* infections, carried out in many countries, have revealed a very high level of poultry gut colonization by these micro-organisms. The contamination of poultry carcasses in abattoirs is considered a main risk factor for human infection (Bang *et al.*, 2001; Petersen *et al.*, 2001). A recent study in Poland showed that 88·5 % of chicken carcasses are contaminated with *Campylobacter* species (Daczkowska-Kozon, 2002). However, according to data from the National Food and Nutrition Institute in Poland, chicken meat is consumed by only 18 % of children under 5 years (Szponar, personal communication), which may suggest an alternative source of *Campylobacter* infection in this group of patients. Origins of human *Campylobacter* infections other than poultry meat have been postulated recently by German authors who found a difference in antimicrobial susceptibility patterns between human and poultry strains (Luber *et al.*, 2003).

Some properties of *Campylobacter* species, such as the ability to adhere and colonize, as well as invasiveness for enterocytes and synthesis of one or more toxins, appear essential in the process of infection (Konkel *et al.*, 2001). Research on the molecular mechanisms of *Campylobacter* infection pathogenesis has been stimulated by the publication of the *C. jejuni* NCTC11168 genome sequence (Parkhill *et al.*, 2000). Several potential markers of bacterial virulence have been identified (Bang *et al.*, 2003; Datta *et al.*, 2003; Fouts *et al.*, 2005). One of these is the *cadF* gene, which encodes a 37 kDa protein belonging to the group of outer-membrane proteins (OMPs) that functions as an adhesin responsible for certain steps of invasion (Konkel *et al.*, 1999). Another interesting region, designated an invasion-associated marker (*iam*), has been identified in some *C. jejuni* and *C. coli* strains (Carvalho *et al.*, 2001). So far, no product attributed to this region has been

found. The virulence of *Campylobacter* species is also associated with the production of a cytotoxin that consists of three subunits of molecular mass 30, 29 and 21 kDa, which are encoded by the *cdtA*, *cdtB* and *cdtC* genes, respectively. All three subunits are necessary for the cytotoxin activity known to be lethal for host enterocytes (Purdy *et al.*, 2000; Lara-Tejero & Galan, 2001).

Since the distribution of potential virulence markers among Polish *Campylobacter* isolates has never been analysed, our study was aimed at comparing the prevalence of the *iam*, *cadF* and *cdtABC* genes in *C. jejuni* and *C. coli* isolates from children and from chicken carcasses.

METHODS

Bacterial strains. *Campylobacter* isolates were obtained from anal swabs from children with diarrhoea presenting at the Children's Memorial Health Institute, Warsaw, Poland, during 2002–2004, and swabs from chicken carcasses obtained in supermarkets and slaughterhouses in various regions of Poland in the same period. Isolation was performed by direct spreading of the swab-collected specimens on solid selective media (Preston agar and Blazer-Wang agar). Inoculated media were incubated for 48 h at 42 °C in microaerobic conditions (Campy-Pak BBL envelopes). *Campylobacter* identification and discrimination among thermophilic *Campylobacter* species were based on colony morphology, Gram's staining and biochemical reactions. Species identification was confirmed by PCR with specific primers (listed in Table 1), as described elsewhere (Linton *et al.*, 1997; On & Jordan, 2003). Reference strains *C. jejuni* ATCC33560 and *C. coli* ATCC33559 were used as controls.

Detection of potential virulence markers by PCR. The presence of the *cadF*, *cdtA*, *cdtB* and *cdtC* genes and the *cdt* cluster in all *Campylobacter* isolates was determined with the primers listed in Table 2, as described elsewhere (Konkel *et al.*, 1999; Eyigor *et al.*, 1999; Bang *et al.*, 2003). Three sets of primers were used for detection of the *iam* marker: a pair published previously (Carvalho *et al.*, 2001), and two pairs designed in this study (Table 2), selected from the *iam* locus sequence of *C. jejuni* strain (GenBank accession no. AF023133). The PCR conditions for all *iam*-PCRs were the same as in the original study by Carvalho *et al.* (2001).

RESULTS AND DISCUSSION

Among the isolates, 57 were identified as *C. coli* and 115 as *C. jejuni* by both classical methods and PCR. Thus, only these 172 well-defined isolates were subjected to further analysis. Eighty isolates were from children (62 *C. jejuni* and 18 *C. coli*), and 92 came from chicken carcasses (53 *C. jejuni* and 39 *C. coli*).

Analysis of the prevalence of the *cadF* gene revealed that nearly all of the *Campylobacter* isolates carried this marker, regardless of their strain origin (Table 3). Similar observations indicating that the *cadF* gene is present in *Campylobacter* species isolated from human specimens as well as from chicken carcasses and droppings have recently been reported by other authors (Konkel *et al.*, 1999; Dorrell *et al.*, 2001; Bang *et al.*, 2003; Datta *et al.*, 2003). The product of this gene is an adhesin and fibronectin-binding protein involved in the process of invasion, influencing microfilament organization in host cells (Monteville *et al.*, 2003). It was shown that *cadF*-negative strains were not able to colonize chicken guts (Ziprin *et al.*, 1999). Thus, the *cadF* gene, which appears to be essential for chicken gut colonization, may presumably have a similar role in the pathogenesis of human infection.

Another marker potentially associated with the severity of *Campylobacter*-induced enteritis, called an invasion-associated marker (*iam*), has been described by Carvalho *et al.* (2001). Because the *iam* sequence was detected in 85 % of *Campylobacter* isolates from children with diarrhoea, as compared to only 20 % of isolates from asymptomatic patients, they suggested that this locus could be used as a marker of invasive *Campylobacter* strains. In this study, the *iam* sequence was found in isolates from only 20 % of patients with diarrhoea, and its prevalence varied depending not only on the origin but also on the species of the

Table 1. Primers for identification of *Campylobacter* isolates

Primers	Sequence (5′⟶3′)	Product (bp)	Reference
Multiplex PCR*			
Mu 1	CATCTTCCCTAGTCAAGCCT	773 (*C.j.*†)	On & Jordan (2003)
Mu 2	AAGATACTCTAGCAAGATGG		
Mu 3	AGGCAAGGGAGCCTTTAATC	364 (*C.c.*†)	
Mu 4	TATCCCTATCTACAATTCGC		
Identification of *C. jejuni*			
HipOR2	AGCTAGCTTCGCATAATAACTTG	500	Linton *et al.* (1997)
HipOF2	GAAGAGGGTTTGGGTGGT		
Identification of *C. coli*			
CC 1	GGTATGATTTCTACAAAGCGAG	500	Linton *et al.* (1997)
CC 2	ATAAAAAGACTATCGTCGCGT		

*Muliplex PCR for simultaneous identification of *C. jejuni* and *C. coli*.

†*C.j.*, *C. jejuni*, *C.c.*, *C. coli*.

Table 2. List of primers for the detection of the *iam* marker, *cadF* and *cdt* genes, and respective references

Genes	Sequences (5′→3′)	Product (bp)	Reference
cadF	TTGAAGGTAATTTAGATATG	400	Konkel *et al.* (1999)
	CTAATACCTAAAGTTGAAAC		
cdtA	CCTTGTGATGCAAGCAATC	370	Eyigor *et al.* (1999)
	ACACTCCATTTGCTTTCTG		
cdtB	GTTAAAATCCCCTGCTATCAACCA	495	Bang *et al.* (2003)
	GTTGGCACTTGGAATTTGCAAGGC		
cdtC	CGATGAGTTAAAACAAAAAGATA	182	Eyigor *et al.* (1999)
	TTGGCATTATAGAAAATACAGTT		
cdtABC	GGAAATTGGATTTGGGGCTATACT	1215	Bang *et al.* (2003)
	TTGCACATAACCAAAAGGAAG		
iam	GCGCAAAATATTATCACCC	518	Carvalho *et al.* (2001)
	TTCACGACTACTATGCGG		
	GGCGCTTTAGGGAAGCTG	1360	This study
	CTTTAAATTGAATCACGGG		
	TGAGGAGCTAAGGGTGCAAA	270	This study
	AATACTGATATTTTCCACAT		

Table 3. PCR detection of the *cadF* gene and the *iam* marker in 115 *C. jejuni* and 57 *C. coli* isolates

Isolate group (source/species)	No. of positive isolates (%)	
	cadF	***iam***
Children/*C. jejuni* ($n = 62$)	61 (98·4)	1 (1·6)
Children/*C. coli* ($n = 18$)	18 (100)	15 (83·3)
Children/both ($n = 80$)	79 (98·8)	16 (20)
Chickens/*C. jejuni* ($n = 53$)	53 (100)	29 (54·7)
Chickens/*C. coli* ($n = 39$)	39 (100)	39 (100)
Chickens/both ($n = 92$)	92 (100)	68 (74)

Campylobacter isolate (Table 3). More than 50 % of *C. jejuni* and 100 % of *C. coli* isolates from chicken carcasses were *iam*-positive. In contrast, only one *C. jejuni* strain (1·6 %) and 83·3 % of the *C. coli* isolates from children possessed this sequence. Since three different primer sets were used for *iam* amplification in all isolates tested, and all except two gave the same results, the lack of the detectable *iam* locus in some isolates seems to reflect a true absence of this sequence rather than inadequate PCR conditions. The low prevalence (20 %) of the *iam* sequence in *Campylobacter* isolates from children with diarrhoea, which was identical to that observed by Carvalho *et al.* (2001) in asymptomatic patients, indicates that it is not a universal marker of the severity of *Campylobacter* infection.

Another marker analysed in our study was the cytotoxin-encoding gene cluster. The cytopathic effect of the cytotoxin is associated with damage to nuclear DNA, resulting in the inhibition of the cell cycle in G2 or M phase (Whitehouse *et al.*, 1998). It has also been shown that cytolethal distending toxin (CDT) is involved in inducing the release of pro-inflammatory cytokine IL-8 from intestinal epithelial cells (Hickey *et al.*, 2000). The latter observation suggests that cytolethal distending toxin may also play a part in the development of the inflammatory process in humans. Recent studies analysing the distribution of separate *cdtA*, *cdtB* and *cdtC* genes or the *cdt* cluster in *C. jejuni* and *C. coli* indicate that their prevalence in isolates from poultry and other sources exceeds 90 % (Bang *et al.*, 2001; Datta *et al.*, 2003). Microarray testing of the *cdt* cluster showed that these genes were present in all *C. jejuni* isolates from human samples (Dorrell *et al.*, 2001; Volokhov *et al.*, 2003). A similar frequency was also observed in our study of chicken and human *C. jejuni* isolates (Table 4). However, in *C. coli* isolates from children with diarrhoea, detection rates of these genes were much lower (Table 4). Because *cdt* genes have recently been shown to be conserved among different *Campylobacter* strains (Fouts *et al.*, 2005), and we used separate primer sets

Table 4. PCR detection of *cdt* genes in 115 *C. jejuni* and 57 *C. coli* isolates

Isolate group (source/species)	No. of positive isolates (%)			
	cdtA	*cdtB*	*cdtC*	*cdtABC*
Children/*C. jejuni* ($n = 62$)	61 (98·4)	60 (97)	61 (98)	61 (98)
Children/*C. coli* ($n = 18$)	1 (5·6)	15 (83)	1 (5·6)	1 (5·6)
Children/both ($n = 80$)	62 (77·5)	75 (93·8)	62 (77·5)	62 (77·5)
Chickens/*C. jejuni* ($n = 53$)	53 (100)	53 (100)	53 (100)	53 (100)
Chickens/*C. coli* ($n = 39$)	34 (87·2)	38 (97·4)	38 (97·4)	34 (87·2)
Chickens/both ($n = 92$)	87 (94·6)	91 (98·9)	91 (98·9)	87 (94·6)

for detection of individual *cdtA*, *cdtB* and *cdtC* genes and the *cdt* cluster, which increased the reliability of PCR results, the low detectability of *cdt* genes in human *C. coli* isolates seems to result from a true lack of these markers.

The differences in distribution of *cdt* genes and the *iam* sequence between human and chicken isolates observed in our study, together with the low consumption of poultry by young children in Poland, suggests that *Campylobacter* infections in these patients may have additional sources other than contaminated chicken meat.

ACKNOWLEDGEMENTS

This study was supported in part by research grant KBN PB 371/P05/2002/23.

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